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Effect of Growth Substrates on Production of New Soluble Glucose 3-Dehydrogenase in *Halomonas (Deleya)* sp. α-15

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Abstract

Halomonas (Deleya) sp. α-15 produces new co-factor binding soluble glucose 3-dehydrogenase (G3DH), which oxidizes the third hydroxy group of pyranose. This study investigated the condition of efficient production of G3DH using *Halomonas (Deleya)* sp. α-15. This enzyme was inducible, and α-methyl-D-glucoside, isopropyl-thiogalactopyranoside (IPTG) and lactose were revealed to be suitable carbon sources for G3DH induction. Maximum G3DH production was achieved by using minimal medium containing 0.8% (w/v) lactose with a productivity of 470U/l.

Index Entries: Glucose 3-dehydrogenase; glucose dehydrogenase; marine bacteria; *Halomonas* sp.

Introduction

Various glucose oxidoreductases have been reported and utilized for the enzymatic determination of glucose. Among them, only few enzymes that oxidize hydroxy group of pyranose and its derivatives except C-1 position have been reported. Such enzymes can be applied for the production of sugar derivatives *(1,2)* and the measurement of 1,5-anhydro-D-glucitol (1,5AG) *(3–5)*, which is a major polyol in human blood and has been becoming a good clinical marker for diabetes. These include glucose 3-dehydrogenases (G3DHs) from *Agrobacterium tumefaciens (6)*, *Flavobacterium saccharophilum (7)*, and *Cytophaga marinoflava (8)*. G3DHs from *F. saccharophilum* and *C. marinoflava* are membrane-binding proteins that

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require detergent for both during the isolation of enzyme and to express and maintain their activity. Because the presence of detergent prevents easy handling of enzyme and sensitive assay, soluble G3DH is more desirable in practical use. G3DH of *A. tumefaciens* is a soluble enzyme and can oxidize the third hydroxy group of pyranose. In *A. tumefaciens*, G3DH plays an important role in sucrose metabolism. This enzyme oxidizes sucrose to 3-ketosucrose *(9)*. But this strain is known as plant germ, therefore massive production of G3DH using this strain has an inherent problem. The studies on large-scale G3DHs production have not yet been reported in spite of their potential needs in clinical and chemical use.

We previously reported the isolation of a co-factor binding soluble G3DH which oxidizes the third hydroxy group of pyranose from a Gram-negative marine bacterium designated strain α-15, which was identified as *Deleya* sp. *(10)*. Recently, genus *Deleya* was transferred to a genus *Halomonas (11)*. This enzyme is a monomeric enzyme with Mw 67kDa. This enzyme can oxidize monosaccharides like 1,5AG or α-methyl-D-glucoside and disaccharides like lactose or sucrose. On the other hand, the role in metabolism and regulation mechanism of G3DH in α-15 are still unknown.

In this article, we describe the investigation of the condition for the efficient production of G3DH using *Halomonas (Deleya)* sp. α-15. G3DH activity is revealed to be induced when α-methyl-D-glucoside, isopropyl-thiogalactopyranoside (IPTG) or lactose is used as a carbon source.

Material and Methods

Bacterial Strain and Culture Media

Halomonas (Deleya) sp. α-15 *(10)* was used through out these experiments. BSα medium contained polypepton (1% [w/v]), yeast extract (0.1% [w/v]), NaCl (3% [w/v]), KH_2PO_4 (0.2% [w/v]) and α-methyl-D-glucoside (0.4% [w/v]). YM9Sα medium contained Na_2HPO_4 (0.6% [w/v]), KH_2PO_4 (0.3% [w/v]), NH_4Cl (0.1% [w/v]), yeast extract (0.02% [w/v]), $MgSO_4$ (0.02%), $CaCl_2$ (0.002%), NaCl (3% [w/v]) and various concentration of α-methyl-D-glucoside. YM9Slac medium was YM9Sα medium containing lactose instead of α-methyl-D-glucoside.

Batch Fermentations

Seed cultures (150 mL × 2) were prepared in two 500-mL conical-shaped flasks and incubated in a reciprocating shaker overnight at 30°C, 120 rpm. Batch culture was carried out at 30°C in a 10-L jar fermenter (Mitsuwa Bio Systems, Japan) containing 7 L of medium by inoculating 300 mL of aforementioned seed culture. Aeration and agitation were controlled as 1 vvm and 800 rpm, respectively. pH was not controlled.

Selection of Carbon Source as an Inducer

The cultures were prepared in 3 mL of YM9S media containing 0.4% (w/v) of each carbon source and incubated in a reciprocating shaker overnight at

30°C, 80 rpm. The cells were harvested at their stationary phase and measured G3DH activity and protein concentration.

Analytical Methods

Growth was monitored by measuring the optical density (OD) at 660 nm. Lactose concentration in a culture was measured using α-15 G3DH, because it can catalyze the oxidation of lactose *(10)*, as follows. One mL of culture was centrifuged for 5 min at 5000*g* and removed cells. Ten µL of this supernatant, 190 µL of 10 m*M* potassium phosphate buffer (pH 7.0) containing α-15 G3DH, 60 µ*M* 2,6-dichlorophenol indophenol (DCIP), and 1 m*M* phenazine methosulfate (PMS) were mixed and measured the rate of absorbance decrease at 600 nm.

Protein concentration was determined as follows. The centrifuged cells were washed with 3% NaCl three times and resuspended in 500 µL of 10 m*M* potassium phosphate buffer (pH 7.0). Thirty µL of this sample was mixed with 10 µL of 10% SDS and boiled. Then the protein concentration was measured using DC Protein Assay Kit (BioRad, Hercules, CA).

Bacterial G3DH production was measured as follows. The cell suspension was mixed with 80 µL of 10 m*M* potassium phosphate buffer (pH 7.0), 60 µ*M* DCIP, and 1 m*M* PMS and measured the rate of absorbance decrease at 600 nm with a molecular coefficient of DCIP as 16.3/m*M*/cm at pH7. One unit was defined as the G3DH activity that oxidizes 1 µmol substrate in 1 min.

Results

Effect of Carbon Source on the Production of G3DH

We first investigated the effect of medium component on the G3DH specific activity. *Halomonas (Deleya)* sp. α-15 was cultivated in a 10-L jar fermenter containing either 7 L of BSα medium (complex medium) or YM9Sα medium (minimal medium). The results are shown in Fig. 1 and Table 1. Using YM9Sα medium, after 20 h the amount of produced G3DH was at the maximum (60 U/l), with specific activity of 0.15 U·mg/protein. Using BSα medium, after 11.5 h the amount of produced G3DH was at the maximum (80 U/l), with specific activity of 0.04 U·mg/protein, about 1/3 achieved in YM9Sα. The amount of produced G3DH using BSα medium was only 30% higher than in YM9Sα. Considering the further purification steps, the preparation of cells with higher G3DH specific activity is preferable. Therefore, we chose minimal media containing appropriate carbon source, α-methyl-D-glucoside in this case, for further studies.

We have been using α-methyl-D-glucoside as the carbon source for α-15 cultivation, because the bacterium was isolated from marine environment using minimal medium containing α-methyl-D-glucoside as a sole carbon source. However, α-methyl-D-glucoside is not a practical carbon source considering its commercial price. Therefore, we investigated alternative cost-effective carbon sources to α-methyl-D-glucoside, for G3DH production.

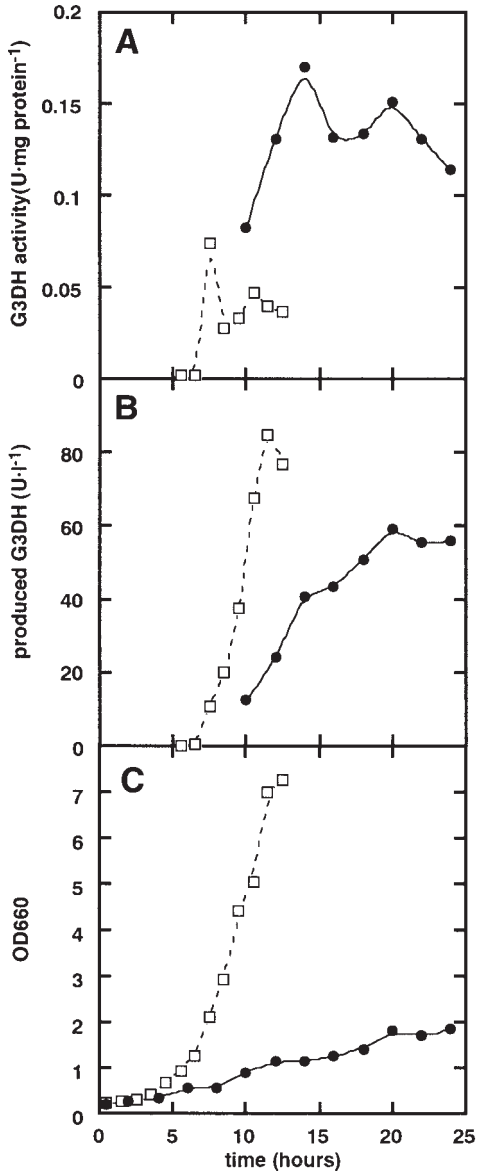

Fig. 1. The effect of medium on cell growth and G3DH production in α-15. **(A)** G3DH activity: —●—, YM9Sα(0.4%); – –□– –, Bs_{α}. **(B)** G3DH production: —●—, YM9Sα(0.4%); – –□– –, Bs_{α}. **(C)** Cell growth: —●—, YM9Sα(0.4%); – –□– –, Bs_{α}.

The results are shown in Table 2. Utilizing IPTG or lactose as a carbon source, high G3DH production could be achieved.

Lactose was more advantageous in both cost and efficiency for G3DH production compared with α-methyl-D-glucoside and IPTG. Therefore, for the G3DH production, lactose was found as the most suitable carbon source.

Table 1
G3DH Production Using Jar Fermenter[a]

Media	G3DH activity (U·mg/protein)	Protein concentration (mg/L)	Produced G3DH (U/L)
Bs_{α}[b]	0.04	2134	84.8
YM9Sα[b]	0.15	392	59.2
YM9Slac(0.4%)	0.21	406	80.6
YM9Slac(0.8%)	0.20	2407	473.0
YM9Slac(1.6%)	0.18	479	88.0

[a]Bacterial cultures were carried out in a 10-L jar fermenter containing 7 L of each medium.
[b]Containing 0.4% α-methyl-D-glucoside.

Table 2
Effect of Carbon Source on G3DH Production[a]

Carbon source[b]	G3DH activity (U·mg/protein)	Protein concentration (mg/L)	Produced G3DH (U/L)
α-methyl-D-glucoside[c]	0.57	572	326.0
IPTG[d]	0.74	233	172.0
D-Glucose[c]	0.09	546	49.1
D-Mannose[d]	0.05	313	15.7
D-Galactose[d]	0.03	549	16.5
D-Xylose[d]	0.06	412	24.7
Maltose[d]	0.07	656	45.9
Sucrose[d]	0.03	849	25.5
Isocitric acid[d]	0.04	552	22.1
Oxaloacetate[d]	0.02	491	10.0
Lactose[c]	0.86	637	548.0

[a]Bacterial cultures were carried out in test tube (3 mL).
[b]All cultures contained 0.4% of each carbon source.
[c]Harvested after 20 h.
[d]Harvested after 24 h.

Effect of Lactose Concentration in Production of G3DH

The effect of lactose concentration in production of G3DH was investigated. Using the jar fermenter, α-15 was cultivated in YM9Slac media which contained either 0.4%, 0.8%, or 1.6% lactose, respectively. The results are shown in Fig. 2 and Table 1. The highest G3DH production was achieved using YM9Slac (0.8%) medium. The G3DH production using YM9Slac (0.8%), was eightfold of that achieved in YM9Sα. In contrast, cell growth and G3DH production in YM9Slac (1.6%) (OD = 2.85, 88.0 U/L) were lower than in YM9Slac (0.8%).

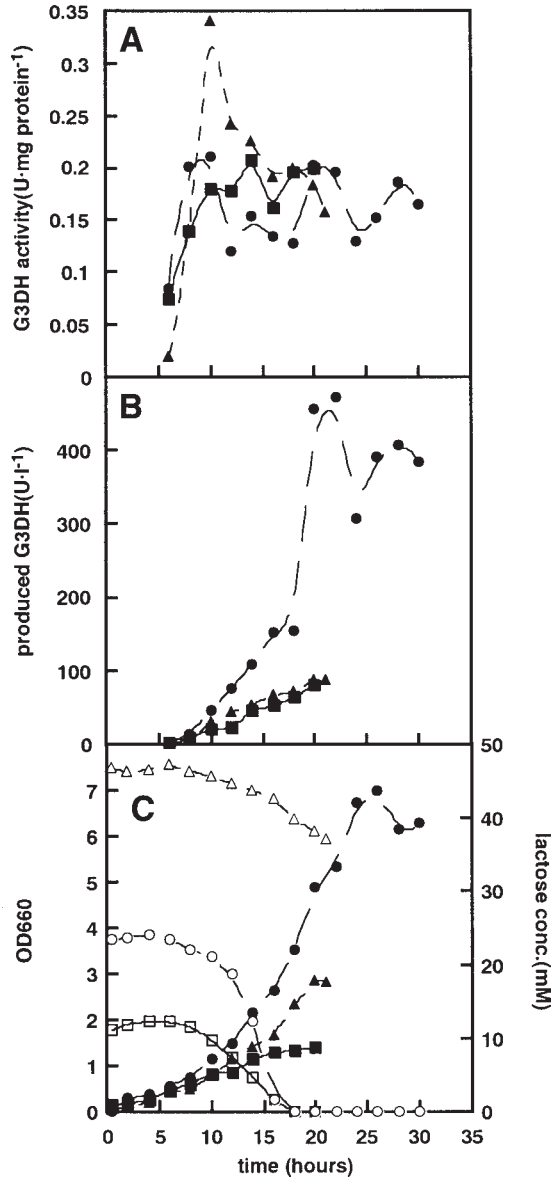

Fig. 2. The effect of lactose concentration on cell growth and G3DH production in α-15. **(A)** G3DH activity: —■—, YM9Slac(0.4%); – –●– –, YM9Slac(0.8%); – –▲– –, YM9Slac(1.6%). **(B)** G3DH production: —■—, YM9Slac(0.4%); – –●– –, YM9Slac(0.8%); – –▲– –, YM9Slac(1.6%). **(C)** Cell growth and lactose concentration. Cell growth: —■—, YM9Slac(0.4%); – –●– –, YM9Slac(0.8%); – –▲– –, YM9Slac(1.6%). Lactose concentration: —□—, YM9Slac(0.4%); – –○– –, YM9Slac(0.8%); – –△– –, YM9Slac(1.6%).

Discussion

We previously reported the isolation of a co-factor binding soluble G3DH from a Gram-negative marine bacterium designated strain α-15,

which was identified as *Halomonas (Deleya)* sp. *(10)*. Because the bacterium was isolated from marine environment using a minimal medium with α-methyl-D-glucoside as a sole carbon source, the cultivation of the bacteria was carried out using the same medium. When the α-15 cultivation was carried out using media either complex media with α-methyl-D-glucoside or minimal media with D-glucose, D-mannose, D-galactose, D-xylose, maltose, or sucrose, the expressed G3DH level was lower than 10% of which achieved in minimal medium containing lactose. Although the aforementioned saccharides are all the substrates of this enzyme, α-15 G3DH could only be produced at a high level when IPTG, α-methyl-D-glucose or lactose is used as a carbon source. Therefore, G3DH is an inducible enzyme. Lactose, β-D-galactopyranosil-(1-4)-D-glucose and IPTG can be regarded as C-1 blocked galactose derivatives. α-methyl-D-glucoside is C-1 blocked glucose derivatives. But both sucrose and maltose, which can be regarded as C-1 blocked glucose derivatives, have no ability to induce G3DH. Therefore, C-1 blocked structure was not necessary for G3DH induction.

G3DH of *A. tumefaciens* plays an important role in sucrose metabolism *(4)*. *A. tumefaciens* oxidizes sucrose to 3-ketosucrose using G3DH. α-15 G3DH can also oxidize sucrose, but α-15 G3DH was not induced by sucrose. In this point, α-15 G3DH has a different role in metabolism from that of *A. tumefaciens* G3DH. Little amount of glucose was detected from α-15 culture through the cultivations using lactose (data not shown). This fact suggest that α-15 imports lactose intracellular without hydrolysis.

During the G3DH production by *Halomonas (Deleya)* sp. α-15 in jar fermenter using YM9Slac (0.8%), lactose concentration became zero at the late-log phase (Fig. 2). This result suggested that the availability of a carbon source—lactose, in this case—may restrict both cell growth and G3DH production. As the cell growth and G3DH production was repressed when YM9Slac (1.6%) was used, high carbon-source concentration at the initial status will not be favorable. The further investigation of fed-batch culture, where lactose concentration will be kept at an adequate level, will improve the G3DH productivity.

Conclusion

In this paper we investigated the conditions for G3DH production of *Halomonas (Deleya)* sp. α-15. The enzyme was found to be inducible, and the cultivation using minimal medium containing lactose as a carbon source was found to be suitable for its massive production.

Maximum G3DH production in a jar fermenter was achieved by using minimal medium containing 0.8% (w/v) lactose. However, under this condition, G3DH production stopped when the lactose concentration became zero in late-log phase. This suggested that the availability of carbon source may restrict both cell growth and G3DH production.

References

1. Asano, N., Katayama, K., Takeuchi, M., Yoshikawa, M., and Matsui, K. (1989), *J. Antibiot.* (Tokyo) **42,** 585–590.
2. Stoppok, E., Matalla, K., and Buchholz, K. (1992), *Appl. Microbiol. Biotechnol.* **36,** 604–610.
3. Yabuuchi, M., Masuda, M., Katoh, K., Nakamura, T., and Akanuma, H. (1989), *Clin. Chem.* **35,** 2039–2043.
4. Fukuyama, Y., Tajima, S., Oshitani, S., Ushijima, Y., Kobayashi, I., Hara, F., Yamamoto, S., and Yabuuchi, M. (1994), *Clin. Chem.* **40,** 2013–2016.
5. Tanabe, T., Umegae, Y., Koyashiki, Y., Kato, Y., Fukahori, K., Tajima, S., and Yabuuchi, M. (1994), *Clin. Chem.* **40,** 2006–2012.
6. Hayano, K. and Fukui, S. (1967), *J. Biol. Chem.* **242,** 3655–3672.
7. Takeuchi, M., Ninomiya, K., Kawabata, K., Asano, N., Kameda, Y., and Matsui, K. (1986), *J. Biochem.* (Tokyo) **100,** 1049–1055.
8. Tsugawa, W., Horiuchi, S., Tanaka, M., Wake, H., and Sode, K. (1996), *Appl. Biochem. Biotechnol.* **56,** 301–310.
9. Schuerman, P. L., Liu, J. S., Mou H., and Dandekar, A. M. (1997), *Appl. Microbiol. Biotechnol.* **47,** 560–565.
10. Tsugawa, W., Ogasawara, N., and Sode, K. (1998), *Enzyme Microb. Technol.* **22,** 269–274.
11. Dobson, S. J. and Franzmann, P. D. (1996), *Int. J. Syst. Bacteriol.* **46,** 550–558.