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Technical Note

Dihydroxyacetone Production from Raw Glycerol by *Gluconobacter oxydans* NBRC 14819^T

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Dihydroxyacetone (DHA) is the main ingredient in self-tanning products [8]. DHA is also an important precursor for chemical synthesis in the pharmaceutical industry [11], and the production of DHA from glycerol using the acetic acid bacterium *Gluconobacter oxydans* is cost effective and environmentally friendly (Fig. 1). Recent increases in the demand for DHA have led to an increase in studies focusing on improved fermentation processes and genetic engineering of DHA-producing bacteria [7,14]. We previously reported that the loss of *adhA* in *G. oxydans* significantly improved cell growth, as well as the production of DHA in the presence of high glycerol concentrations [3].

Raw glycerol is a by-product in the biodiesel and oleochemical industries. Approximately 10% of the by-product weight is produced by the transesterification of vegetable oils and animal fats during biodiesel fuel production [11]. Raw glycerol is purified and used as a feedstock for various industrial chemicals. However, the purification process is costly and energy intensive. Therefore, it is necessary to develop methods for the direct use of raw glycerol, which often contains methanol and alkali metals. In previous studies, citric acid [9], mannitol [12], D-arabitol [13], and DHA [2,14] were produced using raw glycerol; however, raw glycerol (called 'crude glycerol' in some reports) in those studies contained >80% (w/v) glycerol and <0.5% (w/v) methanol. Multiple forms of raw glycerol are produced, depending on the by-production process [6]. Thus, the use of raw glycerol containing more than 10% (w/v) methanol is important for the development of renewable biomass resources.

This study investigated the production of DHA by *G. oxydans* (type strain NBRC 14819^T). The raw glycerol source (pH 12) was produced via the transesterification of triglycerides (previously provided by Sun Care Fuels Corporation) [1], and contained 66.4% (w/v) glycerol, 30.9% (w/v) methanol, and 0.54% (w/v) sodium salt (analyzed by our group) [4].

Prior to using the raw glycerol, DHA production by *G. oxydans* NBRC 14819^T was assessed using pure glycerol in a 1-L jar fermenter (Model MDL; B.E. Marubishi, Tokyo, Japan). For fermentation experiments, five seed cultures in test tubes (5-mL each), which contain both glucose media and *G. oxydans*, were aerobically grown for 2 days at 30°C with agitation at 200 rpm. Seed cultures (total of 25 mL) were then transferred to a jar fermenter containing 500 mL of glycerol media (pH 6.5), consisting of 100 g/L glycerol, 10 g/L polypeptone, 1 g/L yeast extract, 0.9 g/L KH_2PO_4, 0.1 g/L K_2HPO_4, and 1 g/L $MgSO_4{\cdot}7H_2O$, and aerobically incubated for 3 days. The aeration rate and agitation speed were set to 1.0 volume of air per volume of medium per min (vvm), and 500 rpm, respectively. The temperature was maintained at 30±1°C, and the pH was maintained above 6 using 5 M NaOH.

The concentrations of glycerol, DHA, and glyceric acid (GA) in the cultures were quantified using HPLC with an LC-20AD HPLC pump (flow rate, 1.0 mL/min) and a

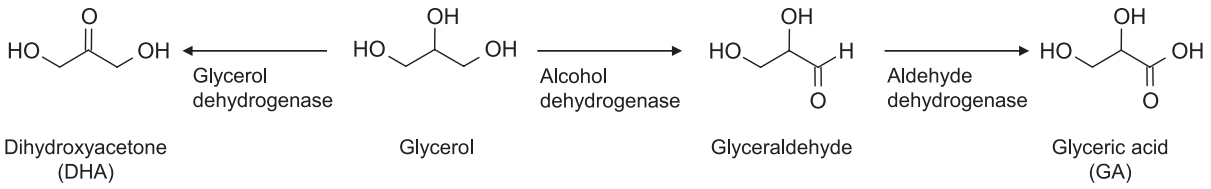

Fig. 1. Bioconversion of glycerol to dihydroxyacetone (DHA) and glyceric acid (GA).

RID-10A detector (Shimadzu). A Shodex® SC1011 Column (Showa Denko, Tokyo, Japan) was used for glycerol and DHA quantification, while a Shodex® SH1011 Column (Showa Denko) was used for GA quantification. A mobile phase of pure water was used for the SC1011 column, while 5 mM H_2SO_4 was used for the SH1011 column. Column temperatures were maintained at 80°C and 60°C for the SC1011 and SH1011 columns, respectively.

G. oxydans produced 66.2 g/L and 71.8 g/L of DHA from 100 g/L of pure glycerol following incubations of 2 and 3 days, respectively. No glycerol accumulated in the 3-day culture, while 3.5 g/L of GA did.

Previously, we developed a fed-batch fermentation method that used glycerol in an alkaline solution to feed the glycerol and control the pH during GA production [5]. Since raw glycerol contains alkaline metals, including sodium and potassium, it can be used as an alkaline pH-control reagent. During DHA production, acids such as GA are also produced, which reduces the pH of the culture. Thus, the use of untreated raw glycerol as a pH-control reagent was investigated during the fed-batch fermentation for DHA production.

Since the raw glycerol used in this study could not be used as the carbon source for *Gluconobacter* strains without a charcoal pretreatment [4], the effect of the initial pure glycerol concentration on DHA production was examined using raw glycerol (pH 12) as the pH-control reagent for maintaining above pH 6 (Fig. 2). For fermentation experiments, the initial glycerol concentrations in the glycerol media were 5, 10, or 20 g/L. For media containing 20 g/L initial glycerol, 82.9 g/L of DHA and 4.3 g/L of GA were produced during the 3-day incubation (Fig. 2A). Glycerol contents were increased to 83.6 g/L because the feeding rate of the raw glycerol exceeded the consumption rate during growth and DHA production. Incubations up to 5 days did not increase DHA concentrations in the cultures. Following 3-day incubations with starting glycerol concentrations of 10 g/L and 5 g/L, 74.0 and 75.2 g/L of DHA and 6.7 and 5.0 g/L of GA were produced, respectively (Figs. 2B and 2C). A gradual accumulation of glycerol was also observed in the culture medium for both experiments. Decreasing the initial glycerol concentration to <5 g/L resulted in poor growth (data not shown).

The culture conditions for DHA production by *G. oxydans* were optimized for an initial pure glycerol concentration of 5 g/L. Figure 3A shows the time course of DHA production using untreated raw glycerol with an aeration rate of 2 vvm (pH 6), and 86.9 g/L of DHA was produced over a 3-day incubation. When the agitation speed was increased from 500 to 700 rpm, DHA production increased to 105.1 g/L over the 3-day incubation (Fig. 3B). Thus, DHA production with concentrations of >100 g/L can be achieved using raw glycerol feedstock. Notably, GA production ranged from 3.4 to 6.6 g/L in all experiments.

We previously showed that methanol decreased GA production by *G. oxydans* because it inhibited membrane-bound alcohol dehydrogenase activity, which catalyze the oxidation of glycerol to glyceraldehyde [10]. In this study, low production of GA was also observed probably because of the presence of methanol in raw glycerol. By contrast, methanol did not inhibit glycerol dehydrogenase, which converts glycerol to

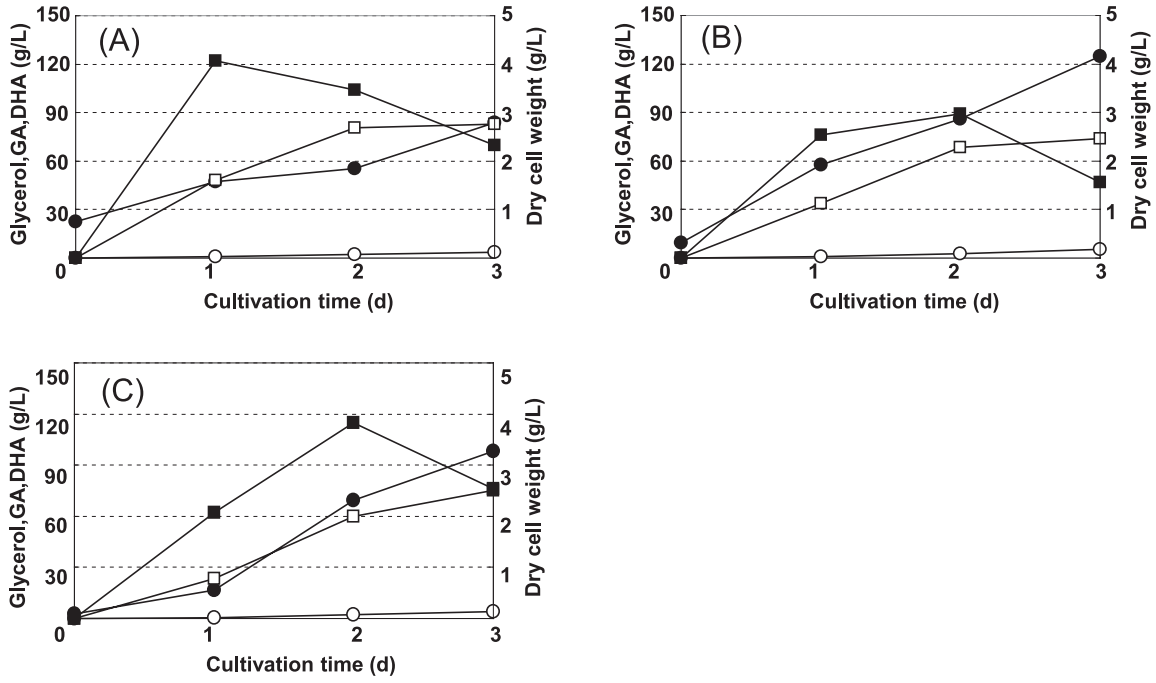

Fig. 2. Time course of DHA production by *Gluconobacter oxydans* NBRC14819^T with initial glycerol concentrations of (A) 20 g/L, (B) 10 g/L, and (C) 5 g/L. Experiments were performed using a 1-L jar fermenter. Cultures were maintained at a pH of 6 using untreated raw glycerol (pH 12). An aeration rate of 1.0 vvm and an agitation speed of 500 rpm were used. Symbols: black circles, glycerol concentration; white squares, DHA concentration; white circles, GA concentration; black squares, dry cell weight.

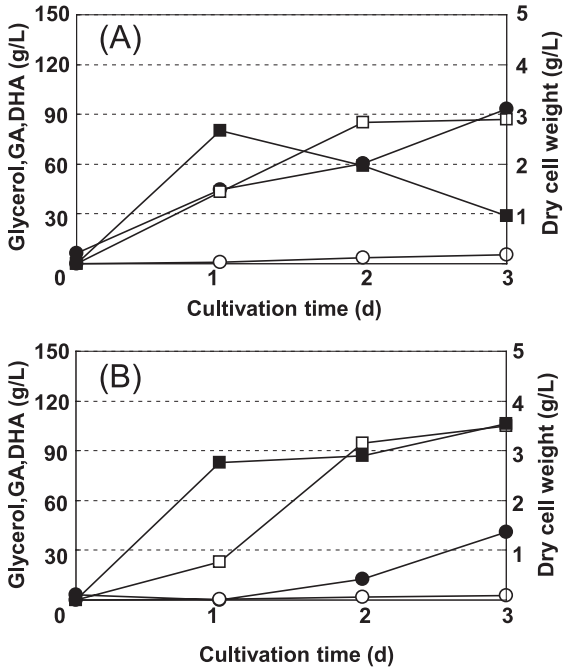

Fig. 3. DHA production by *Gluconobacter oxydans* NBRC14819^T during 3-day incubations with initial glycerol concentrations of 5 g/L. Experiments were performed using a 1-L jar fermenter with (A) a 2.0-vvm aeration rate and 500-rpm agitation speed, or (B) a 2.0-vvm aeration rate and 700-rpm agitation speed. The pH was maintained at 6 using untreated raw glycerol (pH 12). Symbols: black circles, glycerol concentration; white squares, DHA concentration; white circles, GA concentration; black squares, dry cell weight.

DHA. Therefore, DHA can be produced from untreated raw glycerol containing methanol. However, further improvements should be made to the yield and purification of DHA.

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