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1,3-Propanediol production and tolerance of a halophilic fermentative bacterium, *Halanaerobium saccharolyticum* subsp. *saccharolyticum*

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ABSTRACT

1,3-Propanediol (1,3-PD) is widely used in polymer industry in production of polyethers, polyesters and polyurethanes. In this article, a study on 1,3-PD production and tolerance of *Halanaerobium saccharolyticum* subsp. *saccharolyticum* is presented. 1,3-PD production was optimized for temperature, vitamin B_{12} and acetate concentration. The highest 1,3-PD concentrations and yields (0.6 mol/mol glycerol) were obtained at vitamin B_{12} concentration 64 μg/l and an inverse correlation between 1,3-PD and hydrogen production was observed with varying vitamin B_{12} concentrations. In the studied temperature range and initial acetate concentrations up to 10 g/l, no significant variations were observed in 1,3-PD production. High initial acetate (29–58 g/l) was observed to cause slight decrease in 1,3-PD concentrations produced but no effects on 1,3-PD yields (mol/mol glycerol). Initial 1,3-PD concentrations inhibited the growth of *H. saccharolyticum* subsp. *saccharolyticum*. When initial 1,3-PD concentration was raised from 1 g/l to 57 g/l, a decrease of 12% to 75%, respectively, in the highest optical density was observed.

1. Introduction

1,3-Propanediol (1,3-PD) is an interesting chemical due to its wide use among polymer industry in production of polyethers, polyesters and polyurethanes and thus large market volume. In nature, 1,3-PD is derived anaerobically from glycerol by bacteria, including species from genera *Klebsiella* (Chen et al., 2003; Cheng et al., 2004; Huang et al., 2002; Mu et al., 2006), *Enterobacter* (Barbirato et al., 1995), *Citrobacter* (Boenigk et al., 1993), *Lactobacillus* (El-Ziney et al., 1998), and *Clostridium* (Biebl et al., 1992; González-Pajuelo et al., 2004; Heyndrickx et al., 1991; Otte et al., 2009; Papanikolaou et al., 2000; Petitdemange et al., 1995). The biological production route includes glycerol degradation to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase, which is turned to 1,3-PD by 1,3-propanediol dehydrogenase. The intermediate, 3-HPA, is a toxic compound. However, its turnover to 1,3-PD is rapid and no accumulation tends to occur (Malaoui and Marczak, 2001). Glycerol is produced in high amounts as a by-product in biodiesel industry, which makes glycerol an inexpensive substrate for processes.

Recently, a halophilic fermentative bacterium, *Halanaerobium saccharolyticum* subsp. *saccharolyticum* was observed to produce 1,3-PD from glycerol (Kivistö et al., 2010). Halophiles are extremophiles that require salt, in concentrations over 50 g/l up to saturation (340 g/l), for growth. Halophilic microorganisms might have potential for biotechnological applications since hypersalinity as an extremophilic environment serves as a high selection pressure for the bioprocess, thus protecting the process from contaminations. Halophilic fermentative bacteria, though fascinating microorganisms, remain relatively un-studied and halophilic glycerol fermentation for 1,3-PD production has not been studied in more detail thus far. Here we present a study on 1,3-PD production and tolerance of *H. saccharolyticum* subsp. *saccharolyticum* using glycerol as a substrate. 1,3-PD production was studied for the effects of temperature, vitamin B_{12} and acetate concentration.

2. Materials and methods

2.1. Strain, culture conditions and experimental procedure

H. saccharolyticum subsp. *saccharolyticum* strain DSM 6643^T was from the German Collection of Microorganisms and Cell Cultures (DSMZ).

The bacteria were grown in HM100 medium with trace elements and vitamins described earlier (Kivistö et al., 2010) with the following modifications; 2.5 g/l glycerol, initial pH 7.8, and 16 μg/l vitamin B_{12} if not stated otherwise. The glycerol concentration, pH, and vitamin B_{12} concentration mentioned above, together with 100 g/l NaCl were observed to be favorable for 1,3-PD production in previous studies (Kivistö et al., 2010, 2011). The medium was prepared as described by Kivistö et al. (2010), the pH was adjusted

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to 7.8 before inoculation. The pure culture of *H. saccharolyticum* subsp. *saccharolyticum* was inoculated with 1% (v/v) inoculum from liquid pre-culture using aseptic techniques and grown at 37 °C and 150 rpm (expect in the temperature optimization experiment, details explained below) under nitrogen atmosphere for 72 h (1,3-PD production studies) or 163 h (1,3-PD tolerance studies). The pre-culture for acetate experiment was prepared in medium not containing acetate. The pH was not controlled during the incubations.

Studies on 1,3-PD production and tolerance were conducted as batch experiments in 25 ml serum tubes with 10 ml culture volume. The effect of temperature on 1,3-PD production was studied using a temperature-gradient incubator (Test Tube Oscillator, Terratec, Blackmansbay, Tasmania, Australia) with temperature gradient from 30 to 40 °C and oscillation rate of 100 oscillations/min. The vitamin B_{12} (cyanocobalamin, Sigma–Aldrich Co., St. Louis, MO, USA) concentration was optimized for 1,3-PD production with vitamin B_{12} concentration range from 0 to 480 μg/l and the effect of acetate was studied with Na-acetate·$3H_2O$ concentration range from 0 to 58 g/l, i.e. initial acetate concentration range from 0 to 425 mM. The 1,3-PD production experiments were done in duplicate (the effect of temperature) or triplicate (the effects of vitamin B_{12} and acetate) and control cultivations without glycerol were included (with exception of the effect of temperature in which the structure of the temperature gradient machine limited the number of replicates including controls). The 1,3-PD tolerance was studied with initial 1,3-PD concentration ranging from 0 g/l to 57 g/l. The experiment was done in triplicate.

2.2. Analyses

Optical density at the wavelength of 600 nm (OD_{600}) was determined in the end of incubation or regularly during the incubation (vitamin B_{12} optimization, the effect of acetate and 1,3-PD tolerance studies) using an Ultrospec 500 pro spectrophotometer (Amersham Biosciences, Munich, Germany).

The gaseous end products, including H_2 and carbon dioxide (CO_2), were analyzed from the headspace at the end of incubation using a GC-2014 gas chromatograph equipped with Porapak N column (80/100 mesh) and a thermal conductivity detector (Shimadzu, Kyoto, Japan). The temperatures of column and detector oven were 80 °C and 110 °C, respectively. Nitrogen was used as a carrier gas. The gas samples were analyzed in duplicate.

The liquid end products were analyzed using high performance liquid chromatography (HPLC) from samples taken at the end of incubation. Samples of bacterial cultures were centrifuged at 11,000 × *g* for 5 min, the supernatant was diluted to obtain concentrations under 10 mM for each compound analyzed, and finally filtered through a polycarbonate filter (Chromafil PET-45/25, Macherey-Nagel, Düren, Germany). The samples were analyzed for lactate, glycerol, formate, acetate, 1,3-PD, propionate, ethanol, and butyrate at a column temperature of 40 °C with an LC-20AC prominence liquid chromatograph equipped with a Thermasphere TS-130 HPLC column temperature controller (Phenomenex, Torrance, CA, USA), an RID-10A refractive index detector, DGU-20A5 prominence degasser, CBM-20A prominence communications bus module, and SIL-20AC 200 prominence autosampler (Shimadzu, Kyoto, Japan). The column was a 30-cm Rezex RHM Monosaccharide H^+ (8%) (Phenomenex, Torrance, CA, USA). As an eluent, 0.01 N H_2SO_4 was used at a flow rate of 0.600 ml/min.

2.3. Calculations

The metabolites were identified based on retention times and the concentrations (mM) and yields (mol product/mol substrate consumed) were calculated as an average of duplicate or triplicate experiments with standard deviations. The concentrations of liquid end-products are reported as concentrations in the liquid phase and the concentrations of gaseous end-products as the amount of gas in gaseous phase divided by the volume of liquid phase. The background metabolite production (except for background CO_2 production, which was at unidentified level) in glycerol control cultivations, i.e. cultivations without the substrate, was subtracted from the results, unless otherwise mentioned. Carbon recoveries were calculated by dividing the amount of carbon or electrons bound to the end-products with the carbon or electrons gained from the degraded glycerol. The carbon and electron recoveries exclude the carbon and electrons bound to biomass due to difficulties in biomass determination caused by low biomass concentration, precipitation of salts from the medium and challenges in halophilic biomass washing. Inhibition of growth (%) was calculated for the 1,3-PD tolerance results using the optical density (OD) data and the following formula:

$$\frac{OD_{c(t_2)} - OD_{c(t_1)} - (OD_{E_i(t_2)} - OD_{E_i(t_1)})}{OD_{c(t_2)} - OD_{c(t_1)}} \times 100$$

where c is the control, in which initial 1,3-PD concentration 0 g/l, E_i = experiment (i = 1, 2, . . ., x), t_1 = time point 1, and t_2 = time point 2. The inhibition was calculated between time points 7 h (t_1) and 17 h (t_2).

Table 1
1,3-PD yields and carbon balance calculations in the temperature gradient experiment.

Temperature (°C)	1,3-PD yield (mol/mol glycerol)	Carbon recovery[a] (%)
30	0.41 ± 0.04	83.7
31	0.37 ± 0.01	86.0
32	0.35 ± 0.01	85.1
33	0.31 ± 0.01	81.1
34	0.35 ± 0.03	82.1
35	0.36 ± 0.03	85.8
36	0.32 ± 0.01	83.6
37	0.31 ± 0.01	85.0
38	0.32 ± 0.02	82.3
40	0.34 ± 0.01	87.6

[a] Carbon balance calculations exclude biomass production.

3. Results

3.1. Production of 1,3-PD

3.1.1. The effect of temperature on 1,3-PD production

Optical density was measured every 24th hour and the results are shown in Fig. 1A. At the beginning of the experiment, the growth rate increased together with temperature. However, the faster the culture grew in the beginning of incubation, the faster the culture started to flocculate, which was seen as a decrease in optical density.

The concentrations of 1,3-PD, H_2, and glycerol in function of incubation temperature are shown in Fig. 1B, and the 1,3-PD yields with carbon balance calculations are shown in Table 1. The highest 1,3-PD concentration and yield were obtained at the temperature of 30 °C. However, the results (Fig. 1 and Table 1) show that there is no significant difference in 1,3-PD production at different incubation temperatures. H_2 production was its highest at growth temperatures 36–37 °C (Fig. 1). Carbon recovery (excluding the carbon bound to biomass) in the experiment was on average 84% (standard deviation 2%).