J. Microbiol. Biotechnol. (2015), 25(6), 893–902
http://dx.doi.org/10.4014/jmb.1411.11078

jmb

Improved 1,3-Propanediol Synthesis from Glycerol by the Robust *Lactobacillus reuteri* Strain DSM 20016 S

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Received: November 28, 2014
Revised: January 15, 2015
Accepted: January 15, 2015

First published online
January 15, 2015

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Various *Lactobacillus reuteri* strains were screened for the ability to convert glycerol to 1,3-propanediol (1,3-PDO) in a glycerol-glucose co-fermentation. Only *L. reuteri* DSM 20016, a well-known probiotic, was able to efficiently carry out this bioconversion. Several process strategies were employed to improve this process. Co^{2+} addition to the fermentation medium, led to a high product titer (46 g/l) of 1,3-PDO and to improved biomass synthesis. *L. reuteri* DSM 20016 produced also ca. 3 μg/g of cell dry weight of vitamin B_{12}, conferring an economic value to the biomass produced in the process. Incidentally, we found that *L. reuteri* displays the highest resistance to Co^{2+} ions ever reported for a microorganism. Two waste materials (crude glycerol from biodiesel industry and spruce hydrolysate from paper industry) alone or in combination were used as feedstocks for the production of 1,3-PDO by *L. reuteri* DSM 20016. Crude glycerol was efficiently converted into 1,3-PDO although with a lower titer than pure glycerol (33.3 vs. 40.7 g/l). Compared with the fermentation carried out with pure substrates, the 1,3-PDO produced was significantly lower (40.7 vs. 24.2 g/l) using cellulosic hydrolysate and crude glycerol, but strong increases of the maximal biomass produced (2.9 vs 4.3 g/l CDW) and of the glucose consumption rate were found. The results of this study lay the foundation for further investigations to exploit the biotechnological potential of *L. reuteri* DSM 20016 to produce 1,3-PDO and vitamin B_{12} using industry byproducts.

Keywords: 1,3-Propanediol, *L. reuteri*, cobalt, vitamin B_{12}, glycerol

Introduction

1,3-Propanediol (1,3-PDO) is the most widely studied value-added product that can be produced from glycerol. 1,3-PDO is employed in a number of applications, particularly in the plastics industry as a monomer for the synthesis of polyesters, polyethers, and polyurethanes [22]. It is increasingly used for the synthesis of poly(trimethylene terephthalate) (PTT), a new bio-derived polyester, used for the manufacturing of a variety of different products, such as polymers, cosmetics, food, lubricants, medicines, clothing, fibers, and carpeting [7]. Conventionally, 1,3-PDO is produced from petroleum-derived acrolein or from ethylene oxide. Both processes are environmentally unfriendly and produce toxic intermediates [22]. Considering the specificity, the yield, the mild conditions employed, and the availability of cheap substrates, much attention has been placed on the microbial synthesis of 1,3-PDO.

Bacteria that have been studied with regard to 1,3-PDO production include *Clostridium*, *Klebsiella*, *Citrobacter*, *Escherichia*, and *Lactobacillus* [7, 22]. Engineered *E. coli* using glucose as substrate showed the highest production of 1,3-

PDO (135 g/l) [16]. Otherwise, the best natural (non-GMO) producers of 1,3-PDO from glycerol are *Klebsiella pneumoniae* DSM 2026 [15] and *Clostridium butyricum* CNCM 1211 [10], reaching titers of 60–85 g/l. These bacteria are respectively an opportunistic pathogen and a strict anaerobe.

Most lactobacilli are generally regarded as safe (GRAS), and are not subject to special legislative restrictions. Even though they are unable to grow on glycerol as the sole carbon source, when grown on glucose, they use glycerol as the electron acceptor to regenerate NAD^+ for catabolic demands, leading to 1,3-PDO production. Recently *Lactobacillus diolivorans* DSM 14421 was proposed as an alternative host for glycerol bioconversion, with titers to up to 85 g/l [18]. It was also shown that *Lactobacillus reuteri*, a well-known probiotic bacterium [5], converts glycerol to 1,3-PDO in a two-step anaerobic process. In the first step, cobalamin-dependent glycerol dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA). Notably, *L. reuteri* is able to produce *de novo* vitamin B_{12} required in this reaction [24]. In the second step, 3-HPA is reduced to 1,3-PDO by a NADH-dependent oxidoreductase. The highest 1,3-PDO production titer reported for *L. reuteri* in a glucose-glycerol batch co-fermentation is 37.4 g/l for the strain ATCC 55730 [11].

In order to establish a profitable industrial process, cost efficient production with low-cost substrates is required. Agricultural and industrial byproducts could be used for the production of 1,3-PDO. Glycerol (or glycerin) is a byproduct of biodiesel, oleochemical, and bioethanol production processes. Crude glycerol is abundant and inexpensive, and is also a highly reduced molecule, which offers the opportunity to produce fuels and reduced chemicals at yields higher than those obtained using common sugars [2].

For the above-mentioned reasons, in this study we investigated the production of 1,3-PDO by *L. reuteri* DSM 20016 (a vitamin-B_{12}-producing probiotic) also using crude glycerol and cellulosic hydrolysate as feedstocks.

Materials and Methods

Microorganisms and Media

L. reuteri DSM 17509, DSM 20015, DSM 20016, DSM 20053, and DSM 20056 were obtained from the DSMZ culture collection (Germany). Cells were maintained at −80°C in culture broth supplemented with 25% (w/v) glycerol. Cultures were carried out in classical MRS medium [4] or, where indicated, in a modified MRS medium: 10 g/l peptone, 8 g/l meat extract, 4 g/l yeast extract, 1 g/l Tween 80, 2 g/l K_2HPO_4, 2 g/l NH_4-citrate, 0.2 g/l $MgSO_4 \cdot 7H_2O$, and 0.05 g/l $MnSO_4 \cdot 2H_2O$, supplemented with the indicated concentrations of D-glucose and glycerol. Pre-cultures, cultures for screening, and growth experiments in the presence of heavy metals were carried out using 50 or 20 ml tubes, which were incubated anaerobically in jars using an atmosphere generation system (Oxoid AnaeroGen, Basingstoke, England) at 37°C for 24 h.

Growth, Consumption of Carbohydrates, and Synthesis of Metabolites

Cell density was determined by plating onto MRS agar at 37°C for 48 h. Cell density was also monitored using either optical density at 620 nm (OD_{620}) with a Genesys 20 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), or cell dry weight (CDW). For CDW determination, a known volume of fermentation broth was centrifuged for 10 min at 4°C and 3,000 ×*g* in pre-weighed tubes, washed once with water, and dried into a microwave oven at 600 W until stable weight was reached. CDW was routinely determined using a standard curve relating OD_{620} values to CDW; the correlation factor was CDW (g/l) = 0.43 × OD_{620}.

Concentrations of glucose, acetic and lactic acids, glycerol, 1,3-PDO, and ethanol were determined by HPLC analysis using a Waters Alliance 2695 separation module (Waters, Milford, MA, USA) equipped with a Rezex ROA-Organic Acid H+ (8%) 300 mm × 7.8 mm column (Phenomenex Inc., USA), coupled to a Waters 2410 refractive index detector and a Waters 2996 UV detector. Separation was carried out at 65°C with 0.005 M H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min.

Determination of IC_{50} for Substrates and Metabolites

A 96-multiwell plate-based assay was used to assess the effect of substrates (glucose and glycerol, pure and crude) and products (1,3-PDO, lactic and acetic acids, and ethanol) of fermentation on biomass formation. IC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions. Bacteria from an overnight culture were washed with sterile NaCl 0.9% and inoculated at OD_{620} 0.1 in a MRS-based medium containing increasing concentrations of the molecule under analysis. Then 300 µl of the inoculated medium was added to each well and incubated at 37°C in a Tecan Sunrise 96-well Microplate Reader (Tecan, Männedorf, Switzerland). The plate was sealed with a non-breathable film to simulate microaerobic conditions. To measure bacterial growth, optical density was monitored at a wavelength of 600 nm every 16 min for 24 h. Each growth curve was made in triplicate. Standard deviation calculated on duplicate experiments did not exceed 20%. To calculate the IC_{50} for each compound, the maximal biomass reached after 24 h was plotted against the Log10 of the compound concentration.

Bioreactor Cultures

For bioreactor cultivations, a BIOSTAT plus bioreactor system (Sartorius, Göttingen, Germany) equipped with two parallel bioreactors was used, with a working volume of 1 L. Carbon

source, glycerol, and, if indicated, cobalt salts were added (after sterilization) to the modified MRS broth. Cultures were carried out in anaerobic condition, with N_2 gassing (6 l/h). The stirring speed was 200 ± 1 rpm (if not otherwise stated), the temperature set to 37 ± 0.1°C (if not otherwise stated), and the pH controlled at 5.5 with 8 M KOH or NH_4OH. pH and dissolved oxygen values were monitored with two sensors: Easyferm plus K8 (Hamilton, Bonaduz, Switzerland) and OXYFERM O_2 sensor (Hamilton), respectively. For all cultivations, the culture medium was inoculated to a starting OD_{620} of 0.1 from an overnight pre-culture.

Quantification of Vitamin B_{12}

After harvesting, cells were washed twice in 0.1 M phosphate buffer, pH 7.0, and resuspended in 5 ml of extraction buffer consisting of 0.1 M Na_2HPO_4, pH 4.5 (citric acid), and containing 0.1% KCN. The cell suspension was separated in five aliquots of 1 ml each, disrupted at 4°C with 0.3 g of glass beads (0.1 mm diameter) in a mixer mill MM 200 (Retsch, Haan, Germany) at a frequency of 28 Hz for 10 min, and again combined. Extraction buffer was then added up to a final volume of 10 ml and autoclaved (120°C for 15 min) [24]. The extract was clarified by centrifugation (8,000 ×*g* for 10 min) and passed through EASI-EXTRACT Vitamin B_{12} (R-Biopharm AG, Darmstadt, Germany), an immunoaffinity column containing monoclonal antibody with high affinity and specificity to vitamin B_{12}. Subsequently, the vitamin B_{12} immunoaffinity column was washed with 10 ml of water and the vitamin B_{12} was released from the column with 3 ml of methanol [13]. Following evaporation, the samples were reconstituted in mobile phase and analyzed by HPLC-UV at 360 nm on an Kinetex 5 µm C18 150 × 4.6 mm (Phenomenex Inc., USA) analytical column, using a linear gradient elution with a mobile phase consisting of 20 mM $Na_2H_2PO_4$, pH 3 (solvent A) and 100% acetonitrile (solvent B). The linear gradient profile (A:B) started at 95:5 and was decreased up to 75:25 during the first 10 min; then it was increased up to 95:5 in the next 10 min and finally was kept constant for the last 10 min of separation. The total run time was 30 min. The error range (reported as standard error of three independent measurements) was lower than 20%.

Effects of Metals and Raw Substrates

To study the effect of metals on biomass and 1,3-PDO production, 5.6 mM of $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}H_2O$, NaCl, $CoCl_2{\cdot}6H_2O$, $Co(CH_3CO_2)_2$, or increasing concentrations (11.2, 28, and 56 mM) of $Co(CH_3CO_2)_2$ were added to the broth. For the batch bioreactor cultivation, modified MRS was supplemented with D-glucose (or cellulosic hydrolysate) and pharma-grade glycerol (or crude glycerol) at the indicated concentrations. Crude glycerol was supplied by Novance (Paris, France). Spruce lignocellulosic hydrolysate was supplied by Borregaard (Sarpborg, Norway).

Statistical Analysis

Data (three replicates) were subjected to one-way analysis of variance, and paired comparison of treatment means was achieved by Tukey's procedure at a *P* value of <0.05 for significance, using the statistical software GraphPad Prism ver. 5.0 for Windows.

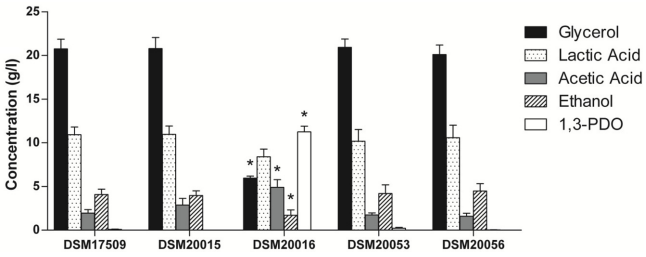

Fig. 1. Concentration of glycerol, lactic acid, acetic acid, ethanol, and 1,3-propanediol (1,3-PDO) in the cultures of five different *Lactobacillus reuteri* strains in MRS supplemented with glycerol (20 g/l), 24 h after the inoculum.

All data are the means ± SD of three independent experiments. *Indicates significant ($p < 0.05$) differences in the concentration of fermentation substrate and products, according to Tukey's test.

Results

Screening of *L. reuteri* Strains for 1,3-PDO Production

The five *L. reuteri* strains available in the DSMZ collection were screened for 1,3-PDO production on MRS (containing 20 g/l glucose) supplemented with 20 g/l glycerol (Fig. 1). Only the type strain DSM 20016 produced a considerable amount of 1,3-PDO (ca. 10 g/l) after 24 h. Glucose-glycerol anaerobic co-fermentation by *L. reuteri* yields mainly lactic and acetic acid, ethanol, CO_2, and 1,3-PDO [8]. In *L. reuteri* DSM 20016, lactic acid and ethanol produced were lower, and acetate was higher than in the non-glycerol-using strains (Fig. 1). Lactate and ethanol formation competes with glycerol-generated 3-HPA for NADH. Glycerol metabolism represents an alternative way to regenerate NAD^+ from NADH and hence facilitates glycolysis in anaerobic conditions. Consequently, if cells are able to convert glycerol into 1,3-PDO, a lower amount of reduced fermentation products (lactate and ethanol) are formed, and a higher amount of oxidized products (acetate) can be obtained increasing ATP yield. Based on the above results, *L. reuteri* DSM 20016 was selected for the further experiments.

Toxicity of Substrates and Products of Fermentation on *L. reuteri*

Since the production of 1,3-PDO is directly related to biomass formation, as reported by Tobajas *et al*. [25], the inhibitory effect on the growth of *L. reuteri* DSM 20016 of fermentation substrates (glucose and glycerol) and products

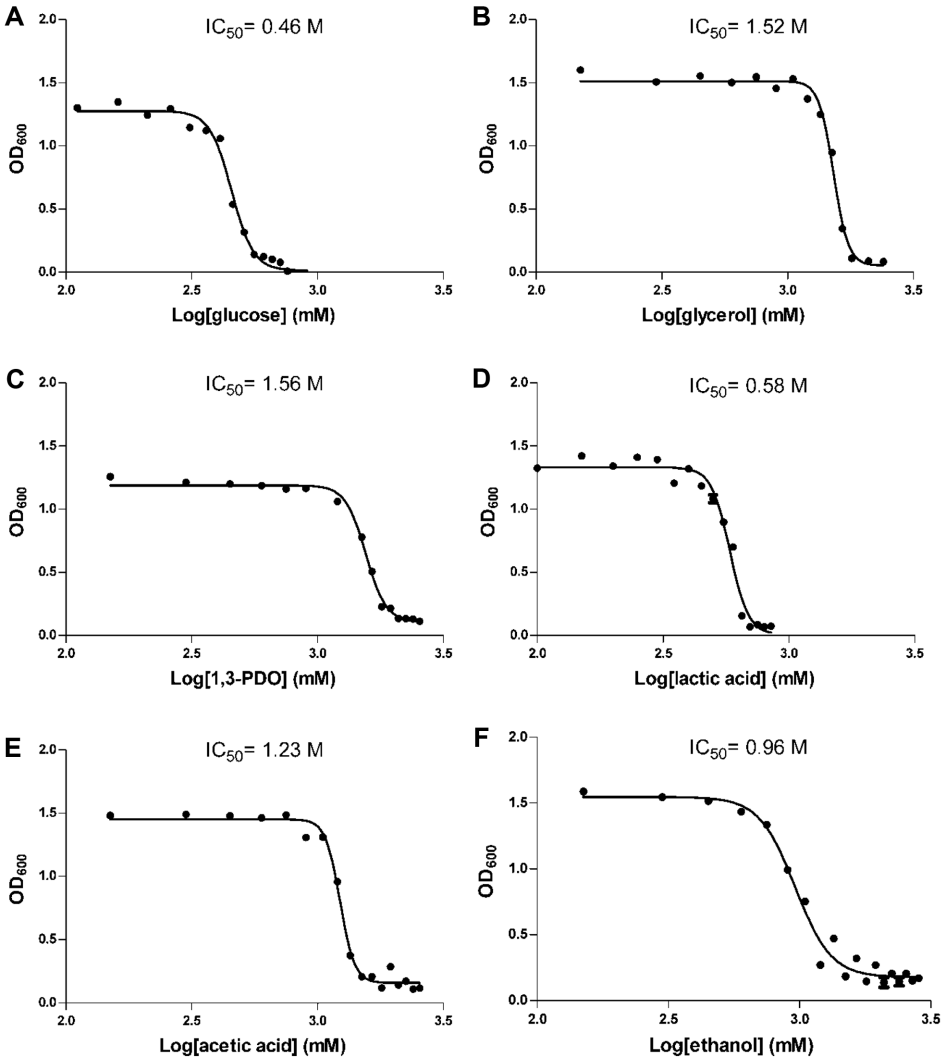

Fig. 2. Inhibition of *Lactobacillus reuteri* DMS 20016 by fermentation substrates and products: (**A**) glucose, (**B**) glycerol, (**C**) 1,3-propanediol (1,3-PDO), (**D**) lactic acid, (**E**) acetic acid, and (**F**) ethanol.
The IC_{50} (the concentration at which a 50% decrease of the biomass accumulation was observed) value for each compound was reported.

(1,3-PDO, lactic and acetic acids, and ethanol) was investigated.

Glucose and glycerol, at high concentrations, inhibited the growth of *L. reuteri* (IC_{50} = 0.46 and 1.5 M, respectively) (Fig. 2). The presence of glycerol increased ($p < 0.05$) glucose tolerance and *vice versa*; this is consistent with the expected mutual synergistic increase of their catabolic rates. As a result, the utilization of glucose up to 0.4 M concentration allowed a concomitant increase of glycerol tolerance up to 2 M (Fig. 3).

The main product of fermentation, 1,3-PDO, displayed the same toxicity as glycerol (IC_{50} about 1.5 M). Amongst the other fermentation products, the highest toxicity was found for lactic acid (IC_{50} = 0.58 M) (Fig. 2).

Development of the Co-Fermentation Process

In order to increase 1,3-PDO production by *L. reuteri* DSM 20016, 1 L anaerobic batch bioreactor cultivation was set up. The effect of several parameters on bacterial conversion of glycerol to 1,3-PDO during batch fermentation was evaluated. Based on the estimated toxicity of fermentation substrates and products on *L. reuteri*, the glucose starting concentration was set at 0.4 M (72.1 g/l). Different glycerol concentrations were tested and the 1,3-PDO titer after 48 h was measured. The best result was obtained using 0.8 M (73.7 g/l) of glycerol, corresponding to a glucose/glycerol molar ratio of 0.5. The temperature, stirring, and pH were also varied; the best results were obtained using the following

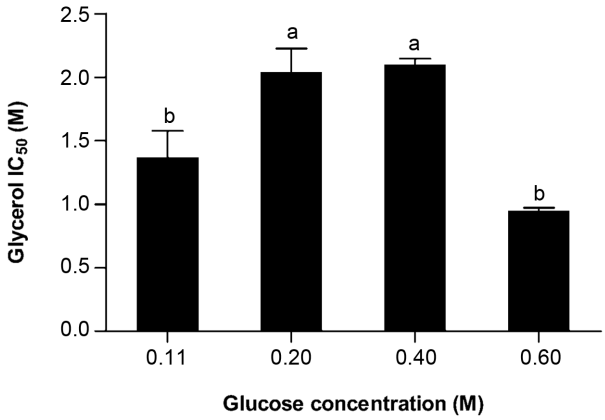

Fig. 3. Glucose and glycerol tolerance of *Lactobacillus reuteri* DSM 20016.

Glycerol IC_{50} (the concentration at which a 50% decrease of the biomass accumulation was observed) was determined in the presence of increasing glucose concentrations (0.11, 0.2, 0.4, and 0.6 M). Reported data represent mean values ± SD from three independent cultures. Columns with different letters indicate significant ($p < 0.05$) differences in the glycerol IC_{50} values, according to Tukey's test.

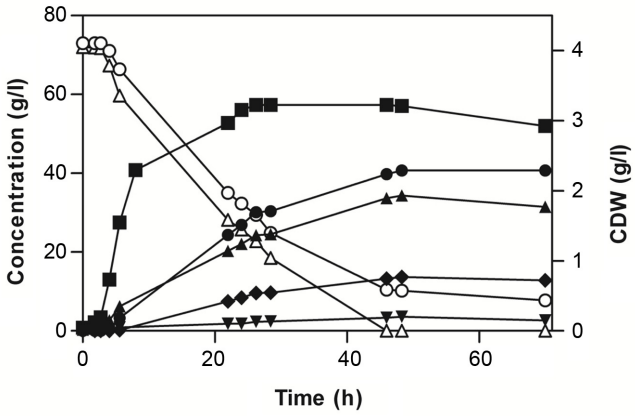

Fig. 4. Representative batch glucose-glycerol co-fermentation profile of *Lactobacillus reuteri* DSM 20016.

Cells were grown in modified MRS supplemented with 0.4 M (72.1 g/l) glucose and 0.8 M (73.3 g/l) glycerol. Cell dry weight (CDW) (■), glucose (△), glycerol (○), lactic acid (▲), acetic acid (◆), ethanol (▼) and 1,3-propanediol (1,3-PDO) (●) are reported. One representative experiment out of at least three independent fermentations under the same conditions is shown. For each data point, standard deviation was lower than 10%.

conditions: 37°C, 200 rpm, and pH 5.5. Interestingly, 1,3-PDO production did not change significantly between 37°C and 41°C; this is a desirable feature in the industrial setting since it may allow the use of higher process temperatures, which may be favorable in reducing the operational cooling cost. No differences ($p > 0.05$) in 1,3-PDO production were found using either 8 M KOH or 5 M NH_4OH for the control of pH (data not shown). The highest 1,3-PDO concentration reached in batch fermentation in a bioreactor using the optimal conditions (0.4 M glucose/ 0.8 M glycerol in modified MRS without sodium acetate, 37°C, 200 rpm, and pH 5.5) was 41 g/l (Tables 1 and 2, Fig. 4). This result represents an improvement of what has been obtained in batch fermentation by Baeza-Jiménez *et al*. [1] (28.7 g/l) for the same strain, and by Jolly *et al*. [11] (37.4 g/l) for a different strain (ATCC 55730) [11].

Lactate was the major byproduct of the co-fermentation process. Lactate is an interesting chemical building block and hence its formation could represent a valuable process. However, *L. reuteri* DSM 20016 produced a racemic mixture of D- and L-lactate. During the exponential growth phase, the L-isoform was prevailing with a ratio of 2–3 over the D-isoform. During the transition and stationary phases, the

Table 1. Biomass, substrates, and metabolites determined at the end of representative glucose-glycerol co-fermentations[a].

Glucose source	Glycerol source	$Co(Ac)_2$	Time (h)	CDW (g/l)	Glucose (g/l)	Glycerol (g/l)	1,3-PDO (g/l)	Lactate (g/l)	Acetate (g/l)	Ethanol (g/l)
Pure glucose 0.4 M	Pure glycerol 0.8 M	-	70	2.9	0.0	7.8	40.7	31.5	12.9	2.6
Pure glucose 0.4 M	Pure glycerol 0.8 M	+	70	3.0	0.0	14.6	46.0	39.2	16.2	3.7
Pure glucose 0.4 M	Crude glycerol 0.8 M	-	70	3.2	0.0	28.9	33.2	31.1	13.5	8.0
Pure glucose 0.4 M	Crude glycerol 0.8M	+	70	3.4	0.0	22.9	33.3	29.2	13.1	5.2
Lignocellulosic hydrolysate glucose 0.35 M	Pure glycerol 0.8 M	-	48	4.9	0.0	41.3	23.2	32.6	7.2	7.9
Lignocellulosic hydrolysate glucose 0.5 M	Crude glycerol 0.8 M	-	48	4.3	8.1	36.5	24.2	45.3	8.4	12.5

[a]All the experiments were carried out at 37°C on modified MRS, pH 5.5, supplemented with glucose and glycerol at the indicated concentrations. Where indicated, pure glycerol was replaced by crude glycerol, pure glucose was replaced by cellulosic hydrolysate, and 5.6 mM of $Co(Ac)_2$ was added. The actual concentrations of glucose and glycerol were determined for all cultivations and used for the calculations and showed a deviation of no more than 10%.

Table 2. Process parameters of representative glucose-glycerol co-fermentations[a].

Glucose source	Glycerol source	$Co(Ac)_2$	Time (h)	μ_{max}	$Q_{p\ max}$	$Q_{p\ average}$	$q_{p\ max}$	$q_{p\ average}$	$Y_{P/S}$	$Y_{P/gly}$
Pure glucose 0.4 M	Pure glycerol 0.8 M	-	70	0.72	1.15	0.59	0.36	0.20	0.49	0.77
Pure glucose 0.4 M	Pure glycerol 0.8 M	+	70	0.73	1.41	0.66	0.37	0.22	0.55	0.90
Pure glucose 0.4 M	Crude glycerol 0.8 M	-	70	0.79	1.06	0.47	0.29	0.16	0.51	0.90
Pure glucose 0.4 M	Crude glycerol 0.8 M	+	70	0.69	0.91	0.47	0.32	0.13	0.42	0.79
Lignocellulosic hydrolysate glucose 0.35 M	Pure glycerol 0.8 M	-	48	Nd	0.96	0.48	0.21	0.10	0.44	0.84
Lignocellulosic hydrolysate glucose 0.5 M	Crude glycerol 0.8 M	-	48	Nd	0.66	0.50	0.20	0.13	0.39	0.80

[a]All the experiments were carried out at 37ºC on modified MRS, pH 5.5, supplemented with glucose and glycerol at the indicated concentrations. Where indicated, pure glycerol was replaced by crude glycerol, pure glucose was replaced by cellulosic hydrolysate, and 5.6 mM of $Co(Ac)_2$ was added. The actual concentrations of glucose and glycerol were determined for all cultivations and used for the calculations and showed a deviation of no more than 10%. μ_{max} (h^{-1}): maximum specific growth rate; Q_p ($g_{1,3\text{-PDO}}/l\ h$): volumetric product formation rate; q_p ($g_{1,3\text{-PDO}}/g_{CDW}\ h$): specific product formation rate; $Y_{P/S}$ ($mol_{C\ 1,3\text{-PDO}}/mol_{C\ sub\ tot}$): molar product yield on total carbon source; and $Y_{P/gly}$ ($mol_{C\ 1,3\text{-PDO}}/mol_{C\ glycerol}$): molar product yield on glycerol. Nd, not detected.

lactate was an equal mixture of the two isoforms (data not shown).

Effect of Cobalt on the Production of 1,3-PDO and Vitamin B_{12} Cell Content

The first step of glycerol biotransformation to 1,3-PDO is catalyzed by glycerol dehydratase, a vitamin-B_{12}-dependent enzyme. Since vitamin B_{12}, also called cobalamin, contains a cobalt ion coordinated in the center of a planar corrin ring, and since *L. reuteri* DSM20016 is able to synthesize this vitamin, we hypothesized that an increase of cobalt concentration in the culture broth could improve cobalamin biosynthesis and thus in turn boost the glycerol degradation pathway. The addition of extra cobalt as trace element (at nano and micromolar concentrations) to the growth broth (MRS tube culture containing 0.16 M glucose and 0.32 M glycerol) did not induce significant changes in biomass and 1,3-PDO production rates during anaerobic glucose-glycerol co-fermentation (data not shown). This result was not unexpected, since MRS contains vitamin B_{12} and is consistent with what has been previously found by Santos *et al*. [19].

However, unexpectedly it was found that the use of higher amounts of cobalt (5.6–56 mM) significantly increased the biomass and 1,3-PDO production compared with the unsupplemented MRS (Figs. 5A and 5B, respectively), without toxic effects on the microorganism.

To find out if cobalt treatment increased its cellular content, vitamin B_{12} was extracted from *L. reuteri* cells in the early stationary phase and purified using an immunoaffinity column. Although the *L. reuteri* capability to produce vitamin B_{12} has been extensively studied, the content of this vitamin in the probiotic biomass has not been previously determined. *L. reuteri* DSM 20016 cells grown on MRS supplemented with glucose and glycerol had a vitamin B_{12} content of about 3 ± 0.6 μg/g CDW. The addition of either 5.6 mM cobalt acetate ($Co(Ac)_2$) or cobalt chloride ($CoCl_2$) did not determine a significant ($p > 0.05$) increase of the cellular content of vitamin B_{12}. However, volumetric cobalamin production increased owing to the higher cell density obtained. It is known that Co^{2+} shows toxic effects in a concentration ranging from micromolar to millimolar levels depending on the microorganism (Table S1). It was found that *L. reuteri* DSM 20016 displays an extremely high resistance to cobalt. Both for $Co(Ac)_2$ and $CoCl_2$, 56 mM was not sufficient to completely inhibit cellular growth after 48 h (Fig. 5A and Table S1). We could not test higher concentrations because of the solubility limits of the cobalt salts. To the best of our knowledge this is the highest resistance to Co^{2+} ever reported for a microorganism.

To evaluate if the stimulating effect was specific for cobalt, other salts were also assayed. Whereas 5.6 mM NaCl did not determine an increase of biomass and 1,3-PDO production, the same concentration of $MnSO_4$, $FeSO_4$, $CoCl_2$, and $Co(Ac)_2$ increased both biomass and 1,3-PDO production. A clear correlation ($R^2 = 0.86$) was found between biomass accumulation, induced by Mn, Fe and Co treatment, and 1,3-PDO production (Fig. 6). Although the addition of $Co(Ac)_2$ up to 28 mM improved 1,3-PDO production (Fig. 5B), it could not be added to fermenters at concentrations higher than 5.6 mM because of safety regulations. The addition of $Co(Ac)_2$ led to an increase of biomass after 24 h compared with control (about 4 g/l vs. 3.3 g/l) (Figs. 4 and 5C) and consequently of 1,3-PDO. As shown in Fig. 5C at longer time points, cellular density was slightly decreased

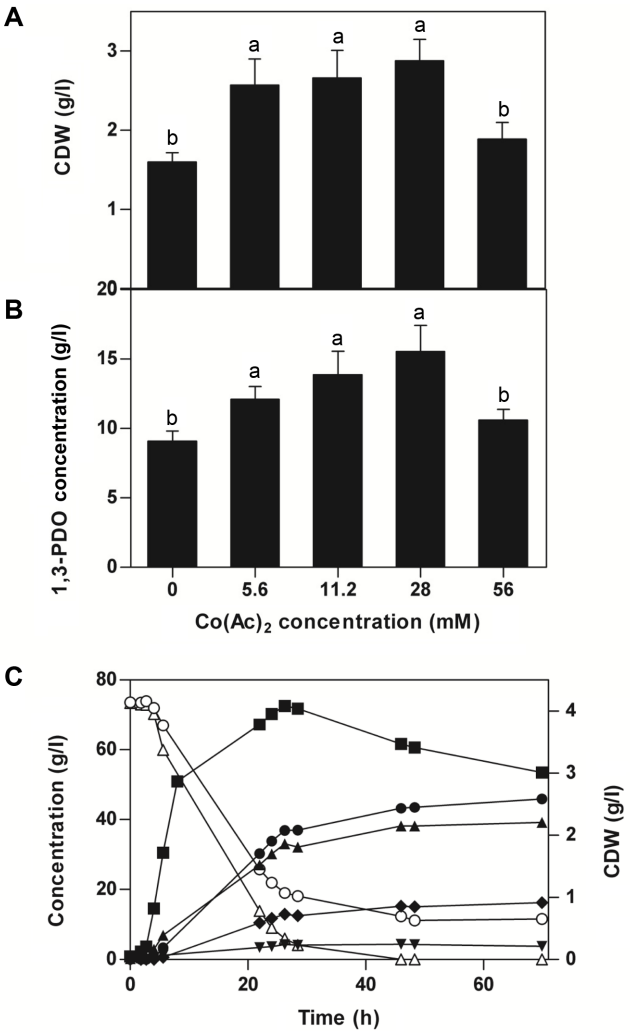

Fig. 5. Effect of cobalt acetate ($Co(Ac)_2$) supplementation on glucose-glycerol co-fermentation of *Lactobacillus reuteri* DSM 20016.

Effects on biomass (**A**) and 1,3-propanediol (1,3-PDO) production (**B**) of $Co(Ac)_2$ addition (5.6, 11.2, 28, and 56 mM) after 48 h of tube culture in MRS broth with 0.16 M glucose and 0.32 M glycerol. The data shown are representative of at least three independent experiments. Columns with different letters indicate significant ($p < 0.05$) differences in the production of biomass or 1,3-PDO production, according to Tukey's test. (**C**) Representative batch co-fermentation profile in modified MRS broth with 0.4 M (72.1 g/l) glucose and 0.8 M (73.3 g/l) glycerol in presence of 5.6 mM $Co(Ac)_2$. Cell dry weight (CDW) (■), glucose (△), glycerol (○), lactic acid (▲), acetic acid (◆), ethanol (▼), and 1,3-PDO (●) are reported. One representative experiment out of at least three independent fermentations under the same conditions is shown. For each datum point, the standard deviation was lower than 10%.

at a higher rate than in controls, most likely due to the lack of glucose and the accumulation of inhibitory fermentation

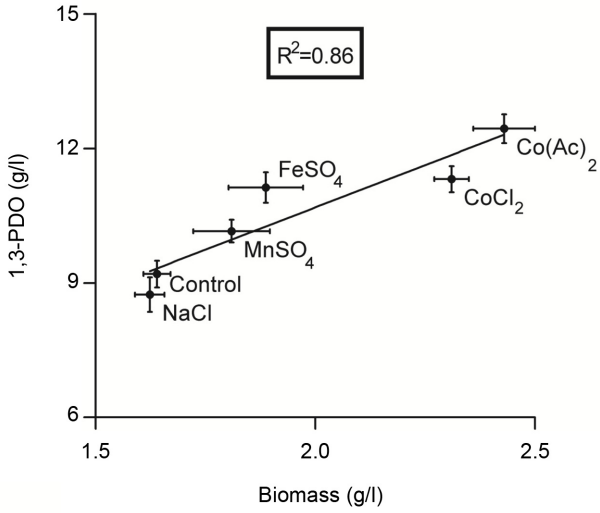

Fig. 6. Correlation of biomass as cell dry weight (CDW) and 1,3-propanediol (1,3-PDO) production. *Lactobacillus reuteri* DSM 20016 cells were grown in MRS broth with glucose 0.16 M, glycerol 0.32 M, and 5.6 mM of $FeSO_4$, $MgSO_4$, NaCl, $CoCl_2$, or cobalt acetate ($Co(Ac)_2$) for 24 h.

Values represent the mean ± SD from at least three independent experiments.

products such as lactic acid and acetic acid whose concentration was higher than the concentration reached in the absence of $CoAc_2$ (cf. Figs. 4 and 5C). Up to 46 g/l 1,3-PDO was obtained at the end of the fermentation (Fig. 5C, Tables 1 and 2) (+13% compared with the standard condition shown in Fig. 4), which is the highest 1,3-PDO concentration ever reported for a *L. reuteri* strain in batch cultures. The yield and productivity also increased as compared with the batch fermentation without $Co(Ac)_2$ (+12% and +11%, respectively).

Production of 1,3-PDO from Crude Glycerol and Cellulosic Hydrolysates

The effect of crude glycerol issued from the biodiesel industry was studied using a microplate absorbance reader. Crude glycerol supplied by Novance (Origin: Rouen 2 - glycerol content 84%) was not significantly ($p > 0.05$) toxic for *L. reuteri* (IC_{50} of 1.46 M vs. 1.52 M measured for pure glycerol). This crude glycerol stock was used for fermentation experiments in a bioreactor. *L. reuteri* DSM 20016 efficiently converted crude glycerol, with the major fermentation product being 1,3-PDO. The final product concentration, and total glycerol and glucose consumption are presented in Table 1. Fermentation performances were only partially affected by the use of crude glycerol. Both the 1,3-PDO titer

and productivity were only decreased by about 18% as compared with the fermentation carried out with pure glycerol and glucose (Tables 1 and 2).

A spruce cellulosic hydrolysate containing 460 g/l of glucose and 32 g/l of xylose, supplied by the Borregaard Biorefinery, was tested as glucose source. Compared with the fermentation carried out with pure glucose and glycerol, a strong increase of the maximal biomass produced (+50% after 24 h) and glucose consumption rate (+43%) was found using cellulosic hydrolysate as glucose source. No significant xylose consumption was detected (data not shown). This is not unexpected, since the genome of *L. reuteri* does not contain any gene displaying significant similarity to the *E. coli* xylose isomerase. The 1,3-PDO produced was however significantly lower (23.2 g/l; −43% compared with the fermentation with pure glucose). This might be explained by the presence in the hydrolysate of hydroxymethyl furfural (about 1 g/l), which was detoxified in the first 6 h of the fermentation process, and most likely of other electron acceptors not detected. This probably lowers the concentration of NADH available for 1,3-PDO production. Importantly, the use of crude glycerol and cellulosic hydrolysates did not determine a significant ($p > 0.05$) variation of the cellular content of vitamin B_{12} (data not shown).

In a fermentation with both crude glycerol and the cellulosic hydrolysate, a lower 1,3-PDO production was obtained (24.2 g/l) compared with the process using pure substrates. The presence of crude glycerol did not affect the increase of the biomass production and of the glucose uptake rate determined by the use of the cellulosic hydrolysate (+27% and +46%, respectively, after 48 h). It is noteworthy that cobalt addition to the fermentations employing raw substrates did not improve the results in terms of 1,3-PDO production (data not shown).

Discussion

Many microorganisms employed in the biotechnological production of 1,3-PDO are obligate anaerobes or facultative pathogens. These constraints have boosted research for finding alternative 1,3-PDO producers. Among them, *L. reuteri* has recently attracted much attention [6, 11]. This microorganism converts glycerol to 1,3-PDO, with glucose as the reducing equivalent source. In our paper, we have found that this feature is strain-specific; among the tested strains, only *L. reuteri* DSM 20016 was able to efficiently convert glycerol to 1,3-PDO. This might be explained by the presence of the propanediol-utilization (*pdu*) operon only in some of the strains of *L. reuteri*. The presence of this metabolic activity in only some strains of *L. reuteri* can be explained by the study of Morita *et al*. [14], who demonstrated that *L. reuteri* JCM 1112T (a strain related to DSM 20016) has a unique cluster of 58 genes for the biosynthesis of 3-hydroxypropionaldehyde (the precursor of 1,3-PDO) and vitamin B_{12}. The genomic features of this gene cluster suggest that it represents a genomic island acquired from an external source. This horizontal gene transfer might have taken place only in some strains of *L. reuteri*. This hypothesis awaits to be tested by sequencing and comparing the genomes of other *L. reuteri* strains.

Through the optimization of substrate concentrations and process variables, we have obtained the highest 1,3-PDO titer ever reported for a batch fermentation for this microorganism (41 g/l). A specific feature of *L. reuteri* DSM 20016 is that 1,3-PDO production is closely linked to biomass accumulation, which is different from the 1,3-PDO synthesis catalyzed by *L. diolivorans*, which takes place also in the stationary phase of growth [17]. This characteristic, probably due to the synergistic inhibitory effect of fermentation products formed during growth, hampers the utilization of a classical fed-batch format in which glucose and glycerol are supplied over time. In fact, we tested different fed-batch fermentation setups (data not shown) but the 1,3-PDO production was not significantly different from that obtained in the batch process. Previously, a higher titer (60 g/l in 140 h) was reported using *L. reuteri* ATCC 55730 in a repeated fed-batch process. However, in this fermentation, a portion of the culture medium was replaced with fresh medium containing glucose and glycerol at regular time intervals [11]; this may hamper the utilization of this process at an industrial level.

The fermentation profile obtained shows that lactic acid was the major byproduct, in a concentration similar to that of 1,3-PDO. Optically pure lactic acid represents an important platform chemical [21]; however, *L. reuteri* DSM 20016 yields a racemic mixture of L- and D-lactic acid. A BLAST search revealed this microorganism has at least three D- and two L-lactate dehydrogenases. This makes the construction of a lactate dehydrogenase null strain or an optically pure lactic acid producer a challenging task.

The synthesis of 1,3-PDO in lactic acid bacteria requires vitamin B_{12}, a cobalt-containing coenzyme. Differently from several other lactic acid bacteria that have auxotrophic requirement for cobalamin, it was demonstrated that *L. reuteri* is able to produce *de novo* vitamin B_{12} [20, 23]. In *L. reuteri* the vitamin B_{12} biosynthetic pathway is neighbored by a cluster of genes encoding the 1,3-PDO operon, cobalt transporters, and the genes encoding the proteins necessary

for the assembly of the metabolosome (microcompartment specialized in glycerol degradation) [23]. In contrast to our starting hypothesis, the addition of cobalt did not directly improve the vitamin B_{12} content of the cells, but it enhanced the biomass formation and consequently the 1,3-PDO production up to 46 g/l. Surprisingly, *L. reuteri* showed an extremely high resistance to cobalt. Other heavy metals were tested, showing a lower but similar effect. Interestingly, cobalt and the other heavy metals tested can be found in the wastewater of many industries, such as mining, metallurgical, and pigments industries. Intriguingly, it has been reported that cobalt greatly stimulates the growth of methanogens and acetogens in anaerobic cultures [9], but this is the first time for the lactic bacteria; this stimulating effect on bacterial growth could represent a possible application before the heavy-metal removal treatments. Moreover, the findings reported in our study open the way for new investigations on the molecular basis of resistance to heavy metals as well as the biochemical mechanisms inducing the increase of biomass synthesis.

The use of a probiotic (vitamin B_{12} producing) catalyst in the conversion of glycerol into 1,3-PDO could improve the process profitability, since the biomass obtained could be used as a probiotic supplement for animal feed or as dietary sources of vitamin B_{12}. The biomass content of this vitamin was quantified (3 μg/g CDW), being lower than that produced by an industrial strain of *Pseudomanas denitrificans* (about 2 mg/g CDW) [3] but about 5 times higher than the titer obtained by a recently created recombinant *E. coli* (0.65 μg/g CDW) [12].

The unexpected high resistance of *L. reuteri* was confirmed also for the raw substrates used as glucose and glycerol source. Whereas the use of crude glycerol caused only a minor drop in 1,3-PDO synthesis, the use of lignocellulosic hydrolysates reduced both the yield and titer, but improved the biomass formation. The decreased 1,3-PDO production can be ascribed to a drain of NADH for the reduction of hydroxymethyl furfural and other electron acceptors such as phenolic compounds present in the cellulosic hydrolysate. It has been found that lactobacilli can reduce external organic electron acceptors, including fructose and HMF [26]. This allows them to get rid of the surplus NADH produced in the glycolitic pathway, in turn increasing biomass synthesis. Based on these findings, cellulosic hydrolysates might be industrially employed as a carbon source in a process oriented to the production of probiotic biomass.

In conclusion, a glycerol-glucose co-fermentation process for the production of 1,3-PDO has been set up using the probiotic cobalamin-producing strain *L. reuteri* DSM 20016. High product titer (46 g/l) and yield (0.9 $mol_{1,3\text{-}PDO}/mol_{glycerol}$) were obtained through process optimization and addition of divalent metal ions, most likely due to improved biomass synthesis. Two waste materials (crude glycerol from biodiesel industry and spruce hydrolysate from paper industry) could be exploited for either product- (1,3-PDO) or biomass-oriented processes. Furthermore, *L. reuteri* DSM 20016 proved to be extremely resistant to toxic heavy metals that can be found in wastewaters originating from some industrial processes. Although we focused on 1,3-PDO production, interesting information was also obtained about the production of vitamin B_{12} and cell biomass. Further pre-industrial studies will be essential to improve such processes.

Acknowledgments

The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 241718 EuroBioRef. This work was partially supported by the "Reti di Laboratori - Produzione Integrata di Energia da Fonti Rinnovabili nel Sistema Agroindustriale Regionale" program funded by the Apulia Region.

The authors thank Borregaard Biorefineries and Novance for providing cellulosic hydrolysates and crude glycerol, respectively. The authors thankfully acknowledge Dr. Maria Gabriella Bitetto and Maria Giovanna de Palma for their technical support. The authors declare no competing financial interest.

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