Bioresource Technology 119 (2012) 133–140

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*

Stefan Pflügl [a,b], Hans Marx [b,*], Diethard Mattanovich [b,c], Michael Sauer [a,b,c]

[a] *School of Bioengineering, FH Campus Wien – University of Applied Sciences, Muthgasse 62, 1190 Vienna, Austria*
[b] *Department of Biotechnology, BOKU – VIBT University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria*
[c] *Austrian Centre of Industrial Biotechnology (ACIB GmbH), Muthgasse 11, 1190 Vienna, Austria*

HIGHLIGHTS

- *Lactobacillus diolivorans* efficiently converts glycerol to 1,3-propanediol.
- Anaerobic fed-batch processes lead to 1,3-propanediol concentrations up to 85 g/l.
- 0.1 mol glucose/mol glycerol is optimal for production in a fed-batch process.
- Vitamin B_{12} addition enhances production of 1,3-propanediol significantly.

ARTICLE INFO

Article history:
Received 26 March 2012
Received in revised form 22 May 2012
Accepted 23 May 2012
Available online 30 May 2012

Keywords:
1,3-Propanediol
Microbial conversion of glycerol
Glycerol dehydratase
Vitamin B_{12}
Biochemical production

ABSTRACT

The aim of this study was to evaluate the natural producer *Lactobacillus diolivorans* as potential production organism of 1,3-propanediol from glycerol. Different cultivation parameters, such as oxygen supply, feeding-strategy, or medium composition have been tested in batch and fed-batch cultivations. The 1,3-propanediol concentration obtained in batch cultivations was 41.7 g/l. This could be increased to 73.7 g/l in a fed-batch co-feeding glucose and glycerol with a molar ratio of 0.1. Yeast extract as part of the MRS cultivation medium could be replaced by nicotinic acid and riboflavin. Furthermore, the addition of Vitamin B_{12} to the culture medium increased production by 15% to a final titer of 84.5 g/l 1,3-propanediol.
© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

1,3-Propanediol has long been considered a specialty chemical due to the high costs connected to its production and the narrow application spectrum. About a decade ago, the application as a monomer in a polymerization process producing polytrimethylene terephthalate (PTT) led to an increased demand for 1,3-propanediol. 1,3-Propanediol can replace classic glycols for the production of polyurethanes, polyesters and polyethers (Kaur et al., 2012; Johnson and Taconi, 2007).

The increased requirement for 1,3-propanediol has also enhanced efforts to produce the substance biotechnologically. Currently, 1,3-propanediol is mainly produced with recombinant *Escherichia coli* strains, using glucose as the sole carbon source, which is usually derived from corn (Nakamura and Whited, 2003). However, the natural carbon source for the production of 1,3-propanediol is glycerol, and the ability of microorganisms to convert glycerol into 1,3-propanediol has first been described over a century ago (Freund, 1881).

Much of the glycerol available today originates as the by-product of oleochemical processes such as biodiesel, soap, fatty acid and fatty alcohol production. For 100 kg biodiesel produced 10–12 kg of crude glycerol are obtained (Sheedlo, 2008; Vasudevan and Briggs, 2008). Since biodiesel production has been increased dramatically over the past few years, also the availability of glycerol increased. Basically, the majority of the market for glycerol requires a value adding and cleaning step in order to establish an economically sound biorefinery process (Posada et al., 2012). However, impurities in crude glycerol may affect product formation and biomass formation during fermentation (Johnson and Taconi, 2007; Jensen et al., 2012).

Quite a large number of microorganisms are capable of converting glycerol into 1,3-propanediol (Sauer et al., 2008; Tokumoto and Tanaka, 2012). For example, the ability of *Klebsiella pneumoniae* to produce 1,3-propanediol has been studied quite extensively (Oh et al., 2011; Cheng et al., 2007; Huang et al., 2012). Mutant strains of this organism obtain titers up to 103 g/l, whereas with wildtype strains in a larger scale typically concentrations of only

* Corresponding author. Tel.: +43 1 3189900401.
E-mail address: hans.marx@boku.ac.at (H. Marx).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.05.121

59 g/l with a volumetric productivity of 0.9 g/l h are reached. However, some reservations against the use of *K. pneumoniae* in an industrial production process arise from the pathogenicity of this organism.

Among non-pathogenic microorganisms there are a number of *Clostridium* strains known to produce 1,3-propanediol. *Clostridium butyricum* has been reported to produce 1,3-propanediol with a titer of 94 g/l when grown on glycerol as the sole carbon source (Wilkens et al., 2012). A process carried out in a 2 m^3 reactor shows a productivity and concentration of up to 2.9 g/l h and 58 g/l, respectively (Günzel et al., 1991). A recombinant strain of *Clostridium acetobutylicum* produces up to 84 g/l in fed-batch cultivation (González-Pajuelo et al., 2005).

Members of the genus *Lactobacillus*, which are harmless, non-pathogenic organisms with a broad field of applications in the food industry, have also been reported to produce 1,3-propanediol. For example, *Lactobacillus reuteri* produces small amounts of 1,3-propanediol (Talarico et al., 1990). However, the work with *L. reuteri* is mainly focused on the production of the antimicrobial substance reuterin, a combination of different forms of 3-hydroxypropionaldehyde (3-HPA), the intermediate in glycerol metabolism towards 1,3-propanediol formation (Vollenweider and Lacroix, 2004; Doleyres et al., 2005). *Lactobacillus diolivorans* has first been described as a novel species by Krooneman et al. (2002), who categorized a strain isolated from silage taxonomically by 16s rRNA analysis. Garai-Ibabe et al. (2008), isolating spoilage organisms from alcoholic beverages, reported strains of *L. diolivorans* to be producers of 1,3-propanediol.

The aim of this study was to characterize the capability of *L. diolivorans* to convert glycerol into 1,3-propanediol. *L. diolivorans* was evaluated regarding the medium composition, the cultivation conditions and the cultivation mode in order to maximize production capacity. Process optimization successfully increased the production capacity of the wildtype *L. diolivorans* to industrially relevant levels. Therefore, we suggest *L. diolivorans* as a superior production host for 1,3-propanediol.

2. Methods

2.1. Microorganism and medium

L. diolivorans DSM 14421 (LMG 19667) was used for all experiments in this study. Cells were maintained at −80 °C in the culture broth supplemented with 10% (w/v) glycerol.

MRS medium as developed by De Man et al. (1960) was used for all cultivations in this study. The composition of MRS was slightly modified (per liter deionized water): 10 g of casein peptone (pancreatic digest), 10 g meat extract, 5 g yeast extract, 33 g glucose$\times H_2O$, 5 g Na-acetate, 1 g Tween 80, 2 g K_2HPO_4, 2.6 g Na_3-citrate$\times 2H_2O$, 1.17 g $(NH_4)_2HPO_4$, 0.2 g $MgSO_4 \times 7H_2O$ and 0.05 g $MnSO_4 \times H_2O$.

During fed-batch cultivations a glucose/glycerol solution with molar ratios of 0.05, 0.1, 0.2 and 0.3 were used as feed medium. The concentration of glycerol in the feed solution was 500 g/l and the concentration of glucose was 48.9, 97.8, 195.6 and 293.4 g/l for 0.05, 0.1, 0.2 and 0.3 mol glucose/mol glycerol, respectively. The actual concentrations of the feed solution were determined for all cultivations and used for the calculations and showed deviations of no more than 10%.

In case cultivations were supplemented with vitamins, Vitamin B_{12}, nicotinic acid or riboflavin were added to the batch and feed medium to final concentrations of 5, 10 and 10 mg/l, respectively.

The antifoam agent used was either 5% (w/v) Glanapon (DG 160, Bussetti & Co. GmbH, Austria) for the batch cultures or 100% (w/v) Struktol® SB 2121 (Schill + Seilacher, Hamburg, Germany) for the fed-batch cultures and was only added when necessary.

2.2. Batch and fed-batch cultures

For all cultivations the fedbatch-pro® bioreactor system (DASGIP AG, Jülich, Germany) with four parallel bioreactors with a working volume of 1200 ml each was used. The reactors were sterilized by autoclaving at 121 °C for 20 min, and the culture medium was added by sterile filtration into the reactor. Cultivations were carried out in anaerobic conditions with N_2 gassing (2.00 ± 0.06 l/h, MX4/4, DASGIP AG, Jülich, Germany) if not otherwise stated. The stirring speed was 400 ± 1 r.p.m. (SC4/RE30, DASGIP AG, Jülich, Germany), the temperature set to 30.0 ± 0.1 °C (TC4, DASGIP AG, Jülich, Germany) and the pH controlled at 5.7 with 8 M KOH. pH and redox reduction potential values were monitored with two sensors provided by the bioreactor system (pH: 405-DPAS-SC-K8S/225 and redox reduction potential: P14805-DPAS-K8S/225, Mettler-Toledo GmbH, Giessen, Germany).

For all cultivations, 700 ml culture medium was inoculated to an OD_{600} of 0.1 with 2% [v/v] inoculum from an exponentially growing preculture. For the fed-batch cultivations, the separately sterilized feed solution was added at a rate of 1.5 ml/h after glycerol was consumed from the batch medium.

2.3. Analytical procedures

Twelve milliliters of samples were taken at regular intervals throughout the whole cultivation duration. Biomass production was determined by appropriately diluting culture broth to an absorbance of 0.1–0.7 at 600 nm determined with a photometer (Ultrospec 1100pro, Amersham Biosciences, Austria), from which, together with the dilution factor, the optical density at 600 nm can be calculated. A correlation between the OD_{600} and the cell dry mass (CDM) was established for *L. diolivorans*. The correlation for the conversion of the OD_{600} into CDM in g/l is given in Eq. (1):

$$\text{CDM (g/l)} = 0.2307 \times OD_{600} + 0.1024 \quad (1)$$

The lowest CDM concentration detectable is 0.125 g/l given by Eq. (1) and the lowest detectable absorbance of 0.1.

The concentrations of glucose, glycerol, 1,3-propanediol, lactic acid, acetic acid and ethanol in the culture broth were determined by HPLC analysis (Shimadzu, Korneuburg, Austria) with an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Hercules, CA, USA) and a refraction index detector (RID-10A, Shimadzu, Korneuburg, Austria). The column was operated at 60 °C temperature, 0.6 ml/min flow rate and 0.004 M H_2SO_4 as mobile phase. HPLC samples were prepared by adding 100 µl of 0.04 M H_2SO_4 to 900 µl culture supernatant. Subsequently the samples were filtrated and 10 µl were injected for analysis. To ensure comparability the standard solutions injected for quantitation were treated in the same way. The detection limit for HPLC measurements is given by the lowest amount which was injected of a standard substance (50 mg/l). The produced CO_2 was quantified (±0.02% + 3% of measured value) by the off-gas sensor provided by the bioreactor system (DASGIP Off-Gas Analyzer GA4, DASGIP AG, Jülich, Germany). Together with the concentrations of the biomass and the produced metabolites carbon balances were set up. The carbon content of the biomass was assumed to be 45% as reported by Mercade et al. (2003) for a *Lactobacillus* strain. Within the margin of error, complete carbon recovery was observed for all cultivations.

2.4. Calculations

The values given for the specific product formation rate q_p for the fed-batch cultivations are average values of the whole feed phase and were calculated with Eq. (2):

$$q_p(f) = \frac{\sum_{f=t_s}^{e} x(f) = \frac{[PD_{t_{s+1}} - PD_{t_s}]}{\frac{[CDM_{t_s} + CDM_{t_{s+1}}]}{2}} + \ldots + \frac{[PD_{t_e} - PD_{t_{e-1}}]}{\frac{[CDM_{t_{e-1}} + CDM_{t_e}]}{2}}}{t_e - t_s} \quad (2)$$

where q_p(f) [$g_{1,3propanediol}/g_{CDM}$ h] is the average specific product formation rate during the feed phase, t_s [h] is the start time of the feed (when glycerol was completely consumed from the batch medium the feed was started and this time was defined as t_s), t_e [h] is the end time of the feed, PD [g/l] represents the 1,3-propanediol concentration for a given time point, CDM [g/l] represents the biomass concentration for a given time point and *x*(*f*) [$g_{1,3\text{-propanediol}}$/$g_{CDM}$] gives the sum of the concentration differences of 1,3-propanediol between two sampling points divided by the average biomass between these two sampling points.

The values given for the volumetric product formation rate Q_p for the fed-batch cultivations are average values of the whole feed phase and were calculated with Eq. (3):

$$Q_p(f) = \frac{\sum_{f=t_s}^{e} y(f) = [PD_{t_s} - PD_{t_{s+1}}] + \ldots + [PD_{t_{e-1}} - PD_{t_e}]}{t_e - t_s} \quad (3)$$

where Q_p(f) [$g_{1,3\text{-propanediol}}$/l h] is the average volumetric product formation rate during the feed phase, t_s [h] is the start time of the feed (when glycerol was completely consumed from the batch medium the feed was started and this time was defined as t_s), t_e [h] is the end time of the feed, PD [g/l] represents the 1,3-propanediol concentration for a given time point and *y*(*f*) [g/l] gives the sum of the concentration differences of 1,3-propanediol between two sampling points.

The values given for the CDM were average values of the whole feed phase and were calculated with Eq. (4):

$$CDM(f) = \frac{\sum_{f=t_s}^{e} z(f) = \frac{[CDMt_s + CDMt_{s+1}]}{2} \times [t_{s+1} - t_s] + \ldots + \frac{[CDMt_{e-1} + CDMt_e]}{2} \times [t_e - t_{e-1}]}{t_e - t_s} \quad (4)$$

where CDM (f) [g/l] is the average biomass concentration during the feed phase, t_s [h] is the start time of the feed (when glycerol was completely consumed from the batch medium the feed was started and this time was defined as t_s), t_e [h] is the end time of the feed, CDM [g/l] represents the biomass concentration for a given time point and *z*(*f*) [g_{CDM} h/l] gives the sum of the average biomass concentrations between two sampling points multiplied by the time span between these two sampling points.

3. Results and discussion

3.1. Characterization of the metabolism of L. diolivorans in batch cultivations

The metabolism of *L. diolivorans* was observed during batch cultivations carried out on MRS medium with glucose as carbon source and in several mixed substrate cultivations with glucose and increasing concentrations of glycerol.

3.1.1. Batch cultivation on MRS medium with glucose as carbon source

When glucose was the only carbon source the formation of lactic acid, ethanol and CO_2 during glucose consumption and formation of biomass was observed (Fig. 1A). The acetic acid concentration was only slightly higher (5.3 g/l) at the end of the cultivation than the initial concentration in the batch medium (4 g/l). Total glucose consumption was reached after approximately 33 h with a final biomass concentration of 3.1 g/l. The highest specific growth rate observed during glucose consumption was μ_{max} = 0.14 h^{-1}. In the stationary phase (no further increase of biomass) no more metabolite formation was observed.

These results concur with the observation that *L. diolivorans* is a heterofermentative lactic acid bacterium as suggested by Krooneman et al. (2002). Like observed, heterofermentative lactic acid bacteria use the heterolactic (phosphoketolase) pathway to catabolize glucose and form 1 mol of lactic acid, ethanol and CO_2 with a net gain of 1 mol ATP per mol glucose catabolized (Garai-Ibabe et al., 2008).

3.1.2. Batch cultivations on MRS medium with glucose and glycerol as carbon sources

Addition of glycerol to the culture medium led to an increase of the maximum specific growth rate μ_{max} and the final biomass concentration (0.21–0.27 h^{-1} and 3.4–4.6 g/l, respectively) as shown in Tables 1 and 2. Furthermore, the time required for glucose consumption decreased in the presence of glycerol. Glucose was again catabolized to lactic acid, ethanol and CO_2. In addition, acetic acid and 1,3-propanediol accumulated during the fermentation (Fig. 1B). With increasing concentrations of glycerol in the batch medium the concentrations of 1,3-propanediol and acetic acid at the end of the process increased (Table 1). Furthermore, for glycerol concentrations higher than 1% (w/v) in the batch medium, a decrease of the concentrations of lactic acid and ethanol after glucose was consumed could be observed. The highest 1,3-propanediol concentration observed was 41.7 g/l for the cultivation with the highest concentration of glycerol in the batch medium (Table 1).

These results are in agreement with the observation that has been reported for two other heterofermentative lactic acid bacte-

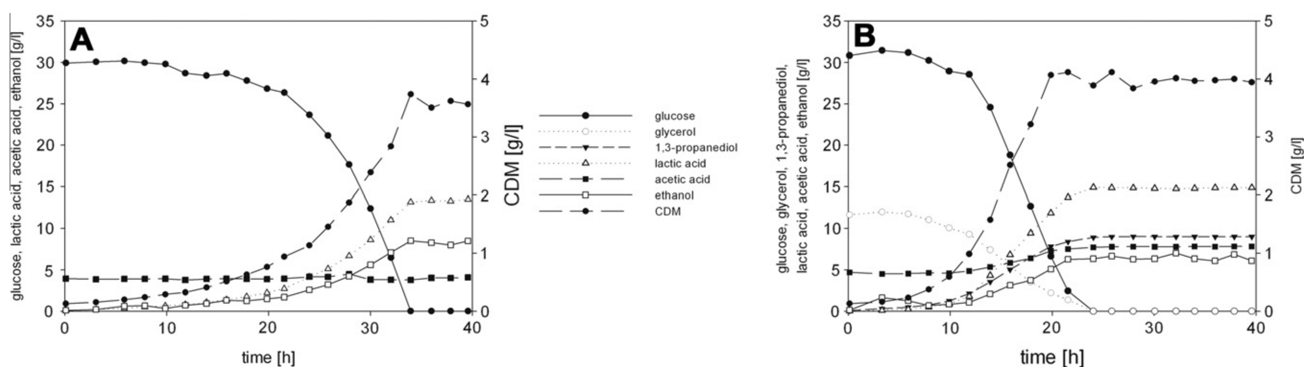

Fig. 1. Time course of the batch cultivations of *L. diolivorans* on MRS with 3% glucose and no glycerol (A) and 3% glucose + 1% glycerol (B).

Table 1
Overview of the biomass and metabolites determined at the end of the batch cultivations of *L. diolivorans* on MRS with 3% (w/v) glucose and different concentrations of glycerol. The column "time" represents the whole cultivation duration. The time for glucose consumption was about 33 h for the cultivation without glycerol, and about 22 h for the cultivations with glycerol in the batch medium. For the cultivation with 1% (w/v) glycerol in the batch medium glycerol was consumed at about 22 h.

Glycerol start in % (w/v)	Time [h]	CDM [g/l]	Glucose [g/l]	Glycerol [g/l]	1,3-Propanediol [g/l]	Lactic acid [g/l]	Acetic acid [g/l]	Ethanol [g/l]
0.0	147	3.1	0.0	0.0	0.3	12.8	5.3	6.2
1.0	147	3.6	0.0	0.0	9.2	14.7	8.4	4.3
2.0	147	3.4	0.0	1.7	14.2	10.8	9.7	3.5
3.0	147	4.0	0.0	2.2	23.8	7.7	13.5	3.4
3.5	139	4.1	0.2	6.9	21.8	8.9	13.0	3.6
5.0	139	4.5	0.2	3.2	34.3	3.9	17.3	2.8
6.0	139	4.6	0.2	8.7	37.9	2.4	18.4	2.6
7.0	139	4.4	0.2	15.3	41.7	1.5	19.4	1.4

Table 2
Overview of the maximum specific growth rate μ_{max} [h^{-1}], the volumetric product formation rate Q_p [$g_{1,3\text{-propanediol}}$/l h], the specific product formation rate q_p [$g_{1,3\text{-propanediol}}/g_{CDM}$ h] and the product yield $Y_{P/S}$ for total carbon source [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate}$] and glycerol carbon [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ glycerol}$] for the batch cultivations of *L. diolivorans* on MRS with 3% (w/v) glucose and different concentrations of glycerol. Since the two substrates used during the cultivations have different molar weights, the product yields considering either only glycerol or glycerol and glucose cannot be compared directly. For that reason the molar amounts of glucose, glycerol and 1,3-propanediol were converted to the molar amount of carbon atoms they contain. The above given product yields were determined with these values.

Glycerol start in % (w/v)	μ_{max} [h^{-1}]	$Q_{p\ max}$ [$g_{1,3\text{-propanediol}}$/l h]	$Q_{p\ average}$ [$g_{1,3\text{-propanediol}}$/l h]	$q_{p\ max}$ [$g_{1,3\text{-propanediol}}/g_{CDM}$ h]	$q_{p\ average}$ [$g_{1,3\text{-propanediol}}/g_{CDM}$ h]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate\ total}$]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ glycerol}$]
0.0	0.14	0.00	0.00	0.00	0.00	0.00	0.00
1.0	0.27	0.77	0.37	0.57	0.35	0.24	0.90
2.0	0.26	0.77	0.10	0.64	0.07	0.32	0.77
3.0	0.26	0.77	0.16	0.64	0.08	0.40	0.78
3.5	0.24	0.85	0.16	0.64	0.08	0.36	0.70
5.0	0.24	0.79	0.25	0.62	0.10	0.47	0.79
6.0	0.21	0.73	0.27	0.55	0.11	0.48	0.75
7.0	0.23	0.69	0.30	0.56	0.11	0.47	0.70

ria, *Lactobacillus buchneri* and *Lactobacillus brevis* (Da Cunha and Foster, 1992). The cofermentation of glucose and glycerol in these organisms leads to a shift from NADH-consuming to NADH-producing reactions during glucose catabolism. Firstly, acetyl phosphate is converted into acetic acid rather than ethanol, thereby 2 mol, of NADH usually consumed during ethanol formation. Acetyl phosphate is then dephosphorylated by the enzyme acetate kinase creating 1 mol of ATP per mol of acetic acid formed. Secondly, part of the available pyruvate is converted into acetyl phosphate and subsequently to acetic acid instead of lactic acid. The enzyme converting 1 mol pyruvate to 1 mol acetyl phosphate and releasing 1 mol CO_2 is pyruvate oxidase, which uses free phosphate to phosphorylate the acetyl group remaining after CO_2 is released. Acetyl phosphate is then metabolized as described above to acetic acid with the gain of 1 mol of ATP. Thirdly, the accumulated lactic acid is reoxidized to pyruvate, which is further metabolized to acetic acid and CO_2 with the gain of 1 mol of ATP.

These ways to generate ATP are suggested to be the source of maintenance energy during the stationary phase (no increase in CDM observed) of the process.

Avoiding the consumption of 2 mol NADH during ethanol formation creates a surplus of NADH. This seems to enable the observed formation of 1,3-propanediol from glycerol. The reductive glycerol pathway has two steps, in the first step glycerol is converted into 3-hydroxypropionaldehyde (3-HPA), which is subsequently reduced to 1,3-propanediol. The second step requires 1 mol NADH per mol 1,3-propanediol formed and prevents the accumulation of toxic 3-HPA (Garai-Ibabe et al., 2008). The described mechanism is also supported by the ratio of the molar concentrations of 1,3-propanediol and acetic acid over time (for the cultivation shown in Fig. 1B) shown in Fig. 2. This correlation suggests that the formation of 2 mol 1,3-propanediol requires the formation of 1 mol acetic acid.

Due to high amounts of acetic acid formed during cofermentations of glucose and glycerol, for subsequent cultivations the acetic acid added to the medium of the batch cultivations was omitted in fed-batch cultures in order to avoid inhibition of the cultures (unless indicated otherwise).

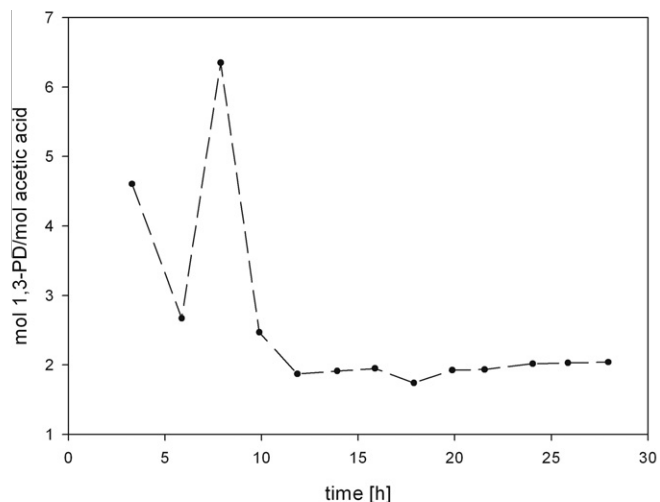

Fig. 2. Ratio between the molar amounts of 1,3-propanediol and acetic acid plotted against the process run time for the cultivation depicted in Fig. 1B. Each dot represents a single ratio of 1,3-propanediol and acetic acid for a corresponding time point.

The highest volumetric and specific product formation rates of all cultivations were 0.85 $g_{1,3\text{-propanediol}}$/l h and 0.64 $g_{1,3\text{-propanediol}}/g_{CDM}$ h, respectively. The maximum product yield coefficients for total carbon source and glycerol carbon were 0.48 $mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate}$ and 0.79 $mol_{C\ 1,3\text{-propanediol}}/mol_{C\ glycerol}$, respectively (Table 2).

Fed-batch cultivations are the preferable cultivation mode for conversion of glycerol into 1,3-propanediol with *L. diolivorans* – despite high product formation rates in batch cultivations. This is due

to the fact that the substrate concentration in batch cultivations cannot be increased above a certain threshold without substrate inhibition occurring (data not shown), thereby limiting the obtainable product concentration.

3.2. The effect of different ratios of glucose/glycerol in the feed medium on the production of 1,3-propanediol

L. diolivorans was investigated in several fed-batch cultivations on MRS medium, with a glucose–glycerol co-fermentation in the batch medium (3% (w/v) glucose, 1% (w/v) glycerol), followed by a feed of a glucose–glycerol solution with different glucose/glycerol-ratios in order to determine the optimal feed composition. The feeding rate was determined based upon the highest specific production formation rate and set to 1.5 ml/h corresponding to a maximal glycerol consumption rate of 0.75 g/h observed during batch cultivation.

The ratios of glucose/glycerol in the feed medium were 0.05, 0.1, 0.2, 0.3 mol/mol. The concentrations of lactic acid and ethanol increased when the ratio between glucose and glycerol was higher, whereas the concentrations of acetic acid were almost constant throughout the whole range of ratios tested (Table S1, Supplementary data). The 1,3-propanediol concentrations were highest for low ratios of glucose and glycerol, whereby 0.1 mol/mol resulted in the highest 1,3-propanediol concentration (68.3 g/l). Higher ratios led to significantly lower 1,3-propanediol concentrations (38.5 g/l for 0.3 mol/mol) and a higher accumulation of glycerol at the end of the process was observed (16.6 g/l for 0.1 mol/mol and 52.1 g/l for 0.3 mol/mol, Fig. 3).

These observations suggest that the amount of glucose at a feed ratio of 0.1 mol/mol is sufficient to provide the cell with (i) enough energy (via ATP) for maintenance and (ii) enough reduction equivalents (NADH) to maintain the conversion of glycerol to 1,3-propanediol after the end of the batch. Higher ratios, where higher amounts of glucose are fed to the culture, suppress the conversion of glycerol to 1,3-propanediol. *L. diolivorans* reacts similarly to *K. pneumoniae*, where in the study of Hiremath et al. (2011) a ratio of 0.1 mol/mol was reported to be optimal for the batch medium regarding the conversion of glycerol to 1,3-propanediol.

The highest average volumetric and specific product formation rates of all cultivations during the feeding phase were 0.52 $g_{1,3\text{-propanediol}}$/l h and 0.09 $g_{1,3\text{-propanediol}}/g_{CDM}$ h. The maximum product yield coefficients for total carbon source and glycerol carbon were 0.57 $mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate}$ and 0.86 $mol_{C\ 1,3\text{-propanediol}}/mol_{C\ glycerol}$ (Table 3).

3.3. The effect of oxygen supply on the production of 1,3-propanediol

Microaerophilic conditions during fermentation of glycerol by *K. pneumoniae* have been reported to be favorable towards 1,3-pro-

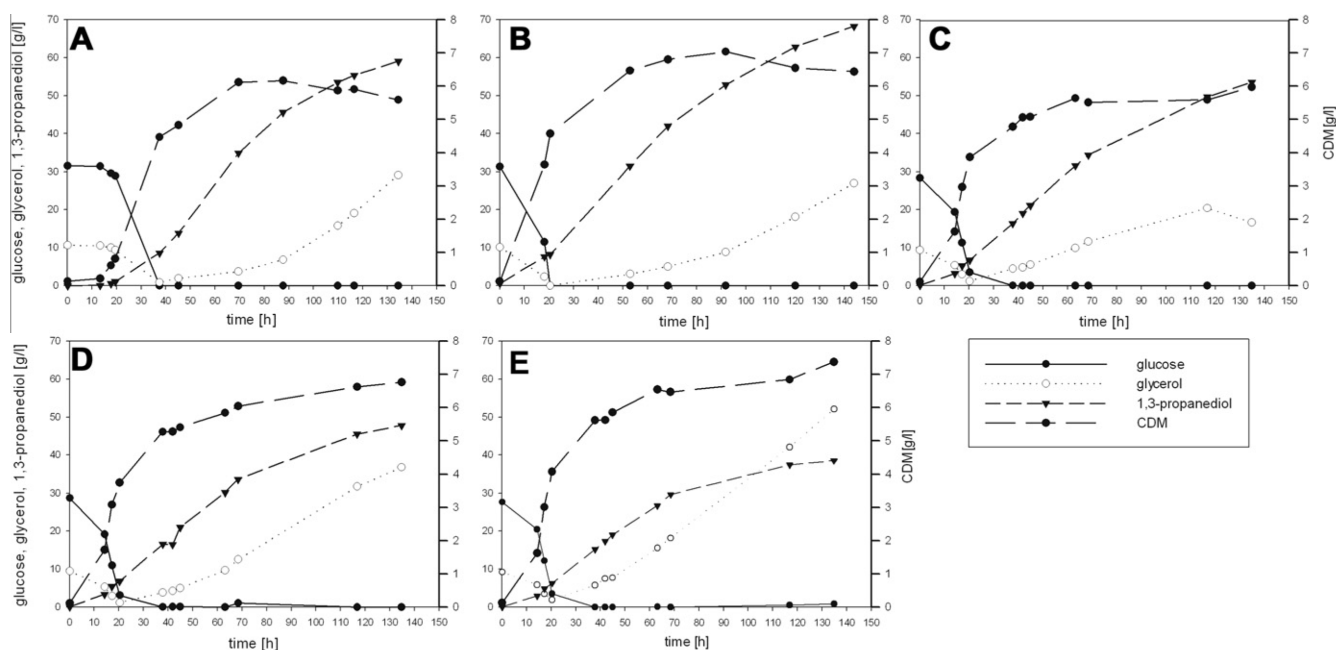

Fig. 3. Time course of the concentrations of glucose, glycerol, 1,3-propanediol and biomass for cultivations with different ratios of glucose/glycerol in the feed medium. All cultivations were carried out on MRS with 3% (w/v) glucose and 1% (w/v) glycerol in the batch medium, followed by a feed with a feeding rate of 1.5 ml/h. (A) 0.05 mol/mol without acetic acid in the batch medium; (B) 0.1 mol/mol without acetic acid in the batch medium; (C) 0.1 mol/mol with acetic acid in the batch medium; (D) 0.2 mol/mol with acetic acid in the batch medium; and (E) 0.3 mol/mol with acetic acid in the batch medium.

Table 3
Overview of μ_{max}, Q_p, q_p, and $Y_{P/S}$ for the fed-batch cultivations of *L. diolivorans* with different ratios of glucose/glycerol in the feed solution.

glc/gly ratio [mol/mol]	$Q_{p\ average\ feed}$ [$g_{1,3\text{-propanediol}}$/l h]	$q_{p\ average\ feed}$ [$g_{1,3\text{-propanediol}}/g_{CDM}$ h]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate\ total}$]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-propanediol}}/mol_{C\ glycerol}$]
0.05 w/o acetate	0.52	0.09	0.57	0.86
0.1 w/o acetate	0.48	0.08	0.51	0.73
0.1	0.41	0.08	0.55	0.84
0.2	0.36	0.06	0.38	0.63
0.3	0.28	0.05	0.27	0.50

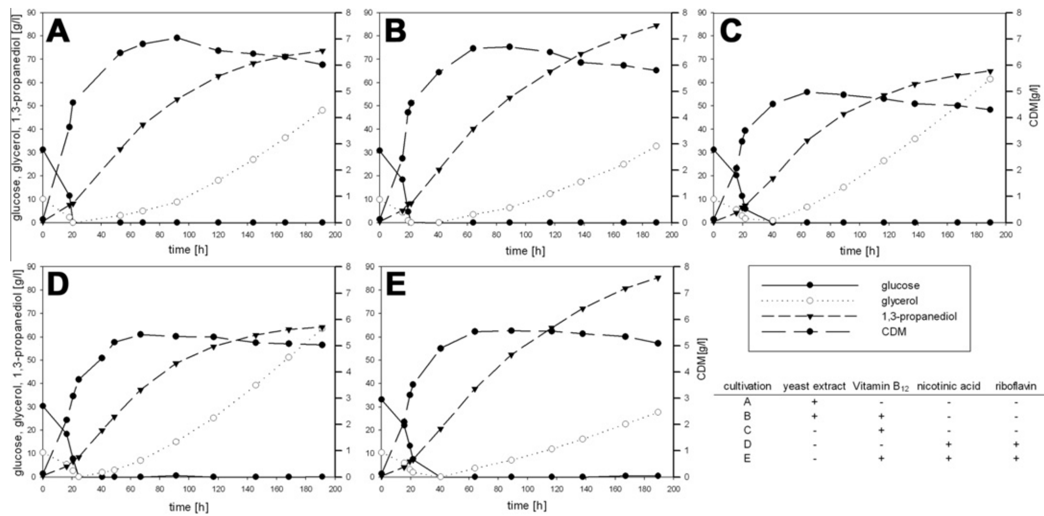

Fig. 4. Time course of the concentrations of glucose, glycerol, 1,3-propanediol and biomass for cultivations where single components were omitted from/added to the batch and feed medium. All cultivations were carried out on MRS with 3% (w/v) glucose and 1% (w/v) glycerol in the batch medium, followed by a feed with a glucose/glycerol solution with a molar ratio of 0.1. The feeding rate was 1.5 ml/h.

panediol production compared to anaerobic culture conditions (Chen et al., 2003). It is suggested that low amounts of oxygen enable a better growth of the culture and therefore increase the overall number of biocatalysts for the conversion of glycerol to 1,3-propanediol and thus increasing the 1,3-propanediol production. Therefore the effect of low oxygen concentrations on the 1,3-propanediol concentration reached during fermentation of glycerol by *L. diolivorans* was evaluated in fed-batch cultivations (Fig. S1, Supplementary data).

The composition of the batch and the feed medium was as described in Section 3.2. The ratio used for the feed was 0.1 mol glucose/mol glycerol.

Three oxygen conditions were tested: anaerobic (100% N_2 gassing), microaerophilic (1% O_2, 99% N_2 gassing) and aerobic (21% O_2, gassing with air). For anaerobic and microaerophilic conditions, glucose and glycerol from the batch medium were consumed. The feed was started after full glycerol consumption was observed. The concentrations of 1,3-propanediol, lactic acid, acetic acid and ethanol at the end of the cultivations were comparable. However, in case of 1,3-propanediol the concentrations were slightly higher for anaerobic than for microaerophilic conditions. Aerobic conditions showed completely different results. Here, only a small amount of glucose from the batch medium was consumed. Therefore, only small amounts of lactic acid, acetic acid and ethanol were formed. Furthermore, no 1,3-propanediol production whatsoever could be observed for these cultivations, with the glycerol of the batch medium remaining in the supernatant. These results clearly indicate that gassing with oxygen neither in microaerophilic nor in aerobic conditions is preferable for 1,3-propanediol production with *L. diolivorans*.

3.4. The effect of Vitamin B12 on glycerol dehydratase activity and 1, 3-propanediol production

Vitamin B_{12} is the co-factor of most glycerol dehydratases (EC 4.2.1.30) known today. Among others, the activity of glycerol dehydratases from *K. pneumoniae* and *L. reuteri* strains was found to depend on Vitamin B_{12} (Wang et al., 2009; Talarico and Dobrogosz, 1990). The sequences of the genes coding for the glycerol dehydratase in *L. diolivorans* are available (Uniprot entry codes: Q6YNN0, Q6YNM9, Q6YNM8). Sequence alignment of the large subunit with sequences from *L. reuteri* and *K. pneumoniae* reveals similarities of above 60% (with a sequence coverage of 97%). However, comparison of the larger of the two subunits of the few known Vitamin B_{12}-independent glycerol dehydratases did not reveal any significant sequence similarity. This suggests that the glycerol dehydratase of *L. diolivorans* depends on Vitamin B_{12} as co-factor. Therefore, it is likely that an efficient synthesis of Vitamin B_{12} by the cell is required in order to maintain a high level of activity of glycerol dehydratase.

Table 4
Overview of μ_{max}, Q_p, q_p, and $Y_{P/S}$ for the fed-batch cultivations where single components were omitted from/to the batch and feed medium. Vitamins, nicotinic acid and riboflavin.

±Vitamin B_{12} ± yeast extract ± vitamins	$Q_{p\ average\ feed}$ [$g_{1,3\text{-}propanediol}$/l h]	$q_{p\ average\ feed}$ [$g_{1,3\text{-}propanediol}$/$g_{CDM}$ h]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-}propanediol}$/$mol_{C\ substrate\ total}$]	$Y_{P/S}$ [$mol_{C\ 1,3\text{-}propanediol}$/$mol_{C\ glycerol}$]
−Vitamin B_{12} + yeast extract − vitamins	0.38	0.06	0.46	0.62
+Vitamin B_{12} + yeast extract − vitamins	0.45	0.07	0.55	0.76
+Vitamin B_{12} − yeast extract − vitamins	0.34	0.07	0.43	0.59
+Vitamin B_{12} − yeast extract + vitamins	0.46	0.09	0.56	0.78
−Vitamin B_{12} − yeast extract + vitamins	0.33	0.07	0.39	0.53

The impact of Vitamin B_{12} on 1,3-propanediol production with *L. diolivorans* was investigated in two fed-batch cultivations. The composition of the batch and feed medium is described in Section 3.2. The molar ratio of glucose to glycerol in the feed medium was 0.1. To ensure a high level of activity by glycerol dehydratase, 5 mg/l Vitamin B_{12} were additionally added to both, the batch and the feed medium.

Addition of Vitamin B_{12} significantly increased 1,3-propanediol concentration from 73.7 to 84.5 g/l (Fig. 4A and B). However, biomass formation was not affected by addition of Vitamin B_{12} (Table S2, Supplementary data). The specific product formation rate was higher for the cultivation with Vitamin B_{12}, explaining the higher end concentration of 1,3-propanediol in comparison to the cultivation without Vitamin B_{12} (Table 3). This strongly indicates that glycerol dehydratase of *L. diolivorans* is indeed Vitamin B_{12} dependent.

3.5. The impact of yeast extract, riboflavin and nicotinic acid on growth and productivity of L. diolivorans

Yeast extract is an expensive medium component, which contains vitamins and amino acids in varying concentrations. Among others, yeast extract contains riboflavin and nicotinic acid. These vitamins play a role in the biosynthesis pathway of Vitamin B_{12}. Furthermore, *L. diolivorans* was found to be auxotrophic for these two vitamins (data not shown). The effect of yeast extract, riboflavin and nicotinic acid on biomass formation and 1,3-propanediol production was studied. The composition of the batch and feed medium described in Section 3.2 was modified according to Fig. 4.

Substitution of yeast extract with Vitamin B_{12} or a combination of nicotinic acid and riboflavin led to decreased biomass and accordingly to decreased 1,3-propanediol concentrations (Fig. 4C and D, Table S2 Supplementary data).

Substitution of yeast extract with a combination of all three vitamins, also led to decreased biomass accumulation. However, the 1,3-propanediol concentration reached was 85.4 g/l (Fig. 4E), which is comparable to the cultivation with yeast extract and Vitamin B_{12}. Surprisingly, the specific product formation rate is higher when yeast extract is replaced by a combination of nicotinic acid, riboflavin, and Vitamin B_{12} (Table 4). These results show that yeast extract can be omitted from the culture medium.

4. Conclusion

L. diolivorans proves to be a top candidate microorganism for industrial production of 1,3-propanediol from glycerol. The wild type strain reaches productivities of up to 0.85 $g_{1,3\text{-propanediol}}$/l h and product concentrations up to 85.4 g/l. Total carbon yields of 1,3-propanediol in a fed-batch process approach 0.57 $mol_{C\ 1,3\text{-propanediol}}/mol_{C\ substrate}$.

The next steps for the bioprocess design include an optimization of the feeding strategy in order to avoid accumulation of glycerol, and overall reduction of the medium costs. Genetic engineering of the strain is being developed to further optimize this microbial cell factory.

Acknowledgements

This work was supported by Vogelbusch GmbH, Vienna, Austria. A patent application comprising the data of this study has been filed. The authors are indebted to Stefanie Müller for excellent technical support, Joachim Gatterer for fruitful discussions, and Gottfried H. Sodeck and Matthias G. Steiger for critically reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.05.121.

References

Chen, X., Zhang, D.-J., Qi, W.-T., Gao, S.-J., Xiu, Z.-L., Xu, P., 2003. Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions. Appl. Microbiol. Biotechnol. 63, 143–146. http://dx.doi.org/10.1007/s00253-003-1369-5.

Cheng, K.-K., Zhang, J.-A., Liu, D.-H., Sun, Y., Liu, H.-J., Yang, M.-D., Xu, J.-M., 2007. Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. Process Biochem. 42, 740–744. http://dx.doi.org/10.1016/j.procbio.2007.01.001.

Da Cunha, M.V., Foster, M.A., 1992. Sugar-glycerol cofermentations in lactobacilli: the fate of lactate. J. Bacteriol. 174, 1013–1019.

De Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of Lactobacilli. J. Appl. Microbiol. 23, 130–135. http://dx.doi.org/10.1111/j.1365-2672.1960.tb00188.x.

Doleyres, Y., Beck, P., Vollenweider, S., Lacroix, C., 2005. Production of 3-hydroxypropionaldehyde using a two-step process with *Lactobacillus reuteri*. Appl. Microbiol. Biotechnol. 68, 467–474. http://dx.doi.org/10.1007/s00253-005-1895-4.

Freund, A., 1881. Über die Bildung und Darstellung von Trimethylenalkohol aus Glycerin. Monatsh. Chem. 2, 636–641. http://dx.doi.org/10.1007/BF01516545.

Garai-Ibabe, G., Ibarburu, I., Berregi, I., Claisse, O., Lonvaud-Funel, A., Irastorza, A., Dueñas, M.T., 2008. Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. Int. J. Food Microbiol. 121, 253–261. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.11.004.

González-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J.C., Vasconcelos, I., Soucaille, P., 2005. Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab. Eng. 7, 329–336. http://dx.doi.org/10.1016/j.ymben.2005.06.001.

Günzel, B., Yonsel, S., Deckwer, W.-D., 1991. Fermentative production of 1,3-propanediol from glycerol by *Clostridium butyricum* up to a scale of 2 m^3. Appl. Microbiol. Biotechnol. 36, 289–294. http://dx.doi.org/10.1007/BF00208143.

Hiremath, A., Kannabiran, M., Rangaswamy, V., 2011. 1,3-Propanediol production from crude glycerol from Jatropha biodiesel process. New Biotechnol. 28, 19–23.

Huang, Y., Li, Z., Shimizu, K., Ye, Q., 2012. Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of Klebsiella pneumoniae. Bioresour. Technol. 103, 351–359. http://dx.doi.org/10.1016/j.biortech.2011.10.022.

Jensen, T. Ø., Kvist, T., Mikkelsen, M. J., Christensen, P. V., Westermann, P. Fermentation of crude glycerol from biodiesel production by *Clostridium pasteurianum*. J. Ind. Microbiol. Biotechnol. In press. doi:10.1007/s10295-011-1077-6.

Johnson, D.T., Taconi, K.A., 2007. The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. Environ. Prog. 26, 338–348. http://dx.doi.org/10.1002/ep. 10225.

Kaur, G., Srivastava, A.K., Chand, S., 2012. Advances in biotechnological production of 1,3-propanediol. Biochem. Eng. J. 64, 106–118. http://dx.doi.org/10.1016/j.bej.2012.03.002.

Krooneman, J., Faber, F., Alderkamp, A.C., Oude Elferink, S.J.H.W., Driehuis, F., Cleenwerck, I., Swings, J., Gottschal, J.C., Vancanneyt, M., 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Syst. Evol. Microbiol. 52, 639–646.

Mercade, M., Duperray, F., Loubière, P., 2003. Transient self-inhibition of the growth of *Lactobacillus delbrueckii* subsp. bulgaricus in a pH-regulated fermentor. Biotechnol. Bioeng. 84, 78–87. http://dx.doi.org/10.1002/bit.10751.

Nakamura, C.E., Whited, G.M., 2003. Metabolic engineering for the microbial production of 1,3-propanediol. Curr. Opin. Biotechnol. 14, 454–459. http://dx.doi.org/10.1016/j.copbio.2003.08.005.

Oh, B.-R., Seo, J.-W., Heo, S.-Y., Hong, W.-K., Luo, L.H., Kim, S., Park, D.-H., Kim, C.-H., 2011. Optimization of culture conditions for 1,3-propanediol production from glycerol using a mutant strain of Klebsiella pneumoniae. Appl. Biochem. Biotechnol. 166, 127–137. http://dx.doi.org/10.1007/s12010-011-9409-6.

Posada, J.A., Rincón, L.E., Cardona, C.A., 2012. Design and analysis of biorefineries based on raw glycerol: addressing the glycerol problem. Bioresour. Technol. 111, 282–293. http://dx.doi.org/10.1016/j.biortech.2012.01.151.

Sauer, M., Marx, H., Mattanovich, D., 2008. Microbial production of 1,3-propanediol. Recent Pat. Biotechnol. 2, 191–197. http://dx.doi.org/10.2174/187220808786240999.

Sheedlo, M.J., 2008. A review of the processes of biodiesel production. MMG 445 Basic Biotechnol. eJ. 4, 61–65.

Talarico, T.L., Dobrogosz, W.J., 1990. Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. Appl. Environ. Microbiol. 56, 1195–1197.

Talarico, Todd.L., Axelsson, L.T., Novotny, J., Fiuzat, M., Dobrogosz, W.J., 1990. Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol:NAD+ oxidoreductase. Appl. Environ. Microbiol. 56, 943–948.

Tokumoto, H., Tanaka, M., 2012. Novel anaerobic digestion induced by bacterial components for value-added byproducts from high-loading glycerol. Bioresour. Technol. 107, 327–332. http://dx.doi.org/10.1016/j.biortech.2011.12.051.

Vasudevan, P.T., Briggs, M., 2008. Biodiesel production–current state of the art and challenges. J. Ind. Microbiol. Biotechnol. 35, 421–430. http://dx.doi.org/10.1007/s10295-008-0312-2.

Vollenweider, S., Lacroix, C., 2004. 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. Appl. Microbiol. Biotechnol. 64, 16–27. http://dx.doi.org/10.1007/s00253-003-1497-y.

Wang, X.-D., Zhang, X.-E., Guo, Y.-C., Zhang, Z.-P., Cao, Z.-A., Zhou, Y.-F., 2009. Characterization of glycerol dehydratase expressed by fusing its alpha- and beta-subunits. Biotechnol. Lett. 31, 711–717. http://dx.doi.org/10.1007/s10529-009-9911-x.

Wilkens, E., Ringel, A.K., Hortig, D., Willke, T., Vorlop, K.-D., 2012. High-level production of 1,3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a. Appl. Microbiol. Biotechnol. 93, 1057–1063. http://dx.doi.org/10.1007/s00253-011-3595-6.