Brief report

1,3-Propanediol production from glycerol by a newly isolated *Trichococcus* strain

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Summary

A coccal bacterium (strain ES5) was isolated from methanogenic bioreactor sludge with glycerol as the sole energy and carbon source. Strain ES5 fermented glycerol to 1,3-propanediol as main product, and lactate, acetate and formate as minor products. The strain was phylogenetically closely related to *Trichococcus flocculiformis*; the rRNA gene sequence similarity was 99%. However, strain ES5 does not show the typical growth in chains of *T. flocculiformis*. Moreover, *T. flocculiformis* does not ferment glycerol. Strain ES5 used a variety of sugars for growth. With these substrates, lactate, acetate and formate were the main products, while 1,3-propanediol was not formed. The optimum growth temperature of strain ES5 ranges from 30–37°C, but like several other *Trichoccoccus* strains, strain ES5 is able to grow at low temperature (< 10°C). Therefore, strain ES5 may be an appropriate catalyst for the biotechnological production of 1,3-propanediol from glycerol at low ambient temperature.

Introduction

There is quest to find new and better catalysts to produce interesting chemicals from organic waste products and by products. Glycerol is a by product of biodiesel production. The increasing demand for biodiesel production made that the market price for glycerol has dropped considerably (Yazdani and Gonzalez, 2007). Therefore, glycerol is

an interesting compound for the production of valuable compounds, such as 1,3-propanediol (PDO) (Choi, 2008; Saxena *et al.*, 2009; Khanna *et al.*, 2011). PDO is an organic chemical that is growing in importance as it can replace ethylene glycol and butylene glycol for the synthesis of polyesters and polyurethanes, and it can be used as solvent, antifreeze agent or protective agent (Zeng and Biebl, 2002; Liu *et al.*, 2007; 2010; Saxena *et al.*, 2009; Khanna *et al.*, 2011). The possibility to produce PDO biotechnologically from glycerol is known for a long time. Several anaerobic and facultatively anaerobic bacteria with the ability to produce PDO from glycerol have been described. Currently, *Klebsiella pneumoniae, Clostridium butyricum* and *Citrobacter freundii* are known as high PDO producers (Homann *et al.*, 1990; Biebl *et al.*, 1998; Zeng and Biebl, 2002; Liu *et al.*, 2010).

The most common mechanism of glycerol fermentation involves a reductive pathway and an oxidative pathway (Liu *et al.*, 2010). In the reductive pathway, a vitamin B_{12}-dependent glycerol dehydratase catalyses glycerol conversion to 3-hydroxypropionaldehyde, and this compound is further reduced to PDO by 1,3-propanediol oxidoreductase. Besides this common vitamin B_{12}-dependent pathway, Raynaud and colleagues (2003) have described a vitamin B_{12}-independent glycerol dehydratase in *Clostridium butyricum*. In the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone by a NAD^+-linked glycerol dehydrogenase, which is then converted to dihydroxyacetone phosphate by an ATP-dependent dihydroxyacetone kinase. Dihydroxyacetone phosphate is an intermediate of the glycolysis that can be further converted to acetate and to other products, depending on the type of bacterium.

We started research to find novel bacteria for biotechnological purposes and we obtained a glycerol-fermenting bacterium that was morphologically different from the known glycerol-fermenting bacteria. Upon rRNA gene analysis the strain turned out to be a *Trichococcus* strain.

Trichococcus is a genus that was created by Scheff and colleagues (1984). By the reclassification of some species of the genera *Lactosphaera* and *Ruminococcus*, an emended genus description was published (Liu *et al.*, 2002). Currently, the genus *Trichococcus* includes five
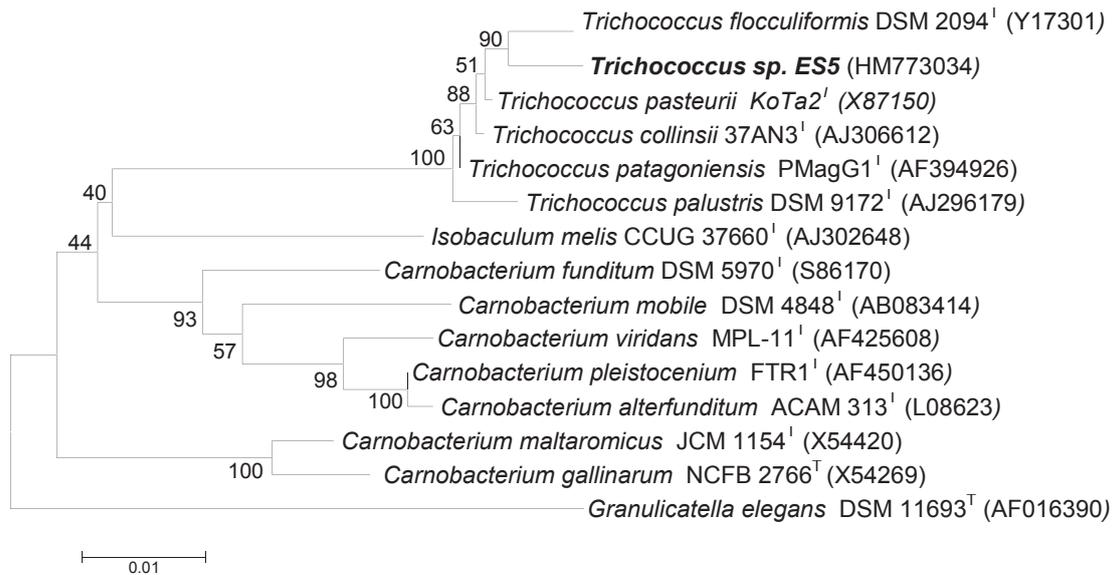
Fig. 1. Comparison of the rRNA gene similarities of strain ES5, other *Trichococcus* strains. The bar represents 0.01% sequence difference.

established species: *Trichococcus flocculiformis*, *T. palustris*, *T. pasteurii, T. collinsii* and *T. patagoniensis*. *Trichococcus* species are able to grow at low temperatures; *T. patagoniensis* even below 0°C. All species have the same morphology; the typical coccoid-shaped cells occur singularly, in pairs, in short chains or as irregular conglomerates. This pleomorphic nature is a common characteristic within the genus. *Trichococcus* species are known as facultative anaerobes capable to create redox conditions to reduce resazurin in aerobic media during growth. Genotypically, all species of this genus have a high (99–100%) 16S rRNA gene sequence similarity (Liu *et al.*, 2002), but DNA–DNA hybridization showed relatedness values of below 70% (enough for separation of strains into novel species). All of the *Trichococcus* strains are thought to be very similar phenotypically. They are all oxidase and catalase negative, and can grow with a wide variety of sugars and other substrates.

Here we describe the isolation and physiological properties of a bacterium that fermented glycerol to PDO as the main product. The bacterium grows in mineral media, supplemented with vitamins and it has a broad pH and temperature range and a high salt tolerance, which makes it a suitable catalyst for biotechnological production of PDO.

Results and discussion

Isolation and phylogenetic position of strain ES5

Strain ES5 was isolated from methanogenic granular sludge by direct dilution of crushed sludge in mineral media supplemented with 20 mM pure glycerol as carbon and energy source and incubated at 30°C. This method allows to obtain the most abundant glycerol-fermenting bacteria present in the sludge. The bacterium that was enriched and isolated was coccus-shaped and different from the known glycerol-fermenting bacteria. Analysis of the rRNA gene of the bacterium revealed its close relatedness with *Trichococcus flocculiformis* (DSM 2094^T); the rRNA gene sequence similarity was 99% (Fig. 1). However, these two bacteria are morphologically distinct even when grown in the same medium with glucose as substrate (Fig. 2). With all substrates tested, strain ES5 appeared as single cells or in small chains of up to four cells. By contrast, with the substrates tested *T. flocculiformis* typically forms very long chains of coccal cells.

Growth properties of strain ES5

Strain ES5 grows in mineral media, supplemented with vitamins. The specific growth rate in mineral media with 20 mM glycerol is about 0.31 h^{-1} (doubling time about 2.2 h). The strain required ammonium as nitrogen source. In media without ammonium chloride no growth was observed. Yeast extract was not needed for growth, but addition of yeast extract stimulated growth and higher optical densities were achieved (Table S1). In media with 0.02 and 0.2 g l^{-1} yeast extract, specific growth rates of about 0.39 and 0.47 h^{-1} (doubling times of about 1.8 and 1.5 h) were determined respectively. Fast growth occurred over a broad pH range from 6.5 to at least 9.0. Below pH 6.5 no growth was observed. The strain was moderately salt tolerant (Table S3); the specific growth rate with 34 g l^{-1} extra NaCl added was 0.16 h^{-1} (doubling time is 4.3 h).
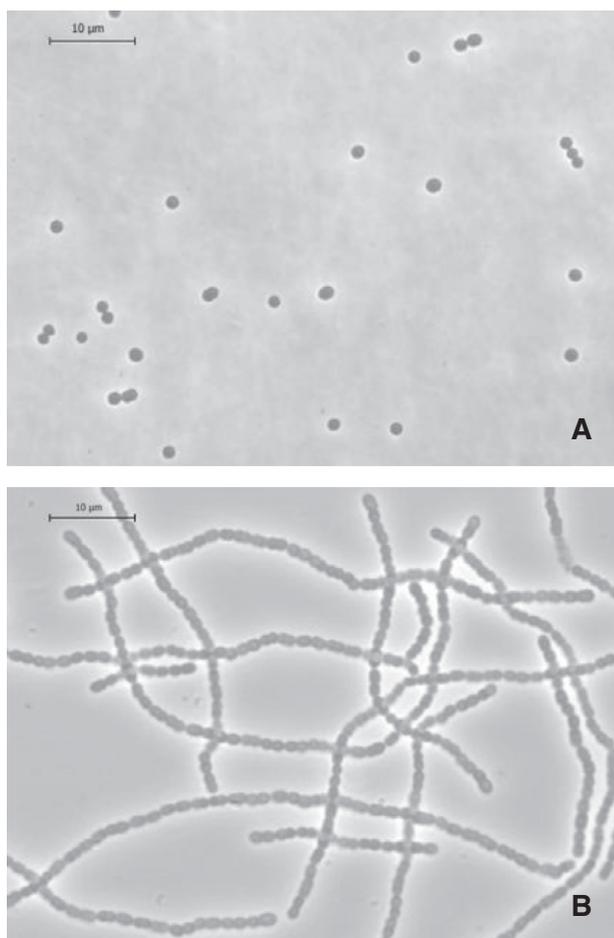
Fig. 2. Microscopic picture of strain ES5 grown in bicarbonate buffered medium (A) and of *Trichococcus flocculiformis* DSM 2094[T] (B). Both strains were cultured with 10 mM glucose and 0.1 g l^{-1} yeast extract.

The temperature range was very broad (Table S2). Optimal growth was determined around 35°C. At 40°C slow growth was observed, while the strain was not able to grow at 45°C. Strain ES5 is well able to grow at low temperature. At 10 and 2°C, the specific growth rates were 0.05 and 0.015 h^{-1} (doubling times 14 and 45 h) respectively. The strain grew best in the standard sulfide-reduced medium. There was only slow growth (specific growth rate: 0.07 h^{-1}) when sodium sulfide was omitted or when oxygen (0.5%) was introduced. At oxygen levels higher than 1% in the gas phase, no growth was observed. The strain could not grow in media without the supplementation of vitamin B$_{12}$. Other vitamins were not necessary or only needed in very low amounts; cells could be transferred several times in mineral media without added vitamins, but with vitamin B$_{12}$. In batch culture, the bacterium was able to grow with at least 500 mM glycerol in the medium, but as during glycerol fermentation the pH dropped to 6.4 only about 80 mM glycerol of the added glycerol was fermented in our media. Glycerol was fermented to PDO (main product) and lactate, acetate and formate (Table 1). The carbon and redox balance for glycerol fermentation was always higher than 90%, when taking into account the cell mass formation and assuming that the formation of one acetate also yields one formate or CO$_2$. Strain ES5 was able to grow well and produce PDO with crude glycerol diluted to concentrations of 170 mM (Biovegetal) and 33 mM (Biodiesel Kampen BV). With the latter crude glycerol slightly delayed growth was observed at a concentration of 75 mM glycerol, while strong inhibition occurred at a concentration of 125 mM glycerol. Methanol present in crude glycerol was not degraded by strain ES5.

Strain ES5 was able to use pyruvate and several sugars for growth. These compounds were fermented to lactate, acetate, ethanol and formate (Table 1), which are the common fermentation products formed by *Trichococcus* species (Liu *et al.*, 2002). PDO was not formed as end product with these substrates. The fermentation of a mixture of glycerol and glucose yielded approximately the same products as the sum of products formed with each of the substrates separately.

Table 1. Growth, substrate utilization and product formation by strain ES5 grown in bicarbonate-buffered sulfide-reduced medium with 0.1 g l^{-1} yeast extract.

Substrate degraded	OD$_{600}$ (biomass)	Products				
		1,3-Propanediol	Lactate	Formate	Acetate	Ethanol
19.0 ± 0.11 pyruvate	0.47 ± 0.001 (4.8)	–	–	11.4 ± 0.06	15.5 ± 0.22	–
9.9 ± 0.23 glucose	0.96 ± 0.004 (9.8)	–	10.7 ± 0.25	4.5 ± 0.20	1.3 ± 0.09	3.8 ± 0.25
10.1 ± 0.14 glucose and 9.6 ± 0.08 glycerol	1.32 ± 0.01 (13.5)	7.6 ± 0.17	11.1 ± 0.22	4.3 ± 0.05	3.8 ± 0.10	2.1 ± 0.07
20.0 ± 0.25 glycerol	0.50 ± 0.01 (5.1)	13.3 ± 0.11	0.2 ± 0.12	2.2 ± 0.66	3.3 ± 0.1	–
39.1 ± 0.42 glycerol	0.87 ± 0.01 (8.9)	25.2 ± 0.06	1.9 ± 0.06	4.0 ± 0.01	5.7 ± 0.03	–
57.8 ± 0.18 glycerol	1.08 ± 0.02 (11.0)	37.4 ± 0.02	5.6 ± 0.06	4.0 ± 0.07	7.1 ± 0.12	–
75.8[a] glycerol	1.12[a] (11.5)	47.2[a]	8.9[a]	3.9[a]	8.0[a]	–

a. Single experiment.
All concentrations with standard error of the measurement are in mM. Besides OD$_{600}$, the calculated amount of produced biomass expressed as mmol C per litre is given (between brackets).

Table 2. Comparison of anaerobic substrate utilization by strain ES5 and *Trichococcus* strain R210 and *Trichococcus flocculiformis* DSM 2094^T.

Substrate	Strain ES5	Strain R210[a]	DSM 2094[b]
Glycerol	+	−	−
Citrate	−	+	+
Pyruvate	+	+	+
Malate	−	+	−
Lactate	−	−	−
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Arabinose	−	+	+
Xylose	+	n.d.	+
Maltose	+	+	+
Sucrose	+	+	+
Cellobiose	+	+	+
Mannitol	+	+	−
Sorbitol	−	n.d.	−
Xylitol	−	n.d.	−
Olive oil	−	n.d.	n.d.

a. Except for glycerol data were obtained from Stams and colleagues (2009).
b. Except for glycerol, data were obtained from Liu and colleagues (2002).
All incubations were performed with an anaerobic bicarbonate-buffered mineral medium supplemented with 0.1 g l^{-1} yeast extract and sodium sulfide as reducing agent. Incubations were performed without shaking.
n.d., not determined.

Comparison with other glycerol-fermenting PDO-forming bacterial strains

Glycerol fermentation is a unique property of *Trichococcus* strain ES5; it clearly is no common substrate for *Trichococcus*. As described, *T. pasteurii* (Schink, 1984), *T. palustris*, *T. flocculiformis* and *T. collinsii* (Liu *et al.*, 2002) and *T. patagoniensis* (Pikuta *et al.*, 2006) are not able to use glycerol for growth. We tested *T. flocculiformis* and *Trichococcus* strain 210 on the ability to ferment glycerol in our media, but they were not able to grow with glycerol (Table 2). In addition, strain ES5 and these two strains differ in the ability to ferment citrate and arabinose (Table 2).

   The ability to ferment glycerol and form PDO as a main product is known for a number of anaerobic and facultatively anaerobic bacteria. Much research has been done on product formation in batch cultures, fed-batch cultures and continuous cultures (Liu *et al.*, 2010). Therefore, it is difficult to compare the glycerol-fermenting abilities of the different strains with strain ES5 from literature data. In Table 3, product formation of some bacteria that were grown in batch culture is compared. The yield of PDO per amount of glycerol fermented by strain ES5 is comparable to that of other strains. Besides PDO, other products are formed. All the other strains were grown in the presence of 1 g l^{-1} yeast extract. It is not clear from the literature if these strains only grow in the presence of yeast extract or if yeast extract can be omitted as is the case for strain ES5. Also the need for vitamins of these strains is unknown. In any case, the newly isolated *Trichococcus* strain is able to ferment glycerol without yeast extract and is performing equally well as other strains with respect to PDO yield per glycerol fermented. The only vitamin that seems to be needed is vitamin B$_{12}$. This suggests that the glycerol dehydratase that is active in this bacterium also belongs to the family of B$_{12}$-dependent glycerol dehydratases (Lin, 1976; Biebl *et al.*, 1999; Liu *et al.*, 2010). The low nutrient requirement and the broad range of environmental conditions at which the bacterium can grow and produce PDO, make it attractive for biotechnological applications. Further research is needed to get insight into the exact nutrient requirements of strain ES5 and to obtain information about its performance and robustness under pilot and industrial scale fermentation conditions. Strain ES5 has been deposited in the German Collection of Microorganism and Cell Cultures as *Trichococcus* strain ES5 (DSM 23957).

Experimental procedures

Isolation of strain ES5

Strain ES5 was isolated from methanogenic sludge of an anaerobic reactor that treats wastewater of paper mills. A detailed description of the treatment plant was given by Janssen and colleagues (2009). Granular sludge was crushed and diluted in a bicarbonate-buffered medium described by Stams and colleagues (1993). Medium was

Table 3. Comparison of product formation by glycerol fermenting PDO-producing bacteria grown in batch culture. Data are given in molar percentages.

Species	Strain	PDO	Lactate	Ethanol	Acetate	Reference
Strain ES5	DSM	65	10	0.0	12	This study
Citrobacter freudii	DSM 30039	33	0.0	1	12	Homann *et al.* (1990)
Klebsiella oxytoca	NRCC 3006	55	11	39	11	Homann *et al.* (1990)
Klebsiella pneumoniae	DSM 2026	73	12	4	8	Homann *et al.* (1990)
Clostridium pasteurianum	DSM 525	21	0.0	14	4	Biebl (2001)
Clostridium butyricum	DSM 2478	58	0.0	2	3	Biebl *et al.* (1992)
Caloramator viterbensis	DSM 13723	11	0.0	0.0	5	Seyfried *et al.* (2002)

The clostridia also form butanol (*C. pasteurianum*) and butyrate (*C. butyricum*) as products.

prepared in 120 ml bottles, containing 50 ml medium and a gas phase of 1.7 bars N_2/CO_2 (80/20). Glycerol (20 mM) was the sole energy and carbon source. The bottles were incubated without stirring at 30°C in the dark. After incubation for several days the highest dilution with growth was diluted again in the same medium. This procedure was repeated three more times. The culture that was obtained was morphologically pure and designated strain ES5. Further growth tests in complex media and microscopic observation confirmed purity.

Two types of crude glycerol were kindly provided by Pedro Pereira of Biovegetal (Portugal) and by Paul Mooij of Biodiesel Kampen BV (The Netherlands). The former was colourless and contained about 89% (w/v) glycerol and 0.1% methanol, and it had a pH of about 5. The latter was brown in colour, contained about 39% glycerol and 13% methanol, and had a pH of about 13. Prior to use it was neutralized and centrifuged.

Phylogenetic analysis

DNA was extracted by first mechanically disrupting the cells by bead-beating, followed by purification using the Bio101 FASTDNA isolation kit, according to manufacturers' protocols (MP Biomedicals, Solon, OH, USA). PCR was performed with the bacterial primers 27 f and Ba1492r (Park *et al.*, 2006) by using the GoTaq DNA Polymerase Kit (Promega, Leiden, The Netherlands) to amplify the bacterial 16S rRNA gene. PCR products were purified with the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Sequencing of the complete 16SrRNA gene was performed at Baseclear (http://www.baseclear.com/). A total 1436 nucleotides of the 16S rRNA gene were sequenced. The sequences were checked for reading errors with the alignment programs of BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Closely related 16S rRNA gene sequences were identified using the BLASTN search program (http://ncbi.nlm.nih.gov/blast). The rRNA gene sequence of strain ES5 is deposited in GenBank data base under the accession number HM773034.

Physiological characterization

The spectrum of substrates used by strain ES5 was determined in the medium described above, but with yeast extract (0.1 g l^{-1}) and different substrates added. Growth was quantified by optical density measurements at 600 nm (OD_{600}). The relation between OD_{600} and the amount of organic matter content from the cells was determined (dried cell pellet – ashed cell pellet). With assumption of $<C_5H_8O_2N>$ as a simplified chemical composition of the cells, it was found that OD_{600} multiplied by a factor 10.2 represented the amount of cells in mmol carbon per litre. Substrate conversion and product formation were measured with a Thermo Scientific Spectrasystem HPLC system equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 30°C and running with 0.005 M sulfuric acid as eluent. The eluent had a flow of 0.8 ml min^{-1}. The detector was a refractive index detector. Gasses were measured with a Shimadzu GC-2014 Gas Chromatograph equipped with a Molsieve 13× column, 2 m × 3 mm, hold at 100°C. Injections were direct on column

via an injection block hold at 80°C. The carrier gas was Argon with a flow of 50 ml min^{-1}. The detector was a TCD detector, hold at 130°C with a current of 70 mA. Uninoculated media and inoculated media without substrate served as controls. All substrate tests were done at least in duplicate.

Tests with other Trichococcus strains

Trichococcus flocculiformis (DSM 2094^T) was purchased from the German Collection of Microorganisms and Cell cultures (DSMZ) in Braunschweig, Germany. *Trichoccoccus* strain R210 (DSM 22150) was isolated and maintained at our laboratory (Stams *et al.*, 2009). The two strains were grown in the same medium as described for strain ES5.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Effect of yeast extract on growth with 20 mM glycerol at 35°C.
Table S2. Effect of temperature on growth of strain ES5 with 20 mM glycerol supplemented with 0.1 g l^{-1} yeast extract.
Table S3. Effect of extra NaCl on growth with 20 mM glycerol and 0.1 g l^{-1} yeast extract at 30°C.

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