Biotechnology Letters **23:** 463–466, 2001.
© 2001 Kluwer Academic Publishers. Printed in the Netherlands.

Conversion of glycerol to 1,3-propanediol by a newly isolated thermophilic strain

Peter Wittlich*, Alexandra Themann & Klaus-Dieter Vorlop
Institute of Technology and Biosystems Engineering, Federal Agricultural Research Centre (FAL), D-38116 Braunschweig, Germany
Author for correspondence (Fax: 49-531-596-363; E-mail: peter.wittlich@fal.de)

Received 5 December 2000; Revisions requested 20 December 2000; Revisions received 15 January 2001; Accepted 15 January 2001

Key words: anaerobic glycerol conversion, 1,3-propanediol, thermophiles

Abstract

Of 60 different thermophilic enrichment cultures, 16 converted glycerol anaerobically to 1,3-propanediol. Two PD-forming strains were further enriched, isolated, and characterised. For the most active strain, AT1, the optimal cultivation parameters for pH and temperature were determined as 5.8 to 6.0 and 58 °C, respectively. In batch-fermentations with AT1, 6.4 g propanediol per litre was formed with a productivity of 0.17 g l^{-1} h^{-1}.

Introduction

The microbial production of 1,3-propanediol (PD) from glycerol by different mesophilic bacterial strains like *Klebsiella pneumoniae, Clostridium butyricum,* and *Citrobacter freundii* has been studied over the past ten years (Deckwer 1995, Biebl *et al.* 1999, Wittlich *et al.* 1999, Papanikolaou *et al.* 2000). Only scant attention was paid to finding new PD producing strains, although, except for the clostridia, the other strains are potentially pathogenic microorganisms which is a problem in any technical application.

The use of thermophiles for the anaerobic PD production could be advantageous for various reasons: as a rule, most thermophilic bacteria belong to the lowest microbiological risk class. Besides this, the use of thermophiles offers interesting options for making biotechnical PD production more cost-efficient (Canganella & Wiegel 2000). In thermophilic fermentation it is possible to directly use the hot effluents from fat cleavage plants at elevated temperatures of about 60 to 65 °C. Facilities for cooling large fermenters are partly or completely dispensable. Energy costs for heating up the fermentation broth for *down-stream-processing* by means of distillation are lowered. Moreover a potential by-product of PD production, i.e., ethanol, can probably easily be removed *in situ.*

Materials and methods

Cultivation medium

Enrichment cultures were done on reinforced clostridial medium (RCM) from Merck (Darmstadt, Germany), supplemented with 10 g glycerol l^{-1}.

For isolation of pure cultures the following mineral salt medium (MSM) was used: 10 g glycerol, 2.5 g $Na_2HPO_4 \cdot 2H_2O$, 1.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.1 $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, 1 mg resazurin, 0.5 g L-cysteine, 10 ml vitamin solution of DSMZ-medium 141 (DSMZ 2000), 10 ml trace element solution of DSMZ-medium 144 (DSMZ 2000), 3 g yeast extract, all per l, pH value set to 7 with NaOH.

For activity tests a content of 10 g 2-(*N*-morpholino)ethanesulfonic acid (MES) per l was used instead of phosphates and pH was set to 6.5 with NaOH.

In batch fermentation experiments with AT1 the concentration of all nutrients was doubled, buffering was done with 2 g MES per l.

Screening, enrichment and isolation

For enrichment of thermophilic PD formers an aliquot of sample material was incubated anaerobically at

60 °C in 30 ml RCM with glycerol. After a few days, growth was controlled via measuring turbidity and the supernatant of positive samples was examined. Cultures showing PD forming activity were successively transferred to MSM. From cultures showing stable PD forming activity in this medium pure cultures were gained by plating on MSN with 16 g agar l^{-1}.

Activity tests

Tests for determination of pH, temperature, and substrate concentration optima as well as tests regarding product inhibition were done in glass vials with butyl rubber stoppers in a volume of 30 to 50 ml without pH control. Incubation was done in a water bath.

Fermentations

Batch fermentations were carried out at a volume of 300 ml in a double-walled glass fermenter. pH value was controlled by adding NaOH solution, temperature was set via an external heating circulator. Anaerobic conditions were maintained by slight sparging with nitrogen. Data acquisition was done with the data processing software DASYlab (datalog, Mönchengladbach, Germany).

Analytical methods

The contents of glycerol and the conversion products were determined using HPLC with a cation type HPX87H column (Biorad, Munich, Germany) and a refraction index detector. Analysis was done at 60 °C (column) with 5 mM sulfuric acid as the eluent at a flow rate of 0.7 ml min^{-1}.

Results and discussion

Screening for PD forming bacteria

Of more than 60 samples from various sites worldwide which were tested for presence of thermophilic PD-forming microorganisms, 16 were able to utilise glycerol anaerobically, but only two of these cultures formed PD and kept on forming the diol after transferring to mineral salt medium. Pure cultures were gained and further characterised. Strain AT1 was the most active with regard to PD formation. It is a strictly anaerobic, Gram-positive, endospore-forming bacterium and hence probably belongs to the genus *Clostridium*.

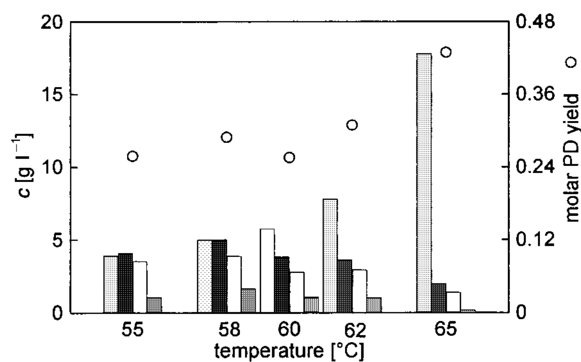

Fig. 1. Influence of temperature on the product formation and the PD yield of AT1 after 112 h. Start: 20 g glycerol l^{-1}, pH 6.5, volume 30 ml. Glycerol ( ), propanediol ( ), n-butyrate ( ), and ethanol ( ).

Some clostridial strains are described as butyrate producers, e.g., *C. thermobutyricum* (Wiegel *et al.* 1989), *C. thermopapyrolyticum* or *C. thermosaccharolyticum* (Canganella & Wiegel 1993).

Strain AT1 was characterised in terms of general parameters like pH and temperature optimum, nutrient requirements, product inhibition, and substrate optimum in various activity tests.

Temperature and pH value optimum

The optimum temperature was determined as 58 °C (Figure 1).

Neither cell growth nor PD formation was found even after more than four days at temperatures of 37 and 69 °C. Higher temperatures led to higher PD yields.

In anaerobic vial experiments AT1 showed a rather broad pH optimum with an optimum at 5.8 to 6.0.

Optimum of glycerol concentration

Differing from mesophilic PD producers that show rather small k_S-values for glycerol of only a few mg l^{-1} (Zeng *et al.* 1994), that of AT1 seems to be fairly high for AT1 (Figure 2). This may be due to transport through the cell membrane or the affinity to AT1's glycerol dehydratase, which is considered to be the key enzyme for PD production in *C. butyricum* (Abbad-Andaloussi *et al.* 1996).

Glycerol tolerance is high since glycerol concentrations up to 80 g glycerol l^{-1} are tolerated with only minor losses in activity, which is comparable to mesophilic strains.

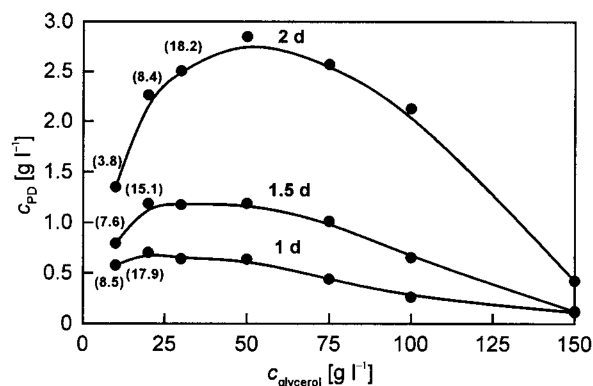

Figure 2. Influence of glycerol concentration on PD formation for AT1. Temperature 60 °C, pH 6.5, volume 30 ml. The numbers in brackets give the residual glycerol concentration c_{Glyc} at this time.

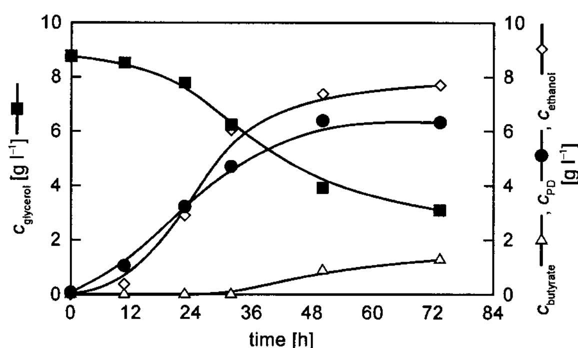

Figure 3. Anaerobic batch fermentation of AT1 on mineral salt medium with glycerol. Temperature 60 °C, pH 6, volume 300 ml.

Batch fermentations

Based on these experiments different batch fermentations with a reaction volume of 300 ml were done (Figure 3).

As expected under pH controlled conditions the PD productivity could be raised compared to experiments in vials. A volumetric productivity of about 0.17 g PD l^{-1} h^{-1}) was reached. The molar yield for PD is 0.39 which is about 1.5-fold less than for mesophilic *C. butyricum*. As the main by-product ethanol is formed and less *n*-butyrate, which is different from vial experiments. Within the fermentation, *n*-butyrate is first formed when PD formation is already levelling off or to put it more generally, when conditions for AT1 get worse, which might be the common aspect of vial and fermentor experiments. The maximum PD concentration that was reached was 6.4 g PD l^{-1}. No limitation of the various medium compounds could be verified after reaching

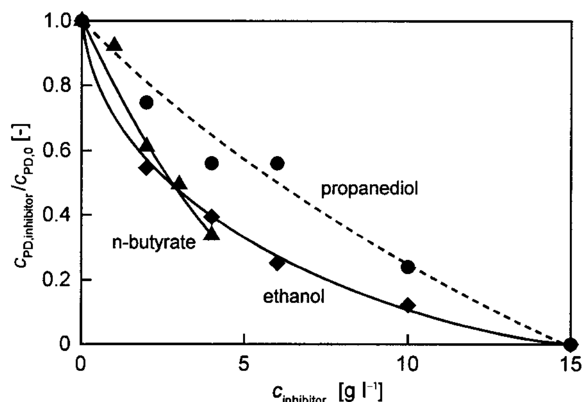

Figure 4. Inhibitory effect of major products of anaerobic glycerol fermentation on PD formation of AT1 after 48 h. Start: 20 g glycerol l^{-1}, temperature 60 °C, pH 6.5, volume 30 ml.

this concentration by adding these nutrients in higher concentration.

Product inhibition

Different products were tested regarding their inhibitory effect on AT1's growth and PD formation activity in vial experiments. Within these experiments a concentration of 15 g l^{-1} turned out to be completely inhibitory for PD or ethanol each (Figure 4).

Thus the cessation of PD formation at 6 to 7 g PD l^{-1} in the fermentation experiments could be due to combined inhibition by PD and by-product(s).

Conclusions

The productivity of AT1 is still low compared to mesophilic strains like *Clostridium butyricum*. The productivity that can be reached yet with mesophilic bacteria strongly depends on the type of cultivation. Values range from about 2 g PD l^{-1} h^{-1} for simple batch fermentation (Reimann & Biebl 1996), about 15 to 20 g PD l^{-1} h^{-1} for continuous culture with cell recycling (Reimann *et al.* 1998) to more than 30 g PD l^{-1} h^{-1} for continuous culture of *C. butyricum* entrapped in LentiKats (Wittlich *et al.* 1999). With mesophilic strains maximum PD concentration of about 65 to 70 g l^{-1} are found. The molar yields of course depend on the cultivation conditions and by-products formed. Usual values for mesophiles are 0.50 to 0.65.

Attempts must be made to evaluate possibilities for raising productivity by immobilisation of the thermophilic strain AT1. Since reaching as high a PD

concentration as possible is crucial for technical applications, the rather strong inhibitory effect of PD and by-products on the PD formation of AT1 has to be examined further. If the product inhibition turns out to be crucial, strain AT1 has to be adapted to higher product concentrations, e.g., by mutation and selection of more tolerant strains.

Parallel to this, more samples have to be screened and new thermophile strains must be found.

Acknowledgement

This work was supported by the Fachagentur Nachwachsende Rohstoffe e.V., Gülzow, Germany.

References

Abbad-Andaloussi S, Guedon E, Spiesser E, Petitdemange H (1996) Glycerol dehydratase activity: the limiting step for the 1,3-propanediol production by *Clostridium butyricum* DSM 5431. *Lett. Appl. Microbiol.* **22**: 311–314.

Biebl H, Menzel K, Zeng AP, Deckwer WD (1999) Microbial production of 1,3-propanediol. *Appl. Microbiol. Biotechnol.* **52**: 289–297.

Canganella F, Wiegel J (1993) The potential of thermophilic clostridia in biotechnology. In: Woods DR, ed. *The Clostridia in Biotechnology*. Stoneham: Butterworth, pp. 393–429.

Canganella F, Wiegel J (2000) Continuous cultivation of *Clostridium thermobutyricum* in a rotary fermentor system. *J. Ind. Microbiol. Biotechnol*. **24**: 7–13.

Deckwer WD (1995) Microbial conversion of glycerol to 1,3-propanediol. *FEMS Microbiol. Rev.* **16**: 143–149.

DSMZ (2000) German Collection of Microorganisms and Cell Cultures, List of media at URL: *http://www.dsmz.de/media/media.htm*

Papanikolaou S, Ruiz-Sanchez P, Pariset B, Blanchard F, Fick M (2000) High production of 1,3-propanediol from industrial glycerol by newly isolated *Clostridium butyricum* strain. *J. Biotechnol.* **77**: 191–208.

Reimann A, Biebl H (1996) Production of 1,3-propanediol by *Clostridium butyricum* DSM 5431 and product tolerant mutants in fedbatch culture: feeding strategy for glycerol and ammonium. *Biotechnol. Lett.* **18**: 827–832.

Reimann A, Biebl H, Deckwer WD (1998) Production of 1,3-propanediol by *Clostridium butyricum* in continuous culture with cell recycling. *Appl. Microbiol. Biotechnol.* **49**: 359–363.

Wiegel J, Seung-Uk K, Kohring GW (1989) *Clostridium thermobutyricum* sp. nov., a moderate thermophile isolated from a cellulolytic culture, that produces butyrate as the major product. *Int. J. Syst. Bacteriol.* **39**: 199–204.

Wittlich P, Schlieker M, Jahnz U, Willke T, Vorlop KD (1999) Bioconversion of raw glycerol to 1,3-propanediol by immobilized bacteria. In: *Proceedings Ninth European Congress on Biotechnology,* July 11–15, Brussels, no. P2762, ISBN 805215-1-5.

Zeng AP, Ross A, Biebl H, Tag C, Günzel B, Deckwer WD (1994) Multiple product inhibition and growth modelling of *Clostridium butyricum* and *Klebsiella pneumoniae* in glycerol fermentation. *Biotechnol. Bioeng.* **44**: 902–911.