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ORIGINAL RESEARCH PAPER

Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1

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Abstract A continuous cultivation process for the fermentative production of succinic acid from glycerol with the recently isolated bacterium *Basfia succiniciproducens* DD1 was developed. Crude glycerol (5.1 g l^{-1}) was used as C-source and NH_4OH as N-source and pH-control agent. The problem of wall growth was solved by transfers of the cultivation broth into an empty identical fermentor. The resulting continuous cultivation process was maintained for more than 80 days. Glycerol-limited steady states were established for dilution rates between 0.004 and 0.018 h^{-1}. Higher dilution rates resulted in glycerol accumulation. Succinic acid concentrations, productivities, yields and specific productivities increased with increasing dilution rates: at 0.018 h^{-1} the highest values were 5.21 g l^{-1}, 0.094 g l^{-1} h^{-1}, 1.02 g g^{-1} and 0.375 g g^{-1} h^{-1}, respectively.

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Introduction

Glycerol, a by-product of biodiesel production, is an inexpensive and abundant carbon source. The growth of the biodiesel industry has resulted in a 10-fold decrease in crude glycerol prices in 2 years (Murarka et al. 2008). Recently, the price of crude glycerol has fallen to about \$0.05 lb^{-1} (Johnson and Taconi 2007; Tang et al. 2009). Given the more reduced state of carbon in glycerol, its conversion to fuels or reduced chemicals can be obtained with higher yields than with common sugars (Dharmadi et al. 2006).

Succinic acid is a main building block that can be produced from renewable resources via fermentation (Patel et al. 2006; Werpy and Peterson 2004). Succinic acid and its salts have several possible applications: e.g. direct use as monomer and in the food, pharmaceutical and cosmetic industries as well as its conversion into different monomers, solvents and other chemicals currently made from petroleum (Bechthold et al. 2008; McKinlay et al. 2007). Bacteria producing impressive succinic acid concentrations and yields have been described: most prominent is *Actinobacillus succinogenes* (106 g l^{-1} and

0.9 g g^{-1}; Guettler et al. 1996). Succinic acid concentrations above 50 g l^{-1} were obtained with glucose as C-source, yeast extract and an additional complex N-source.

Recently, a new bacterium DD1 was isolated from bovine rumen (Scholten and Dägele 2008). It converts D-glucose and other common sugars as well as glycerol into succinic acid. With glucose almost equimolar amounts of succinic, acetic and formic acid were formed. With pure and crude glycerol succinic acid was formed almost exclusively with a yield of 1.2 g g^{-1}. This indicates two advantages of succinic acid production from glycerol instead of sugars: (i) a lower C-source demand per gram of succinic acid, (ii) substantially less by-products (Scholten and Dägele 2008).

Our goal was to establish a robust continuous cultivation process for succinic acid production from crude glycerol with strain DD1 under highly production relevant conditions. The resulting set up allows continuous succinic acid production for several months.

Materials and methods

Bacterial strains

The novel succinic acid-producing bacterial strain DD1 is facultatively anaerobic. It was isolated from rumen content originating from a cannulated Holstein cow, belongs to the family *Pasteurellaceae* (Scholten and Dägele 2008) and was deposited with DSMZ having the deposit number DSM 18541. It is classified as *Basfia succiniciproducens* gen. nov., sp. nov. (Kuhnert et al. 2009).

Media

Seed cultivations were performed in medium A (Scholten and Dägele 2008) with glucose at 50 g l^{-1}. Main cultivations were conducted in medium B containing (per litre) 0.5 g yeast extract, 1 g $(NH_4)_2SO_4$, 0.2 g $CaCl_2{\cdot}2H_2O$, 0.2 g $MgCl_2{\cdot}6H_2O$, 1 g NaCl, 3 g K_2HPO_4 and 5.1 g crude glycerol produced by ecoMotion GmbH Sternberg. In a pretest of different crude glycerols this type resulted in the highest succinic acid productivity and yield.

Cultivation conditions

Seed cultures were grown for 8 h in 100 ml serum bottles with gas-tight butyl rubber stoppers containing 50 ml medium A with 50 g glucose l^{-1} and a CO_2 atmosphere with 0.8 bar overpressure at 170 rpm (shaking diameter: 2.5 cm).

Inoculation of the seed cultures was performed with 1 ml of a working cell bank. To prepare an inoculum without residual glucose, peptone and $MgCO_3$ the seed cultures were centrifuged and the cell pellet was washed once and then resuspended in 50 ml main culture medium without a carbon source.

Continuous cultivations were performed in 500 ml standard fermentors (Sixfors, Infors, Switzerland) containing 300 ml medium which had been gassed overnight with CO_2 to ensure O_2-free conditions. Inoculation was performed with 15 ml of the washed cells. CO_2 flow and stirrer speed were adjusted to 0.1 l min^{-1} and 500 rpm, respectively. pH control was performed with NH_4OH (227 g l^{-1}). The pH setpoint was 6.5 which, in a pretest, gave higher succinic acid production than pH 5.8 and 7.2. The fermentors were equipped with a sampling tube for the effluent. This tube was inserted through the top plate and adjusted to maintain the reaction volume constant at 300 ml. To prevent back-growth from the fermentor into the feed reservoir and from the effluent into the fermentor the respective tubes were connected to sterile traps. The traps were changed when necessary. For the precise control of the feeding (a dilution rate of 0.004 h^{-1} requires a feed stream of 1.2 g h^{-1}) enclosed lab balances with two digits (LP 3200 D, Sartorius, Germany) were used. Whenever first signs of wall growth became visible at the gas–liquid interface the cultivation broth was transferred into an identically equipped and freshly prepared fermentor. This was necessary every 2 weeks.

Sampling and analytics

Broth samples were taken directly from the fermentor or from the effluent of the continuous cultivation and held on ice. Biomass was calculated from OD_{600} values that were performed immediately after sampling. Succinic acid, by-products and residual glycerol were determined via HPLC analysis of the undiluted cell-free supernatants of the cultivation broth. [Cell separation was performed by filtration

(0.22 μm).] An Aminex HPX-87 H column (Biorad, USA) and 5 mM H_2SO_4 were used as stationary and mobile phase, respectively. Biomass concentration X was calculated from OD_{600} values using a linear equation determined from $X = f(OD_{600})$-plots for strain DD1.

Calculation of specific rates

Specific succinic acid productivity p and specific glycerol consumption q were calculated from the steady state values of (i) dilution rate D, (ii) concentration difference between incoming and outgoing stream of the continuous cultivation Δc, and (iii) the biomass concentration X according to:

$$p = D\ \Delta c_{succinic} X^{-1} \quad (1)$$

$$q = D\ \Delta c_{glycerol} X^{-1} \quad (2)$$

Results and discussion

Succinic acid production in the continuous cultivation could be maintained free of contamination and free of any strain instability for more than 80 days. The problem of wall growth (which became visible 18 days after inoculation) was efficiently solved by transfers of the cultivation broth into an identically equipped and freshly prepared alternative fermentor. Steady state concentrations and calculated parameters of the glycerol-limited continuous cultivations are summarized in Tables 1 and 2.

Table 2 Calculated steady state parameters for succinic acid production in the glycerol-limited continuous cultivation of strain DD1 with crude glycerol (5.1 g l^{-1}) as C-source

D (h^{-1})	P (g l^{-1} h^{-1})	$Y_{P/S}$ (g g^{-1})
0.004	0.016	0.79
0.008	0.029	0.71
0.012	0.052	0.84
0.014	0.07	0.97
0.018	0.094	1.02

D dilution rate. Values are means of at least three independent measurements over at least 72 h, sample standard deviation was below 0.001 h^{-1} in all cases

P productivity for succinic acid (g succinic acid l^{-1} h^{-1})

$Y_{P/S}$ yield for succinic acid [g succinic acid (g glycerol)$^{-1}$]

The data show that the steady state succinic acid concentrations increase with increasing dilution rates, D, and hence with increasing specific growth rates, μ. Consequently, succinic acid productivities, P, and yields, $Y_{P/S}$, increase with the specific growth rates, too. The highest specific growth rate which could be maintained under glycerol-limitation was 0.018 h^{-1}. Further even small increases of D resulted in glycerol accumulation. The corresponding highest succinic acid productivity P and yield $Y_{P/S}$ were 0.094 g l^{-1} h^{-1} and 1.02 g g^{-1}, respectively. This productivity P is almost one order of magnitude lower whereas the yield $Y_{P/S}$ is only just slightly lower than the respective values obtained with strain DD1 in batch cultivations in complex medium with 5 g peptone l^{-1} and $MgCO_3$ as buffer (0.9 g l^{-1} h^{-1} and 1.2 g g^{-1}; Scholten and Dägele 2008).

Table 1 Measured steady state concentrations in the glycerol-limited continuous cultivation of strain DD1 with crude glycerol (5.1 g l^{-1}) as C-source

D (h^{-1})	$c_{glycerol}$ (g l^{-1})	$c_{succinic}$ (g l^{-1})	c_{lactic} (g l^{-1})	c_{acetic} (g l^{-1})	X (g l^{-1})
0.004	0	4.01 ± 0.13	0.09 ± 0.01	0.75 ± 0.06	0.21
0.008	0	3.62 ± 0.05	0.45 ± 0.04	1.16 ± 0.07	0.22
0.012	0	4.30 ± 0.07	0.23 ± 0.01	0.99 ± 0.01	0.31
0.014	0	4.97 ± 0.05	0.18 ± 0.02	0.77 ± 0.06	0.3
0.018	0	5.21 ± 0.07	0.33 ± 0.04	0.41 ± 0.03	0.25

D dilution rate. Values are means of at least three independent measurements over at least 72 h, sample standard deviation was below 0.001 h^{-1} in all cases

c Concentration of the respective compound. Values are means of at least three independent measurements over at least 72 h ± sample standard deviation, sample standard deviation for $c_{glycerol}$ was 0 g l^{-1} in all cases

X biomass concentration (g cell dry weight l^{-1}). Values were calculated from OD_{600} optical density measured at 600 nm. Values are means of at least three independent measurements over at least 72 h

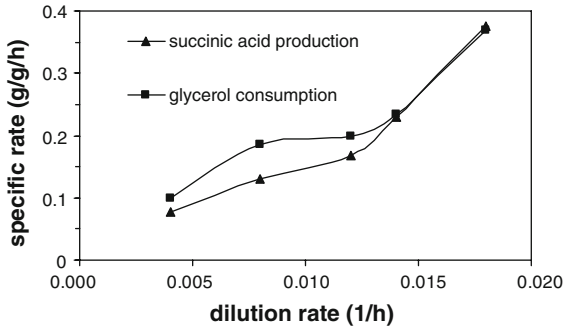

Fig. 1 Specific rates of succinic acid production, p, and glycerol consumption, q, as a function of the dilution rate, D, in the glycerol-limited continuous cultivation of strain DD1

Figure 1 shows the specific rates of succinic acid production p and glycerol consumption q as a function of the dilution rate D and hence as a function of the specific growth rate μ.

Both specific rates increase with increasing dilution rates D and hence with increasing specific growth rates, μ. The highest specific succinic acid productivity, 0.375 g succinic acid (g cell dry weight)$^{-1}$ h^{-1}, is obtained for $D = \mu = 0.018$ h^{-1}. The difference between the two curves at the three lowest specific growth rates ($\mu = 0.004$ h^{-1}, 0.008 h^{-1} and 0.012 h^{-1}, respectively) reflects the fact that $Y_{P/S}$ is clearly below 1 g g^{-1}. In contrast, $Y_{P/S} = 0.97$ and 1.02 g g^{-1} is obtained at the two highest specific growth rates (Table 2). The different concentration of acetic acid, which is the most abundant by-product detected, also reflect this fact (0.75 g l^{-1} to 1.16 g l^{-1} at the lower vs. 0.41 g l^{-1} to 0.77 g l^{-1} at the higher specific growth rates, μ).

The results in Table 2 and Fig. 1 clearly show that succinic acid production by strain DD1 is getting more efficient when the specific growth rate, μ, increases. This indicates that more efficient succinic acid producing mutants of strain DD1 could be selected in a continuous cultivation process by selection for faster growing cells. This could be a tool to further improve rationally developed strains under production-relevant conditions and could be of special interest when certain strain improvements cannot be realized straight forward by a set of defined genetic modifications (e.g. improved product- or pH-tolerance).

Considering possible pathways from glycerol to dihydroxyacetone phosphate (Gonzalez et al. 2008), its conversion to phosphoenolpyruvate (PEP) and the different pathways from PEP to succinic acid (Lee et al. 2006) conversion of glycerol to succinic acid can at best result in the net production of 1 ATP per glycerol via substrate level phosphorylation. Additional ATP generation via electron transport coupled phosphorylation can be expected by fumarate respiration in which fumarate functions as electron acceptor and upon its reduction is converted to succinic acid. However, succinic acid cannot become the only product formed from glycerol. Since this is a redox-balanced pathway, an additional pathway is required to consume the excess of reducing equivalents generated during biomass formation from glycerol. Murarka et al. (2008) have found clear evidence that production of 1,2-propanediol is the means to consume reducing equivalents in *E. coli*.

To make the process suitable for commercial succinic acid production of course both succinic acid concentration and productivity have to be increased substantially. If this is achieved the current process can become more cost efficient than other succinic acid processes because of (i) the higher yield for succinic acid $Y_{P/S}$ which can be achieved with glycerol instead of common sugars [maximum theoretical values without biomass formation: 1.28 g g^{-1} with glycerol (Dharmadi et al. 2006) versus 1.12 g g^{-1} with glucose (McKinlay et al. 2007)], (ii) the lower price of crude glycerol from biodiesel production, (iii) the cheaper down stream processing since lower amounts of by products have to be separated and (iv) gaseous ammonia for pH-control being cheap, making an additional N-source unnecessary and resulting just in negligible dilution of the broth. Another important feature of our process is the formation of ammonium succinate which can be converted chemically into the C4-chemicals tetrahydrofuran, 1,4-butanediol, gamma-butyrolactone (Bauduin et al. 2007) and pyrrolidones (Fischer et al. 2006) without isolation of succinic acid.

Strain DD1 was isolated from the rumen of a cow and belongs to the family *Pasteurellaceae* (Scholten and Dägele 2008). Many *Pasteurellaceae* members contain virulence factors being cytotoxic for eukaryotic host cells and RTX toxins are the most prominent representatives (Frey and Kuhnert 2002). Hence, special attention was given to examine DD1 for virulence factors. In cytotoxicity assays with *Mannheimia haemolytica* as positive control no toxicity

towards bovine, human and fish cells was observed for strain DD1. In addition, no RTX toxin genes were detected in Southern-blot hybridizations as well as in silico screening of the genome sequence of DD1 (Kuhnert et al. 2009). Thus, pathogenicity has conclusively been ruled out for DD1. Its rational improvement is now under way.

Conclusions

The established continuous cultivation process for the fermentative production of succinic acid from crude glycerol with strain *Basfia succiniciproducens* DD1 has the following attractive characteristics:

I. Great process stability allowing continuous cultivations over periods of at least several months
II. Potential to achieve attractive production cost for fermentative succinic acid, chemicals made from it and polymers containing succinic acid
III. Pathogenicity of the production strain has conclusively been ruled out

To seriously evaluate if the continuous cultivation approach is an option for commercial succinic acid production of course the final production strain has to be tested. Because of the lower surface-volume-ratio the problem of wall growth should not be so relevant in the production scale.

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