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Crude glycerol as feedstock for the sustainable production of *p*-hydroxybenzoate by *Pseudomonas putida* S12

Research Paper

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Crude glycerol is a promising renewable feedstock in bioconversion processes for the production of fuels and chemicals. Impurities present in crude glycerol can however, negatively impact the fermentation process. Successful crude glycerol utilization requires robust microbial production hosts that tolerate and preferably, can utilize such impurities. We investigated utilization of crude, unpurified glycerol as a substrate for the production of aromatic compounds by solvent tolerant *Pseudomonas putida* S12. In high-cell density fed-batch fermentations, *P. putida* S12 surprisingly performed better on crude glycerol than on purified glycerol. By contrast, growth of *Escherichia coli* was severely compromised under these high cell density cultivation conditions on crude glycerol. For *P. putida* S12 the biomass-to-substrate yield, maximum biomass production rate and substrate uptake rate were consistently higher on crude glycerol. Moreover, production of *p*-hydroxybenzoate by engineered *P. putida* S12palB5 on crude glycerol showed a 10% yield improvement over production on purified glycerol. *P. putida* S12 is a favorable host for bioconversion processes utilizing crude glycerol as a substrate. Its intrinsic stress-tolerance properties provide the robustness required for efficient growth and metabolism on this renewable substrate.

Introduction

In recent years, biodiesel has become an established renewable fuel, which can for a large part be attributed to financial stimuli from local governments. As the subsidies are being abolished, however, valorization of the crude glycerol waste (approximately 0.08 kg of glycerol per liter of biodiesel, *i.e.*, around 10% by weight [1]) is becoming essential for biodiesel to become a profitable proposition [2–5]. A promising outlet for crude glycerol is its use as a renewable feedstock in bioconversion processes for the production of fuels and (base) chemicals [6,7]. However, the presence of impurities in crude glycerol such as methanol, free fatty acids, and salts (especially sodium or potassium) can have a negative impact on the fermentation process [8–11]. Efficient utilization of crude glycerol therefore requires robust fermentation hosts that tolerate the impurities associated with this renewable fermentation feedstock.

In this study, we evaluated the use of crude glycerol as potential carbon source for the bioproduction of substituted aromatics. These compounds can be produced by engineered solvent-tolerant *Pseudomonas putida* strains [12,13] or engineered *Escherichia coli* strains [14,15]. For *E. coli*, crude glycerol has been reported to be an inferior substrate [16], whereas other authors have stated that it is a suitable substrate for growth and production [17–19]. This paradox mainly results from differences in the concentration of crude glycerol applied. At lower concentrations, the impact of inhibiting

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impurities is obviously also lower, leading to conflicting crude glycerol tolerance results.

P. putida S12 can tolerate diverse types of aggressive compounds, ranging from organic solvents and antibiotics to heavy metals [20–23]. It was therefore expected that *P. putida* S12 would outperform *E. coli* on crude glycerol as the substrate. In the present study, *P. putida* S12 and *E. coli* were grown on purified and on crude glycerol in high-cell density fed-batch cultivations. In addition, the production of *p*-hydroxybenzoate from crude glycerol by an engineered *P. putida* S12 strain was evaluated.

Materials and methods

Bacterial strains and culture conditions

The following strains were used in this study: wild type *P. putida* S12 (ATCC 700801), *E. coli* DH5α (Invitrogen) and a *p*-hydroxybenzoate producing strain *P. putida* S12palB5 [24]. Shake flask experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of a phosphate buffered mineral salts medium [24], supplemented with 40 mM of either purified glycerol (MMGly) or crude glycerol (MMRGly). For *E. coli*, thiamine (25 mg l^{-1}) was added to the minimal medium. To cultures of *P. putida* S12palB5, 0.1 mM of sodiumsalicylate and 10 μg ml^{-1} of gentamicin were added. Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.2 with cells from a late-log phase preculture on MMGly. The *P. putida* S12 strains were grown at 30 °C, while *E. coli* DH5α was grown at 37 °C.

Fed-batch experiments were performed in 2-l fermentors (New Brunswick Scientific) using a BioFlo110 controller. The initial batch phase was started with 50 ml of a preculture on MMGly. For the batch phase, 1 l of mineral salts medium was used with the following composition (per l): (crude) glycerol, 18.4 g; $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 3.88 g; $NaH_2PO_4{\cdot}H_2O$, 1.63 g and 20 ml of trace element solution [12]. After depletion of the initially supplied glycerol, the glycerol feed was started and controlled to support maximum growth. The feed solution contained (per l): (crude) glycerol, 736 g and $MgCl_2{\cdot}6H_2O$, 10 g. For *P. putida* S12palB5, gentamicin (10 mg l^{-1}) and sodiumsalicylate (0.1 mM) were added to the batch culture and the feed solution. For *E. coli*, 25 mg of thiamine was added during the batch phase and after 30 h of cultivation another 25 mg of thiamine was added to the culture broth.

The initial stirring speed was set to 200 rpm and air was supplied to the head space at 1 l min^{-1} using a M+W Instruments D-5111 mass-flow controller. Dissolved oxygen tension (DO) was continuously monitored with an InPro model 6900 probe and maintained at 30% air saturation by automatic adjustment of the stirring speed to a maximum of 1000 rpm. When the maximum stirring speed was reached, air was replaced with purified oxygen at a flow of 0.2 l min^{-1} and the maximum stirring speed was set to 800 rpm. The pH was maintained at 7.0 by automatic addition of 25% NH_4OH and the temperature was kept at 30 °C for *P. putida* S12 strains and 37 °C for *E. coli* DH5α. Samples were drawn during the culture to determine cell density and concentrations of glycerol, acetate and *p*-hydroxybenzoate.

Analytical methods

Cell densities were determined at 600 nm with an Ultrospec 10 cell density meter (Amersham Biosciences). An OD_{600} of 1 corresponds to 0.49 g $liter^{-1}$ of cell dry weight (CDW). Glycerol and acetate were analyzed by ion chromatography (Dionex ICS3000 system), using an IonPac ICE AS1 column with 100 mM methyl sulphonic acid as eluent for glycerol or an IonPac ICE AS6 column with 0.4 mM heptafluorobutyric acid as the eluent for acetate. The *p*-hydroxybenzoate was analyzed by HPLC (Agilent 1100 system) using a Zorbax 3.5 μm SB-C18 column (4.6 mm × 50 mm) and a diode-array detector set at 254 nm as described previously [12]. Analysis of metals in crude glycerol was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 5300dv, PerkinElmer Inc.). Before analysis, crude glycerol was diluted 100 times in 0.5 M nitric acid. The calibration samples for the different elements were supplemented with purified glycerol to a final concentration of 10 g l^{-1} to exclude artifacts due to the presence of glycerol in the crude glycerol sample.

Chemicals

Crude glycerol (~80% glycerol content; sample number A-5106) was kindly provided by Cremer Oleo GmbH & Co. KG, Hamburg, Germany. Analytical grade (purified) glycerol was purchased from Fisher Emergo BV, The Netherlands. Glycerol, both crude and purified, was sterilized by autoclaving for 18 min at 105 °C. Stock solutions for sodiumsalicylate and thiamine were prepared and

TABLE 1

Metal concentrations in crude glycerol and pure glycerol media in mg l^{-1}

	Na	K	Ca	Mg	Fe	Mo	Zn	Cu	Mn	Co
Crude glycerol (80% (w/v))[a]	39100	512	48.9	45.1	3.5	2.3	1.5	<1[d]	<1[d]	ND[e]
MMGly[b] (with trace elements)	314	1738	0.27	12.0	1.0	0.08	0.45	0.05	0.34	0.10
MMRGly[c] (with trace elements)	443	1740	0.43	12.1	1.0	0.09	0.46	0.05[f]	0.34[f]	0.10[f]
MMGly[b] – trace elements	314	1738	–	–	–	–	–	–	–	–
MMRgly[c] – trace elements	443	1738	0.16	0.1	–	0.01	0.01	–[f]	–[f]	–[f]
MMGly[b] + only Fe and Mg salts	314	1738		12.0	1.0	–	–	–	–	–
MMRgly[c] +only Fe and Mg salts	443	1740	0.16	12.1	1.0	0.01	0.01	–[f]	–[f]	–[f]

[a] Measured with elemental analysis.
[b] Calculated based on the mineral salts medium composition.
[c] Calculated based on the mineral salts medium composition and on the crude glycerol composition for 40 mM of crude glycerol.
[d] Below detection limit.
[e] ND: not determined.
[f] Assuming that this element is not present in crude glycerol; MMGly: mineral salts medium with 40 mM glycerol; MMRGly: mineral salts medium with 40 mM crude glycerol.

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filter sterilized just before use. All other medium components were autoclaved for 20 min at 121 °C.

Results and discussion

Crude glycerol: composition and evaluation as substrate in shake flask cultures

Crude glycerol contains various impurities like methanol, salts and fatty acids [1]. In the present study, crude glycerol from rapeseed oil was used with the following specifications: 80% (w/v) glycerol, max 10% (w/v) ash, max 3% (w/v) non-glycerol organic matter, max 0.5% (w/v) methanol and water. This composition was in line with the values published by Thompson and He [1]. The actual glycerol concentration was experimentally verified to be 1.0 kg l^{-1} which corresponds to a glycerol content of 79% (w/v). The ash content was analyzed and found to consist mainly of sodium salts (sodium ion concentration 39 g l^{-1}, *i.e.*, 3.1% (w/v)), while other metals were present only in trace amounts (Table 1).

To test whether crude glycerol could serve as substrate for *P. putida* S12 and *E. coli* DH5α, shake flask cultivations were performed on 40 mM of either purified or crude glycerol. No pronounced growth differences were observed between cultures on crude or purified glycerol for both *P. putida* (Fig. 1) and *E. coli* (data not shown). At the diluted crude glycerol concentrations applied, the absence of growth-impeding effects can likely be attributed to low inhibitor concentrations, in agreement with previous observations [18,19]. We calculated that the change in metal composition of the growth medium by the addition of 40 mM crude glycerol was negligible (Table 1). Despite a minor effect on overall medium metal content, here we empirically established that crude glycerol could replace the trace element solution in the medium, with the exception of magnesium and iron, to sustain growth of *P. putida* S12 (Fig. 1).

Fed-batch cultivation of P. putida *S12 on purified and crude glycerol*

The diluted crude glycerol did not have an adverse effect on bacterial growth in shake flask cultures. However, concentrated feed streams used in industrial fermentations are likely to result in the accumulation of feedstock-associated fermentation inhibitors to toxic levels. Therefore, high-cell density fed-batch cultivations were performed with concentrated feeds of both crude and purified glycerol.

Remarkably, wild type *P. putida* S12 showed better performance on crude compared to purified glycerol: a higher final biomass density (60 *vs* 44 g l^{-1}), maximum growth rate and substrate uptake rate were achieved (Fig. 2a; Table 2). Also the biomass-to-substrate yield (Y_{xs}) was higher on crude glycerol when calculated for the amount of glycerol consumed. Notably, the crude glycerol preparation contained 3% (w/v) non-glycerol carbon. Assuming that the non-glycerol carbon consists of C_{22} fatty acids

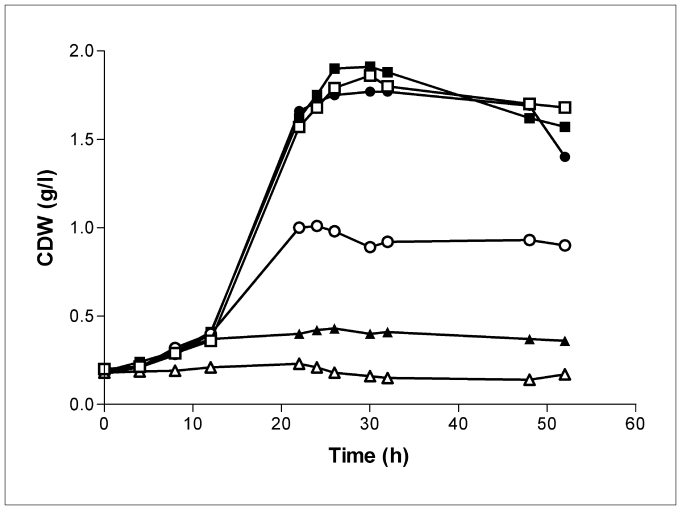

FIGURE 1

Growth of *P. putida* S12 on mineral medium with 40 mM purified (open symbols) and crude glycerol (closed symbols) in shake flask cultivations. With addition of mineral salts (□/■), no addition of mineral salts (△/▲) or only addition of Fe and Mg salts (○/●). See Table 1 for the concentrations of the mineral salts in the media.

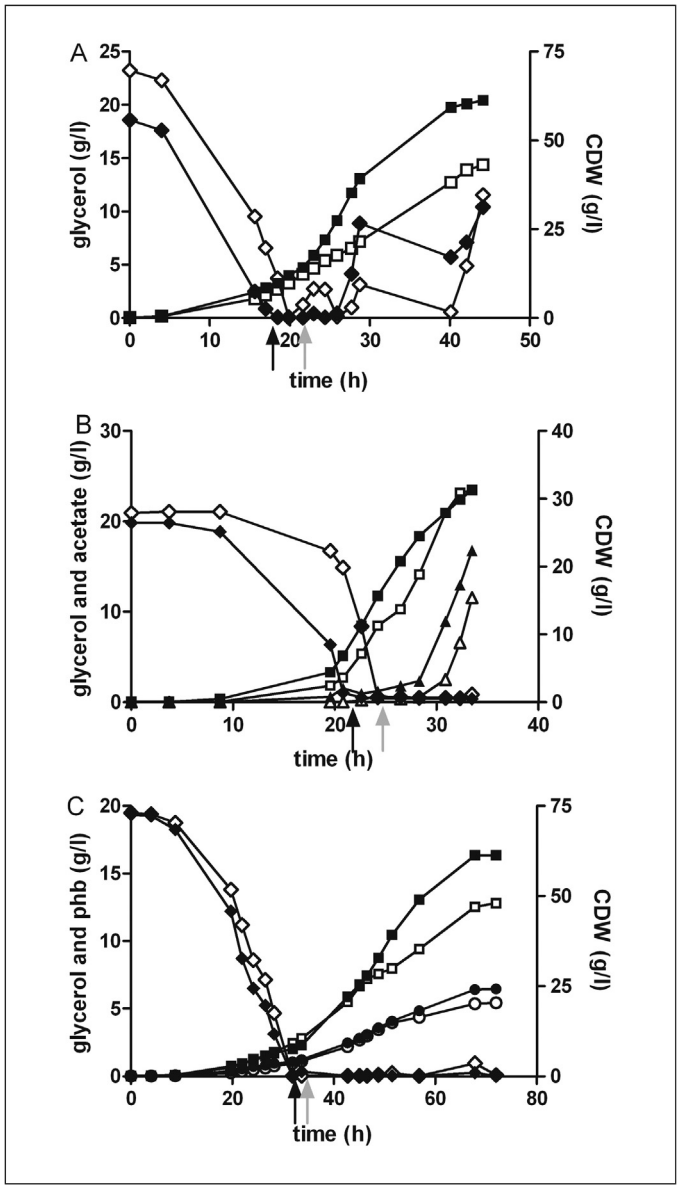

FIGURE 2

Fed-batch cultivations on purified (open symbols) and crude glycerol (closed symbols) of *P. putida* S12 **(a)**, *E. coli* DH5α **(b)** and *P. putida* S12palB5 **(c)**; cell dry weight (CDW) (□/■),glycerol (◇/◆),acetate (△/▲) and *p*-hydroxybenzoate (○/●). The arrows indicate the start of the glycerol feed (filled: crude glycerol, grey: purified glycerol). The data presented are from a single representative experiment.

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TABLE 2

Characteristics of fed-batch cultivations of *P. putida* S12, *P. putida* S12palB5 and *E. coli* DH5α on purified and crude glycerol as substrate

	P. putida S12		*P. putida* S12palB5		*E. coli* DH5α	
	Purified	Crude	Purified	Crude	Purified	Crude
CDW (g l^{-1})	43.7 ± 0.9	59.9 ± 2.1	47.3 ± 1.0	55.6 ± 7.9	32.8 ± 2.1	30.9 ± 0.7
Y_{xs} (Cmol%)[a]	54.8 ± 0.5	58.8 ± 0.6	43.2 ± 0.8	50.7 ± 0.3	45.8 ± 0.9	42.0 ± 0.6
Y_{xs*} (Cmol%)[b]		54.9 ± 0.6		47.4 ± 0.3		39.2 ± 0.6
Y_{ps} (Cmol%)[a]			5.9 ± 0.2	6.6 ± 0.1		
Y_{ps*} (Cmol%)[b]				6.1 ± 0.1		
Y_{px} (Cmol%)			13.6 ± 0.2	12.9 ± 0.2		
$r_{x,max}$ (Cmol h^{-1})	0.10 ± 0.01	0.17 ± 0.02	0.09 ± 0.01	0.10 ± 0.02	0.18 ± 0.03	0.12 ± 0.01
$r_{s,max}$ (Cmol h^{-1})[a]	0.19 ± 0.01	0.26 ± 0.02	0.16 ± 0.01	0.19 ± 0.01	0.34 ± 0.02	0.43 ± 0.04
$r_{p,max}$ (Cmol h^{-1})			0.012 ± 0.0002	0.012 ± 0.001		
p-Hydroxybenzoate (g l^{-1})			5.3 ± 0.2	6.0 ± 0.6		
Acetate (g l^{-1})					11.5 ± 1.2	16.2 ± 0.8
Na^+ (mM)[c]	12.4 ± 0.4	285 ± 5	11.4 ± 0.2	246 ± 63	11.8 ± 0.7	170 ± 4

Data are from two representative fed-batch cultivations, errors represent the maximum deviation from the mean.

[a] Yield calculated from the consumed amount of glycerol.

[b] Corrected yield, calculated based on total amount of carbon present; assumed that the 3% (w/v) organic matter (non-glycerol) has all been utilized and consists only of fatty acids with an average chain length of 22 [1] which resulted in 7% more available carbon. Furthermore, no carbon contribution was expected from the 0.5% methanol present, since it has assumed to be evaporated during the autoclaving process.

[c] Calculated based on the amount of crude glycerol added and the elemental composition of crude glycerol (see Table 1).

[1], the actual amount of total available carbon is higher by 7%. Moreover, previous reports have indicated that fatty acids may stimulate growth in *P. putida* [25,26]. When the biomass-to-substrate yield was corrected for consumption of non-glycerol carbon, the yields on crude and purified glycerol were comparable (55 Cmol%; Table 2).

The improved performance of *P. putida* S12 on crude glycerol demonstrated that salts or other impurities from crude glycerol did not decrease, but rather stimulated, the performance of *P. putida* S12 under the conditions tested. *P. putida* S12 has been found to be rather insensitive to NaCl concentrations below 500 mM (unpublished data). The accumulation of salts to a maximum of 6.5 g l^{-1}(283 mM) of sodium in fed-batches on crude glycerol (Table 2) clearly did not negatively affect growth of *P. putida* S12.

Fed-batch cultivation of E. coli *DH5α on purified and crude glycerol*

By contrast to *P. putida* S12, performance of *E. coli* DH5α was clearly affected on crude glycerol in high cell-density fed batch cultivation (Fig. 2b). The biomass yield (calculated either on a glycerol or on a total carbon basis (46 *vs* 39 Cmol%; Table 2)) and growth rate were lower on crude glycerol (Table 2). Final biomass density was comparable (Fig. 2b; Table 2), even though the substrate uptake rate was higher for crude glycerol. Substantial accumulation of acetate was observed on both purified (11.5 g l^{-1}) and crude glycerol (16.2 g l^{-1}, Table 2). Not only the amount of accumulated acetate, but also the acetate yield was higher in the crude-glycerol cultures: 0.15 g g^{-1} of glycerol, *vs* 0.12 g g^{-1} on purified glycerol.

The reduced performance of *E. coli* on crude glycerol can be attributed at least in part to the extensive acetate formation. The accumulation of acetate not only may lower the substrate availability for biomass formation, but also may exert growth inhibiting effects [27]. The formation of acetate by aerobically-grown *E. coli* is a well-known phenomenon that relates to the inability of the respiratory system to keep up with carbon metabolism, resulting in the fermentation of excess carbon to acetate [28]. Acetate formation can be overcome, but only at the cost of a reduced growth rate. By lowering the specific growth rate to below a crucial value, conditions may be avoided at which the respiratory capacity is limiting [28,29]. Thus, high cell density cultivations of *E. coli* on glycerol are possible up to 180 g l^{-1} CDW [27], but the cultivation time is increased due to the lower growth rate that must be imposed. This fact has been disregarded in previous studies on *E. coli* utilization of crude glycerol.

In addition to acetate formation, the accumulation of feedstock-associated inhibitors (particularly sodium) also play an important role in the reduced performance of *E. coli* on crude glycerol with concentrated feed streams. Inhibitory compounds may exert a direct inhibiting effect on growth, but may also contribute indirectly to acetate formation. The final salt concentrations at which growth ceased in fed batch cultivations of *P. putida* S12 were much higher than those in the *E. coli* cultures (Table 2). Thus, *E. coli* was more readily affected than *P. putida* by the accumulation of salts and/or other impurities from crude glycerol. Although the effect of acetate accumulation cannot be considered separately from intrinsic inhibitory effects on the performance of *E. coli*, it is clear that *P. putida* S12 is better able to use crude glycerol as a substrate. Presumably, the intrinsic tolerance of *P. putida* S12 to diverse types of aggressive compounds [20–23] contributes to the superior performance of this bacterium.

Production of p*-hydroxybenzoate from crude glycerol*

p-Hydroxybenzoate (pHB) is used for the production of liquid crystal polymers (LCPs), high-performance plastics that are widely employed in electronic devices. Hence, pHB is an industrially highly relevant compound, but it can additionally be regarded

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as a model compound for aromatic compounds that are derived from aromatic amino acids such as *t*-cinnamate, *p*-coumarate and *p*-hydroxystyrene [13,30,31]. Previously, we have constructed and optimized a *P. putida* S12-derived strain for the production of *p*-hydroxybenzoate from (model) renewable feedstocks such as glucose, purified glycerol [24] and xylose [32]. In view of the favorable performance of wild type *P. putida* S12 on crude glycerol, we have evaluated the use of this renewable feedstock for the production of *p*-hydroxybenzoate.

The *p*-hydroxybenzoate producing strain *P. putida* S12palB5 showed similar behavior to wildtype *P. putida* S12 with regard to performance on crude *vs* purified glycerol (Fig. 2c). A higher final biomass density (56 *vs* 47 g l^{-1}; Table 2) was achieved, in addition to a higher growth rate and an increased substrate uptake rate. Also the biomass-to-substrate yield was slightly elevated even when corrected for the consumption of non-glycerol carbon (47 *vs* 43 Cmol%; Table 2). In addition to more efficient growth, also the *p*-hydroxybenzoate production was positively affected on crude glycerol, in agreement with previous observations that growth and aromatics biosynthesis are tightly coupled in engineered *P. putida* S12 [12,30,33]. The final product concentration (6.0 *vs* 5.3 g l^{-1}; Fig. 2c), as well as the product-to-substrate yield (Y_{ps}), were higher on crude glycerol compared to purified glycerol (Table 2). The product-to-biomass yield (Y_{px}) was slightly lower, in line with an elevated biomass-to-substrate yield on crude glycerol. Apparently, the biomass formation benefited slightly more than pHB production from the increased carbon efficiency on crude glycerol. Nonetheless, the maximum *p*-hydroxybenzoate production rate ($r_{p,max}$) was identical on both carbon sources (Table 2). The biomass to substrate yield Y_{xs} of the pHB-production strain was overall lower than in wild type *P. putida* S12. This clearly indicates that pHB production posed a substantial drain on biosynthetic intermediates. In addition, production of pHB induces a solvent stress-response that results in an additional energy demand [24,34–36]. This is likely to have contributed to the overall slightly reduced combined yield of biomass and product.

Conclusions

Crude glycerol is an excellent substrate for growth and aromatics production by *P. putida* S12 especially at high cell density cultivations, resulting in superior performance (yields and rates) over purified glycerol. With *E. coli*, crude glycerol had an adverse effect on the performance in high cell density cultivations. This may – in part – be alleviated through tight control of fermentation conditions, but only at the cost of decreased growth rate and hence, productivity. The tolerance of *P. putida* S12 towards chemical aggression, its high respiratory capacity, and the absence of overflow metabolites makes this strain intrinsically more suitable as bioproduction host for crude glycerol-based bioconversion processes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SV, HJR designed the study. NG performed all shake flask experiments. SV performed all fermentation experiments. SV and NG performed the analyses. SV, HJR, and JHdW drafted the manuscript. All authors have critiqued and approved the final manuscript.

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