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C_1 compounds as auxiliary substrate for engineered *Pseudomonas putida* S12

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Abstract The solvent-tolerant bacterium *Pseudomonas putida* S12 was engineered to efficiently utilize the C_1 compounds methanol and formaldehyde as auxiliary substrate. The *hps* and *phi* genes of *Bacillus brevis*, encoding two key steps of the ribulose monophosphate (RuMP) pathway, were introduced to construct a pathway for the metabolism of the toxic methanol oxidation intermediate formaldehyde. This approach resulted in a remarkably increased biomass yield on the primary substrate glucose when cultured in C-limited chemostats fed with a mixture of glucose and formaldehyde. With increasing relative formaldehyde feed concentrations, the biomass yield increased from 35% (C-mol biomass/C-mol glucose) without formaldehyde to 91% at 60% relative formaldehyde concentration. The RuMP-pathway expressing strain was also capable of growing to higher relative formaldehyde concentrations than the control strain. The presence of an endogenous methanol oxidizing enzyme activity in *P. putida* S12 allowed the replacement of formaldehyde with the less toxic methanol, resulting in an 84% (C-mol/C-mol) biomass yield. Thus, by introducing two enzymes of the RuMP pathway, co-utilization of the cheap and renewable substrate methanol was achieved, making an important contribution to the efficient use of *P. putida* S12 as a bioconversion platform host.

Keywords *Pseudomonas putida* · Auxiliary substrate · C_1 compounds

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Introduction

The solvent-tolerant *Pseudomonas putida* S12 is used as a platform for the bioconversion of sugars into substituted aromatic compounds (Nijkamp et al. 2005; Nijkamp et al. 2007; Verhoef et al. 2007; Wierckx et al. 2005). These compounds are produced via central metabolite intermediates such as L-tyrosine and L-phenylalanine, the formation of which is directly linked to cellular growth. In such a process, a significant proportion of the available substrate is not used to form biomass and product, but utilized for the generation of free energy (ATP and proton gradient) and reducing equivalents (NAD(P)H). Since the sugar substrate represents an important cost factor in bioproduction processes (Schmid et al. 2001), the addition of a cheap auxiliary catabolic substrate to generate free energy and/or reducing equivalents may significantly improve the economy of the production process.

Previous studies have shown that co-utilization of thiosulfate or C_1 compounds like formate and formaldehyde leads to an increased yield on the primary carbon source (Baerends et al. 2008; Bruinenberg et al. 1985; Harris et al. 2007; Masau et al. 2001). Another C_1 compound that can be used as auxiliary substrate is methanol. Being more reduced than formate or formaldehyde, methanol can yield

more reducing equivalents per C-mole. Since methanol can be derived from biomass via synthesis gas (Chmielniak and Sciazko 2003) it is a promising renewable auxiliary substrate for biotechnological processes.

The first step in methanol metabolism is the oxidation to formaldehyde, via dehydrogenases or oxidases (Anthony 1986; Sahm 1977). Formaldehyde is extremely toxic due to non-specific reactivity with proteins and nucleic acids. Therefore, rapid and efficient formaldehyde metabolization is a crucial step in the utilization of methanol. Several pathways for the metabolism of formaldehyde have been described. Methylotrophic yeasts may assimilate formaldehyde by the xylulose-5-phosphate cycle. In this pathway, formaldehyde is coupled to xylulose-5-phosphate and converted into dihydroxyacetone and glyceraldehyde-3-phosphate (GA3P) by a specialized transketolase-dihydroxyacetone synthase (Yurimoto et al. 2005a, b). Alternatively, formaldehyde is oxidatively dissimilated to form formate and eventually carbon dioxide and water (Yurimoto et al. 2005a, b).

In bacteria, three formaldehyde metabolic pathways are known. Like yeasts, also bacteria may oxidatively dissimilate formaldehyde to formate and CO_2. Alternatively, formaldehyde may be assimilated via the serine cycle. In this pathway formaldehyde is coupled to L-glycine to form L-serine (L-ser). L-ser is subsequently metabolized via a cyclic pathway that ensures replenishment of L-glycine and permits a drain on glycerate-3-phosphate to generate biomass (Chistoserdova et al. 2003). The third bacterial formaldehyde metabolic route is the RuMP pathway (Fig. 1) (Kato et al. 2006). In this pathway, formaldehyde is coupled to ribulose-5-phosphate (Ru5P) forming hexulose-6-phosphate (Hu6P). Hu6P is isomerized to fructose-6-phosphate (F6P), that can be further metabolized via the Embden–Meyerhof–Parnas (EMP) pathway, the Entner–Doudoroff (ED) pathway, or the pentose phosphate pathway (PPP). When F6P enters the oxidative part of the PPP (after isomerization to glucose-6-phosphate (G6P)), the RuMP pathway constitutes a cyclic oxidation pathway for formaldehyde: G6P yields Ru5P and CO_2 while generating two NADPH. This cyclic oxidative dissimilation of formaldehyde has been described in, a.o., *Methylobacillus flagellatus* KT (Chistoserdova et al. 2000). The RuMP pathway may also constitute a formaldehyde assimilation pathway (Fig. 1). In this case one-third of the F6P formed enters either the EMP or ED pathway and is converted into GA3P and pyruvate. Pyruvate is used for the production of cell constituents whereas GA3P and two-thirds of the produced F6P are used to regenerate Ru5P by a combination of transketolase, transaldolase, and isomerization reactions (Jakobsen et al. 2006; Kato et al. 2006; Large and Bamforth 1988).

Previously, formaldehyde has been successfully applied as auxiliary substrate in yeast; however, its high toxicity hindered efficient co-utilization (Baerends et al. 2008). Expression of formaldehyde dehydrogenase (Fld) and a

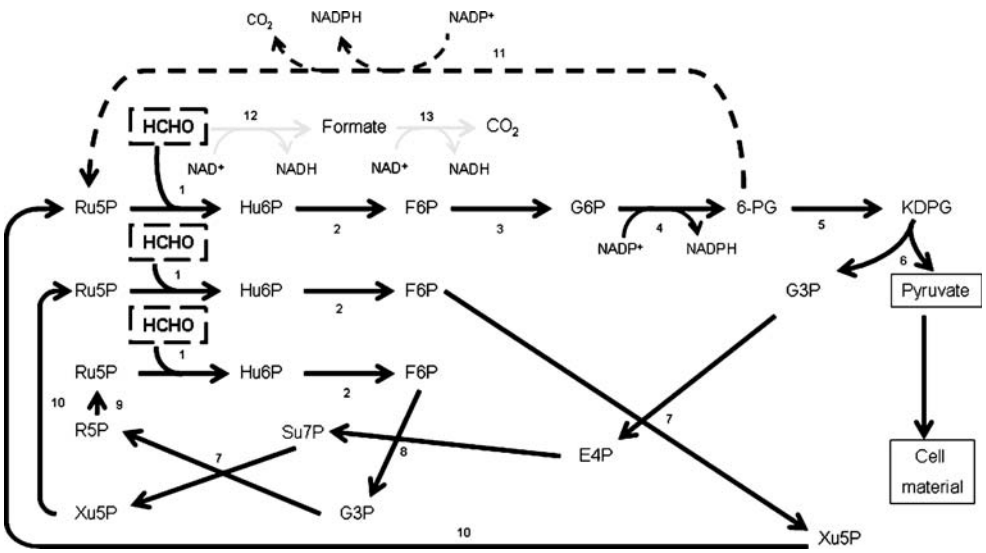

Fig. 1 Assimilation and dissimilation pathways for formaldehyde in *P. putida S12*pJNNhp(t). *1* Hexulose phosphate synthase, *2* hexulose phosphate isomerase, *3* hexose phosphate isomerase, *4* glucose-6-phosphate dehydrogenase, *5* 6-phosphogluconate dehydratase, *6* 2-dehydro-3-deoxy-phosphogluconate aldolase, *7* transketolase, *8* transaldolase, *9* pentose phosphate isomerase, *10* pentose phosphate epimerase; *11* 6-phosphogluconate dehydrogenase, *12* formaldehyde dehydrogenase, *13* formate dehydrogenase. *Ru* ribulose, *Hu* hexulose, *F* fructose, *G* glucose, *Xu* xylulose, *R* ribulose, *E* erythrose, *Su* sedoheptulose, *KDPG* 2-dehydro-3-deoxy-6-phospho-D-gluconate, *HCHO* formaldehyde. *Black arrows* indicate the assimilatory RuMP pathway, *dashed arrows* indicate the dissimilatory RuMP pathway, *gray arrows* indicate the linear oxidation of formaldehyde to carbon dioxide

formate dehydrogenase (Fmd) from the methylotrophic yeast *Hansenula polymorpha* in *Saccharomyces cerevisiae* resulted in an increased biomass yield with formaldehyde as auxiliary substrate (Baerends et al. 2008). An increased tolerance towards formaldehyde was obtained by introducing the *fld* and *fmd* genes.

The aim of the present study was to develop and optimize a *P. putida* S12 strain capable of efficiently utilizing formaldehyde as auxiliary substrate. Since *P. putida* S12 possesses genes encoding formaldehyde and formate dehydrogenase, it was expected that *P. putida* S12 has a basic endogenous capacity to oxidize formaldehyde to formate and CO_2. To further improve this capacity, additional formaldehyde metabolic pathways were constructed by expressing the two key enzymes of the RuMP pathway, 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexulose isomerase (Phi). Biomass yields were determined in chemostat cultures for the engineered strain on different mixtures of glucose and formaldehyde. The RuMP pathway strain showed significantly improved performance over the control strain, achieving a biomass yield-on-glucose of 91% when using formaldehyde as auxiliary substrate. Replacing formaldehyde with the renewable auxiliary substrate methanol resulted in a biomass yield-on-glucose of 84%.

Table 1 Strains and plasmids used in this study

Strains and plasmid	Characteristics	Source or reference
P. putida S12	Wild type	Hartmans et al. 1990
E.coli DH5α	Cloning strain	Invitrogen
B. brevis S1	Wild type	ATCC 12524
P. putida S12pJNN(t)	*P. putida* S12 containing plamid pJNN(t)	This study
P. putida S12pJNNhp(t)	*P. putida* S12 containing plamid pJNNhp(t)	This study
pJNN(t)	Ap^r Gm^r [a], basic expression vector containing the salicylate-inducible *NagR/pNagAa*	Husken et al. 2001; Nijkamp et al. 2005
pJNNhp(t)	Ap^r Gm^r, pJNN(t) containing *hps* and *phi* from *Bacillus brevis* S1, under control of the salicylate-inducible *NagR/pNagAa*	This study
pGEM®-T Easy	Ap^r, used for cloning PCR fragments	Promega
pGEM-HP	pGEM®-T Easy containing *hps* and *phi* from *Bacillus brevis* S1	This study

[a] Ap^r, Gm^r, ampicillin, and gentamicin resistance, respectively

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. Luria broth (LB) (Sambrook and Russel 2001) or phosphate buffered mineral salts medium (MM; Hartmans et al. 1989) were used as indicated. MM was supplemented with 20 mM glucose (MMG) unless otherwise stated. Antibiotics were added as required at the following concentrations: ampicillin, 100 µg/ml; gentamicin, 10 µg/ml (MM), and 25 µg/ml (LB). Heterologous gene expression was induced by addition of 0.1 mM of salicylate unless otherwise stated.

Carbon-limited chemostat cultures were performed either in 1.0 l (working volume) fermentor using a BioFloIIc controller (New Brunswick Scientific) or in 0.7 l (working volume) fermentor using a BioFlo110 controller. MM was used with 10 mM glucose, 10 mg/l gentamicin and 0.1 mM sodium salicylate. Chemostats were inoculated with 50-ml overnight cultures on MMG. The dilution rate (D) was set at 0.05 h^{-1}. After 36 h, the D was increased to 0.2 h^{-1} (glucose/formaldehyde chemostats) or 0.1 h^{-1} (glucose/methanol chemostats). The temperature was maintained at 30°C and the pH was maintained at 7.0 by automatic addition of 2 N NaOH. The stirring speed was controlled by dissolved oxygen (DO) concentration set at 15%. Either air or a mixture of air and oxygen was supplied, using a M + W Instruments D-5111 mass-flow controller (glucose/methanol chemostat), respectively, Brooks mass-flow controllers (5850 E series and 5850 TR series) and a 0154 control unit (Brooks). Dissolved oxygen tension was continuously monitored with an InPro model 6810 probe.

For preparation of formaldehyde solutions, demineralised water was heated to 100°C. During cooling paraformaldehyde (Sigma-Aldrich) was added to obtain a 6 M stock solution. A 5 M NaOH solution was added to until the paraformaldehyde was completely dissolved. Before use, the pH of the formaldehyde solution was set to pH 7 with a 5 M HCl stock solution. The final formaldehyde concentration was measured by HPLC.

Construction of recombinant plasmids

The *hps-phi* genes were amplified from genomic DNA of *B. brevis* S1 (ATCC 12524) using forward (5′-GCCTCGA GGGAATACACACATTTGCTTGTAC-3′) and reverse (5′-CGTCTAGACTATCGAGATTGGCATGTC-3′) primers, designed on the published sequence of *hps* and *phi* (Takashita and Yasueda 2004). The original bicistronic gene organization was maintained as well as the native ribosome binding sites. Standard amplification methods were used with Accuprime Pfx polymerase (Invitrogen). The resulting

PCR fragment was A-tailed for 30 min at 68°C using Supertaq (SphaeroQ). The fragment was subcloned in pGEM®-T Easy (Promega) yielding pGEM-HP. After digestion with NotI, the *hps-phi* fragment was isolated from agarose gel and ligated into NotI digested and dephosphorylated pJNN(t), yielding pJNNhp(t) with the *hps-phi* bicistron under the control of the *nagAa* promoter. After verifying the correct orientation by restriction analysis, pJNNhp(t) was transformed to *P. putida* S12 using a Gene Pulser electroporation device, yielding *P. putida* S12pJNNhp(t).

Plasmid DNA was isolated using QIAprep spin miniprep kit (QIAGEN). DNA fragments were isolated from 0.8% agarose gels with QIAEXII gel extraction kit (QIAGEN). Nucleotide sequencing reactions were performed by MWG Biotech AG. Standard molecular cloning techniques were performed according to Sambrook and Russell (2001).

Analytical methods

Cell densities were measured at 600 nm (OD_{600}) with a Biowave Cell Density Meter (WPA Ltd). Cell dry weight (CDW) was calculated from OD_{600} values where 1 OD_{600} unit corresponds to 0.465 g/l CDW. For calculation of C-mol biomass, CDW was divided by 24 (Roels 1983).

Glucose concentrations were analyzed by HPLC (Waters) using an Aminex HDP-87 N column with 0.01 M Na_2HPO_4 as the eluent and a refractive index detector, or using an ion chromatography (Dionex ICS3000 system). For HPLC-RI, an Aminex HPX-87 N (Bio Rad) column (300×7.8 mm) was applied using 0.01 M Na_2HPO_4 as the eluent. For ion chromatography a CarboPac PA20 column was used with 10 mM NaOH as the eluent.

Organic acids were analyzed by HPLC-UV (Agilent 1100 system) or ion chromatography (Dionex ICS3000). For HPLC-UV, an Aminex HDP-87H (Bio Rad) column (300×7.8 mm) was used with 0.008 N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min. The diode-array detector was set at 210 nm. For ion chromatography an IonPac ICE AS6 column (9×250 mm) was used with 0.4 mM heptafluorobutyric acid as the eluent.

Methanol, formate and formaldehyde were analyzed by ion chromatography (Dionex ICS3000) using an IonPac ICE AS1 column (9×250 mm) with 100 mM methyl sulphonic acid as the eluent. In addition, formaldehyde was analyzed by HPLC-UV (Agilent 1100) after derivatization with 2,4-dinitrophenylhydrazine (DNPH). Equal volumes of DNPH solution (0.5 mg/ml in 3% (*w*/*v*) phosphoric acid) and the sample were mixed and incubated for 10 min at 30°C prior to HPLC analysis. An Eclipse XDB-C8 column (4.6×15 mm) was used with a mixture of 45% acetonitrile and 55% water as the eluent at a flow rate of 1.2 mL/min. The diode-array detector set at 360 nm.

Protein expression was analyzed by SDS-PAGE. Samples were obtained by centrifuging 1 ml of culture at an OD_{600} of 1.5 (mid-exponential growth phase) at 16.000×*g*. The cell pellet was resuspended in 300 µl of loading buffer according to Sambrook and Russell (2001) and heated at 100°C for 10 min. After centrifugation at 16,000×*g* 20 µl of supernatant was applied on the gel for analysis. Routinely, 15% precast Tris–HCl gels (Bio Rad) placed in a Criterion™ electrophoresis cell (Bio Rad) were used. Electrophoresis was performed in running buffer containing 0.025 M Tris (pH 8.8), 0.192 M, glycine, and 3.5 mM SDS in demineralised water as described by Sambrook and Russell (2001) at 130 V. A broad-range unstained protein standard (Bio Rad) was used as a marker. Gels were stained using Coomassie Brilliant Blue R-250 staining solution (Bio Rad).

Enzyme assays

For preparation of cell extracts, 50-ml cultures were harvested at late logarithmic growth phase by centrifugation at 3,200×*g* for 15 min at 4°C. Cell pellets were resuspended in 3 ml of 50 mM potassium phosphate buffer (pH 7.5). All samples were kept on ice during the preparation of extracts. Cells were disrupted using a Branson sonifier with a micro tip at a pulse mode, output set at 4 and effective output set to 60%. After three rounds of sonication (45 s of pulsing and 15 s pause), cell debris was removed by centrifugation at 3,200×*g* for 15 min at 4°C. Supernatants were desalted using a PD10 column (GE healthcare) and used for enzyme assays. Protein concentration was measured using Bradford reagent (Sigma-Aldrich).

Hps- and Phi enzyme activities were determined in a single combined assay as described previously (Arfman et al. 1990; Kato 1990; Orita et al. 2006). The final product formed by these two enzymes from formaldehyde and Ru5P is F6P that is converted into G6P by glucose-6-phosphate isomerase. The formation of G6P was coupled to NADPH formation via glucose-6-phosphate dehydrogenase that converts G6P into glucono-1,5-lactone-6-phosphate. The reaction mixture (0.95 ml) consisted of 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 5 mM ribose-5-phosphate (Ri5P; Sigma-Aldrich), 2.5 mM NADP (Sigma-Aldrich), 10 U phosphoribose isomerase (PRI; Sigma-Aldrich), 10 U phosphoglucose isomerase (Roche), 10 U glucose-6-phosphate dehydrogenase (Sigma-Aldrich). The mixture was preincubated for 5 min at 30°C to achieve equilibrium between Ri5P and Ru5P (catalyzed by PRI). The reaction was started by adding formaldehyde to a concentration of 5 mM. For calculation of the combined Hps- and Phi activity, a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm was used for NADPH. One unit is defined as the overall Hps and Phi activity catalyzing the formation of 1 µmol/min of NADPH at 30°C.

Results

Cloning and functional expression of Hps and Phi in *P. putida* S12

The thermotolerant methylotrophic bacterium *B. brevis* S1 was used as the source of the *hps* and *phi* genes encoding the first two enzymes of the RuMP pathway. Previously, these enzymes have been successfully expressed in the non-methylotrophic mesophile *Escherichia coli* (Yurimoto et al. 2002). Therefore, a similar approach was followed for *P. putida* S12. Heterologous protein expression in the constructed RuMP pathway strain, *P. putida* S12pJNNhp (t), was confirmed by SDS-PAGE (not shown). As the individual activities are difficult to assess, the functionality of the expressed enzymes was confirmed by measuring their combined activity according to Arfman et al. (1990). The overall activity of the two conversions performed by these enzymes was 6,800 U/g protein in cell extract of mid-log phase cells from *P. putida* S12pJNNhp(t). As expected, no activity was observed in the empty vector control strain (*P. putida* S12pJNNmcs(t)).

Formaldehyde as auxiliary substrate: C-limited chemostat cultures at a dilution rate of 0.2 h^{-1}

P. putida S12pJNNhp(t) and an empty vector control strain were cultured in a C-limited chemostat with glucose as the primary carbon source and formaldehyde as auxiliary substrate. The chemostat feed was supplied at a dilution rate of 0.2 h^{-1} and contained a fixed concentration of carbon (60 mM) consisting of a mixture of glucose and formaldehyde the ratio of which was altered during the experiment. The experiment was started with 10 mM glucose (60 mM carbon) and no formaldehyde. After reaching steady state (i.e., after approximately 25 h) 6 mM of glucose-carbon (i.e., 1 mM of glucose) was replaced with 6 mM of formaldehyde-carbon (i.e., 6 mM of formaldehyde). More glucose was replaced with formaldehyde in this step-wise fashion until wash-out of the chemostat cultures was observed.

Both the Hps/Phi-expressing *P. putida* S12pJNNhp(t) and the empty vector control strain reached steady state when up to 50% of the total carbon feed consisted of formaldehyde. No accumulation of glucose or its corresponding acid metabolites gluconate and 2-ketogluconate was observed, confirming that the cultures were C-limited. Also, no formaldehyde or formate accumulated in the cultures of either strain, indicating the presence of endogenous formaldehyde and formate dehydrogenases in *P. putida* S12 by which these compounds were metabolized to completion.

Figure 2a shows the biomass yield-per-glucose (C-mol biomass/C-mol glucose) for both the empty vector control and the RuMP pathway strain. Both strains showed a yield increase with increasing formaldehyde levels (Fig. 2b). However, the performance of the RuMP pathway strain was consistently superior to the performance of the control strain. The yield increase of the RuMP pathway strain relative to the yield increase of the control strain (Fig. 2c) consistently shows that the introduction of the RuMP pathway genes approximately doubled the endogenous yield increase. This effect is independent from the relative formaldehyde concentration within the range tested.

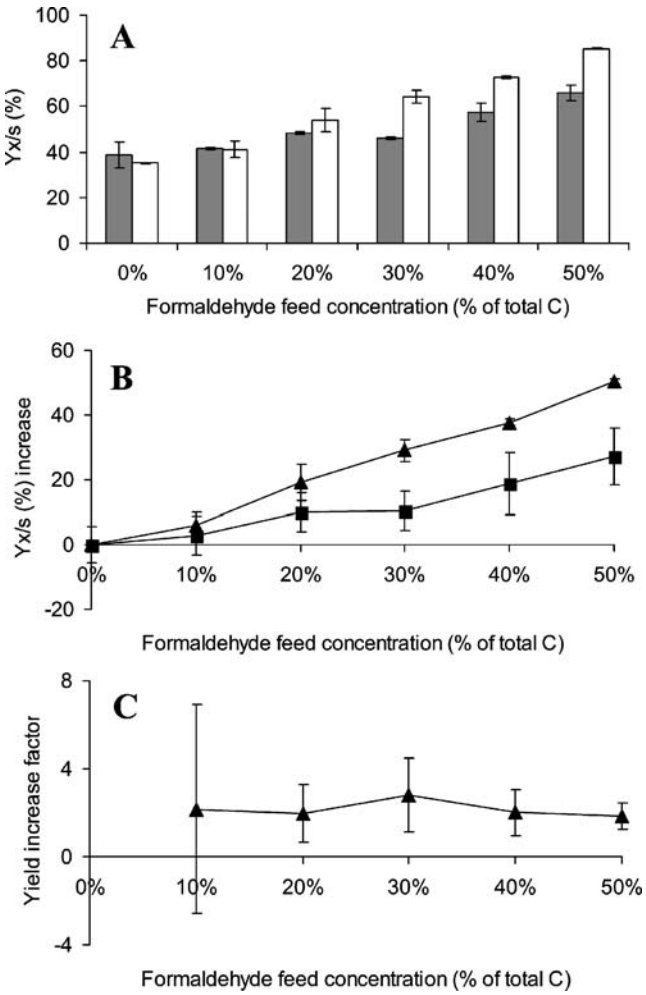

Fig. 2 **a** Biomass yield on glucose in chemostat cultures of strains S12pJNNhp(t) (white bars) and S12pJNN(t) (*gray bars*). Y_{xs} represents the biomass yield (C-mol biomass per C-mol glucose). **b** Biomass yield increase (on glucose) as function of relative formaldehyde concentration in chemostat cultures of strains S12pJNNhp(t) (*triangles*) and S12pJNN (t) (*squares*). The biomass yield on glucose at 0% relative formaldehyde concentration was considered as the baseline yield. The baseline yield was assumed to be independent on relative formaldehyde concentration. **c** Yield increase factor of strain S12pJNNhp(t) over strain S12pJNN(t) as function of relative formaldehyde concentration in chemostat cultures. Data are averages of duplicate experiments; *error bars* denote the maximum deviation from the average of two independent experiments

Formaldehyde as auxiliary substrate: C-limited chemostat cultures at low dilution rate

Both the empty vector control strain and the RuMP pathway strain washed out in chemostats at D=0.2 h^{-1} when the fraction of formaldehyde was increased to 60% of total carbon. Wash-out of chemostat cultures coincided with accumulation of formaldehyde and formic acid. This result suggests that both the endogenous and the engineered formaldehyde metabolic pathway activities were too low to cope with the formaldehyde feed applied. To investigate this hypothesis, chemostat cultures were performed at a lower dilution rate and, consequently, a lower formaldehyde feed rate that is more in agreement with previous studies on continuous culture experiments with methylotrophic bacteria on formaldehyde (Arfman et al. 1992; De Boer et al. 1990; Mitsui et al. 2005).

The experiment was started at a dilution rate of 0.2 h^{-1} and a 7:3 (C-mol) glucose-to-formaldehyde ratio (60 mM total C). When steady state was reached, the dilution rate was set to 0.03 h^{-1}. The formaldehyde feed concentration was kept constant whereas the glucose feed concentration was lowered to 2 mM (12 mM C) to achieve a 4:6 glucose-to-formaldehyde ratio (C-mol; 30 mM C total).

Figure 3 shows that the empty vector control strain did not reach steady state under these conditions. Formaldehyde and formate accumulated and wash-out was observed. In contrast, *P. putida* S12pJNNhp(t) reached steady state with a biomass yield on glucose of 91% (C-mol biomass/C-mol glucose). Since the RuMP pathway strain should theoretically be able to utilize formaldehyde as a sole C-source (see Fig. 1), the glucose feed was stopped. However, formaldehyde and formic acid accumulated and subsequent wash-out of biomass occurred, indicating that the assimilatory pathway activity for formaldehyde was insufficient to sustain growth at the conditions tested.

Methanol as auxiliary substrate: C-limited chemostat cultures at low dilution rate

In the chemostat cultures performed in this study, formaldehyde (and formate) accumulated above a critical formaldehyde feed concentration, probably reflecting the upper limit of the formaldehyde metabolic capacity of the culture. Accumulation of only trace amounts of formaldehyde was always accompanied by wash-out of the culture, underlining the acute and low-threshold nature of formaldehyde toxicity. Thus, for the purpose of better process control, formaldehyde should be replaced with a less toxic auxiliary substrate such as methanol. Utilization of methanol as (co-) substrate, however, requires the ability to oxidize methanol to formaldehyde.

Unexpectedly, formation of NADPH was observed in Hps/Phi enzyme assays with *P. putida* S12 cell extracts when methanol was added as a substrate instead of formaldehyde. The overall activity amounted to 145 U/g of protein in cell extract. Although the activity with methanol was lower by a factor 50 compared to formaldehyde, this result indicated that methanol is oxidized endogenously by *P. putida* S12.

The C-limited chemostat cultures at D=0.03 h^{-1} were repeated, replacing formaldehyde with methanol. The chemostats were started at D=0.1 h^{-1} and a mixed feed of glucose and methanol was applied according to the following regime between the different steady states: glucose/methanol: 10 mM/15 mM; 5 mM/15 mM, and 5/30 mM. After the final steady state, the dilution rate was set at 0.03 h^{-1} and the total feed carbon concentration was decreased to 30 mM with 60% originating from methanol.

P. putida S12pJNNhp(t) reached steady state under these conditions, in contrast to the empty vector control strain (Fig. 4). Approximately 25% of the methanol feed was not metabolized, but no formaldehyde or formate accumulation was observed. The co-utilization of methanol resulted in a

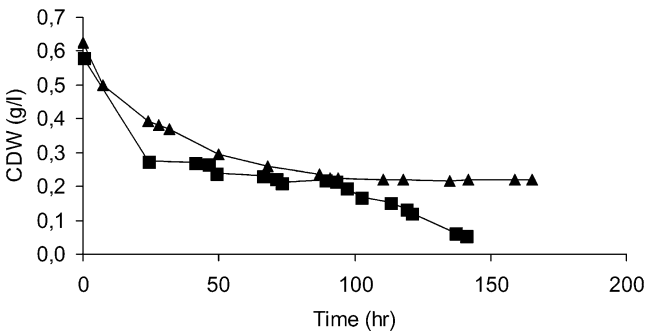

Fig. 3 C-limited chemostat cultures of *P. putida* S12pJNNhp(t) (*triangles*) and *P. putida* S12pJNN(t) (*squares*) at D=0.03 h^{-1}, grown on mineral salts medium with 30 mM of total carbon (60% of all carbon originates from formaldehyde). The data presented are from a single representative experiment

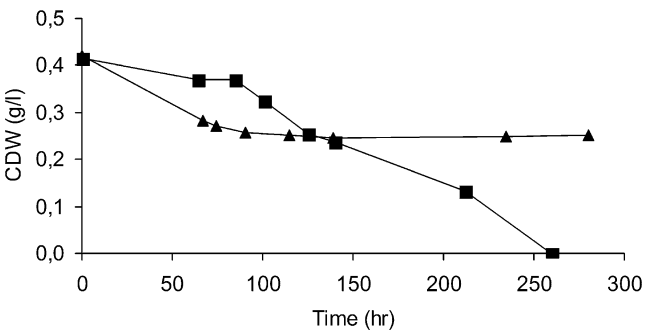

Fig. 4 C-limited chemostat cultures of *P. putida* S12pJNNhp(t) (*triangles*) and *P. putida* S12pJNN(t) (*squares*) at D=0.03 h^{-1}, grown on mineral salts medium with 30 mM of total carbon (60% of all carbon originates from methanol). The data presented are from a single representative experiment

significant improvement of the biomass yield to 84% biomass-per-glucose (C-mol). After stopping the glucose feed, the culture washed out as observed in the glucose/formaldehyde experiments while culture methanol levels increased.

Discussion

P. putida S12 was shown to have an innate capability to co-utilize formaldehyde and glucose, in relative concentrations of up to 50% in C-limited chemostats. This ability is indicative of the presence of an endogenous formaldehyde metabolic pathway in *P. putida* S12. Database searches revealed the presence of several genes coding for formaldehyde and formate dehydrogenases in the *P. putida* S12 genome (manuscript in preparation). Therefore, this pathway probably consists of a linear route that oxidizes formaldehyde, via formate, to CO_2. The observed yield increase of the control strain may be attributed to the reducing equivalents generated during formaldehyde oxidation.

Although *P. putida* S12 disposes of an endogenous formaldehyde detoxification pathway, the introduction of an additional, heterologous formaldehyde metabolic pathway clearly had a positive effect in chemostat cultures grown on a mixture of glucose and formaldehyde. This was reflected by a consistently improved biomass yield on glucose and the ability to grow at elevated relative concentrations of formaldehyde.

The presence of an additional formaldehyde metabolic route may allow for growth at higher relative formaldehyde concentrations, since accumulation of the highly toxic formaldehyde is prevented more efficiently. This unlikely explains, however, the higher biomass yield gain for the strain expressing the RuMP pathway enzymes. Possibly, the improved yield gain relates to a better cofactor balance: when constituting a cyclic oxidation pathway, the RuMP cycle generates 2 mol of NADPH per mol formaldehyde (Fig. 1), whereas the linear oxidation pathway yields 2 mol of NADH. It should be noted, however, that the introduction of the *hps* and *phi* genes will also constitute an assimilatory RuMP pathway (Fig. 1). Thus, (co-)assimilation of formaldehyde may occur, in which case formaldehyde not only serves as a catabolic auxiliary substrate, but also as an assimilatory substrate. It was shown that the presence of the RuMP pathway results in a yield increase that is twice the yield increase attained by linear formaldehyde oxidation. Considering that the same amount of formaldehyde was metabolized in both strains, it may be concluded that the RuMP pathway strain utilizes formaldehyde twice as efficient as the empty vector control strain. Since the linear and the cyclic oxidation pathway yield an equal amount of reducing equivalents (Fig. 1; although of different nature), co-assimilation of formaldehyde is the most likely explanation for the observed additional yield increase found with the RuMP pathway strain. Thus, the biomass yield expressed as yield-per-glucose likely is an overestimation of the actual biomass yield-per-assimilated carbon for the RuMP-pathway strain grown on formaldehyde-glucose mixtures.

The exact relative contributions of linear oxidation, cyclic oxidation, cofactor balance, and assimilation of formaldehyde to biomass yield cannot be determined as it is not possible to selectively shut down either of the pathways. Attempts to knock-out formaldehyde dehydrogenase-encoding genes in order to abolish linear oxidation in *P. putida* S12 have consistently failed (unpublished). No viable double cross-over recombinants could be obtained, suggesting that the linear oxidation pathway apparently is crucial for the detoxification of formaldehyde. Also in non-methylotrophs, formaldehyde is an endogenous metabolite that is produced by oxidative demethylation of DNA (Aas et al. 2003; Falnes et al. 2002) and during methionine, histidine, and choline metabolism (Arnstein 1954). Moreover, the contributions of the cyclic oxidation pathway and the assimilatory pathway cannot be separated since the expression of Hps and Phi constitutes both pathways at a time.

Despite the likely ability of *P. putida* S12pJNNhp to utilize formaldehyde as sole C-source, chemostat cultures washed out when deprived of glucose while maintaining the formaldehyde feed. The activity of the first two enzymes of the RuMP pathway as measured in mid-log phase batch cultures should suffice to assimilate the formaldehyde administered to the low-D (0.03 h^{-1}) cultures. The net Hps/Phi activity amounted to approximately 6,800 U/g protein. Assuming a protein content of 50%, this activity should allow 1 g of cells (dry weight) to cope with a formaldehyde feed of about 200 mmol h^{-1}. However, in the low-D cultures 270 mg of CDW was not able to fully metabolize formaldehyde that was administered at a rate of 0.54 mmol h^{-1}. This observation suggests that the metabolic flux through the endogenous part of the formaldehyde assimilatory cycle may be the bottleneck in preventing formaldehyde accumulation, and subsequent wash-out of cells, when deprived of other C-sources. Alternatively, the activity of the RuMP-pathway enzymes in batch cultures may not be representative for chemostat cultures.

Replacement of formaldehyde with methanol in C-limited chemostat cultures at a low dilution rate also resulted in improved biomass yield. Methanol was not completely utilized, however, and the biomass yield on glucose was slightly lower than for the corresponding formaldehyde experiments. The accumulation of methanol but not formaldehyde/formate suggests that the endogenous methanol dehydrogenase activity is the bottleneck for methanol utilization. This was also indicated by the 50-fold lower activity with methanol as the substrate in the Hps/Phi

enzyme assay. From the *P. putida* S12 genome sequence (manuscript in preparation) no close homologues to established methanol dehydrogenases could be identified. Therefore, it is likely that the low methanol dehydrogenase activity of *P. putida* S12 results from a side activity of a broad-specificity alcohol dehydrogenase.

In conclusion, the enhanced ability of *P. putida* S12pJNNhp(t) to utilize C_1 compounds as auxiliary substrate can be used to substantially improve raw feedstock utilization efficiency. Since both primary and auxiliary substrates can be obtained from biomass, process economy can be improved without compromising sustainability. Although somewhat less efficient than formaldehyde, methanol is the preferred auxiliary substrate as it is less toxic, easier to handle, cheap and renewable. Currently, possibilities are explored to improve the utilization of methanol by increasing the endogenous methanol dehydrogenase activity or by co-expressing a heterologous methanol dehydrogenase.

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