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Overproduction of mannitol dehydrogenase in *Rhodobacter sphaeroides*

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Abstract Mannitol dehydrogenase (MDH) from *Rhodobacter sphaeroides* Si4 was overproduced by constructing a strain that overexpresses the MDH gene and by producing high cell concentrations via fed-batch cultivation in a bioreactor. With the gene of mannitol dehydrogenase (*mtlK*) cloned into the expression vector pKK223-3 expression of MDH in *Escherichia coli* was obtained, but the specific enzyme activity was lower than in *R. sphaeroides* Si4. In order to overexpress *mtlK* in *R. sphaeroides*, plasmid pAK82 was constructed by cloning a DNA fragment carrying *mtlK* into the broad-host-range expression vector pRK415. When pAK82 was introduced into *R. sphaeroides* Si4 the specific mannitol dehydrogenase activity in the strain obtained was 0.48 unit (U)mg^{-1}, 3.4-fold higher than in the wild type. In this way the enzyme yield from cultivation in a bioreactor could be improved from 110 Ul^{-1} to 350 Ul^{-1}. A further increase in productivity was obtained by fed-batch cultivation of *R. sphaeroides* Si4 [pAK82]. Using this cultivation method an optical density of 27.6 was reached in the bioreactor, corresponding to a dry mass of 16.6 g l^{-1}. Since MDH formation correlated with biomass production, the MDH yield could be raised to 918 Ul^{-1}, an 8.3-fold increase in comparison to batch cultivation of the wild-type strain.

Introduction

The application of microbial polyol dehydrogenases for analytical purposes in conventional assays (Berezenko and Sturgeon 1991; Kiba et al. 1991; Lunn et al. 1989; Schmolke et al. 1990; Schneider and Giffhorn 1991) as well as with biosensors (Gautier et al. 1990) is currently being studied intensively. The non-sulphur photosynthetic bacterium *Rhodobacter sphaeroides* Si4 is a promising source of polyol dehydrogenases that can be utilized for biotechnological applications (Schneider and Giffhorn 1989; Kahle et al. 1992). When grown on D-mannitol, D-arabitol or D-glucitol (sorbitol) it expresses a polyol dehydrogenase that oxidizes either of the three polyols to the corresponding keto-sugars. This enzyme has been isolated and characterized (Schneider and Giffhorn 1989). It has been designated as mannitol dehydrogenase (E.C. 1.1.1.67) because it exhibits by far the highest affinity (lowest kinetic constant, K_m value) to D-mannitol.

The applicability of mannitol dehydrogenase (MDH) from *R. sphaeroides* for enzymatic D-mannitol determination (Schneider and Giffhorn 1989), as an enzymatic component of a biosensor (Scheper et al. 1991) and for enzymatic synthesis of D-xylulose from D-arabitol (Schwartz et al. 1994) has been demonstrated. Any application of the MDH requires that the enzyme is easily available in sufficient amounts. Therefore, the optimisation of enzyme production during cultivation of *R. sphaeroides* in a bioreactor is of great interest.

Recently the gene of mannitol dehydrogenase from *R. sphaeroides* Si4 (*mtlK*) has been cloned and sequenced (Schneider et al. 1993), so that the gene can be used for the construction of expression vectors. In the present communication we report the overexpression of *mtlK* in *R. sphaeroides* Si4 and a further improvement in MDH production by fed-batch cultivation of the overproducing strain.

Dedicated to Prof. Dr. Fritz Wagner on the occasion of his 65th birthday.

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Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Rhodobacter sphaeroides Si4	Wild type	Rode and Giffhorn (1983)
Escherichia coli JE85	Phe mutant of *E. coli* DH5α	Jesus Eraso, University of Texas, Houston, USA
E. coli HB101	Res^-, Mod^-, *rec* A13, Sm^r	Boyer and Roulland-Dussoix (1969)
Plasmids		
pRK415	IncP, Tc^r, *lacZα*, *mob*	Keen et al. (1988)
pRK2013	ColE1 replicon, Tra of RK2, Km^r	Ditta et al. (1980)
pUC19	Cloning vector, Ap^r	Yanisch-Perron et al. (1985)
pKK223-3	Expression vector, Ap^r, P *tac*	Pharmacia, Freiburg, Germany
pAK 62	3.4-kb *Bam*HI fragment containing a 2.1-kb *Sma*I-fragment with *mtlK* in pBluescript II KS	This study
pAK74	2.1-kb *Sma*I fragment with *mtlK* in pUC 19 with the start of *mtlK* next to the *Eco*RI site	This study
pAK75	As pAK74, but with opposite orientation of the *Sma*I fragment (start of *mtlK* next to the *Hin*dIII site)	This study
pAK76	*Eco*RI/*Hin*dIII fragment from pAK75 in pKK223-3	This study
pAK77	*Eco*RI/*Hin*dIII fragment from pAK74 in pKK223-3	This study
pAK82	*Eco*RI/*Hin*dIII fragment from pAK75 in pRK415	This study
pAK83	*Eco*RI/*Hin*dIII fragment from pAK74 in pRK415	This study

Media and growth conditions

R. sphaeroides was grown chemoheterotrophically at 30°C in the minimal medium described previously (Schneider and Giffhorn 1989), using 9 $g\,l^{-1}$ of D-mannitol as the carbon source. Fed-batch cultivation was performed in a 2-l bioreactor with aeration (3 $l\,min^{-1}$), agitation (400 rpm), and pH regulation. Cultivation was started in a volume of 1500 ml. After growth for 13 h, 400 ml medium concentrate was added via a peristaltic pump at a rate of 50 $ml\,h^{-1}$. The medium concentrate contained: 9 g D-mannitol; 6 g KH_2PO_4; 6 g NH_4Cl; 2.4 g $MgSO_4 \cdot 7H_2O$; 0.3 g $CaCl_2 \cdot 2H_2O$; 6 ml trace element solution SL4 (ten-fold concentrated, Pfennig and Lippert 1966); 6 ml vitamin solution (ten-fold concentrated, Rode and Giffhorn 1983). *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989). When appropriate, antibiotics were supplemented to the following concentrations: ampicillin, 75 μg/ml; tetracycline, 1 μg/ml for *R. sphaeroides* and 10 $\mu g\,ml^{-1}$ for *E. coli*.

Enzyme assays and protein determination

Cells were harvested by centrifugation and disintegrated in a French press at 15,500 $N\,cm^{-2}$. Polyol dehydrogenase activities were determined as described previously (Schneider and Giffhorn 1989). Protein concentrations were measured by the method of Goa (1953). Crystalline bovine serum albumin was employed as standard.

Genetic techniques and DNA manipulations

Plasmids were mobilized into *R. sphaeroides* by triparental mating with *E. coli* JE 85, containing a mobilizable plasmid, and *E. coli* HB101 [pRK2013] as a helper (Suwanto and Kaplan 1992). The matings were performed for 8 h at 30°C on Luria-Bertani medium (LB) plates (Sambrook et al. 1989).

Standard DNA techniques were used as described by Sambrook et al. (1989). DNA fragments were isolated from agarose gels using the Gene Clean Kit (Bio 101, La Jolla, Calif., USA).

D-Mannitol determination

D-Mannitol concentrations in samples taken from the bioreactor were determined by HPLC on a Ca^{2+}-loaded cation exchange column (Carbohydrate Ca^{2+}, 300 × 7.8 mm, BC-100 from Benson Polymeric, Reno, USA). The column temperature was 85°C. A refractive index detector (Beckman 156 refractive index detector) was used at 35°C. As mobile phase, distilled water was used with a flow rate of 0.8 $ml\,min^{-1}$.

Results

Construction of expression vectors

The gene of mannitol dehydrogenase from *R. sphaeroides* Si4 (*mtlK*) had been cloned and sequenced (Schneider et al. 1993). The DNA sequence obtained reveals that the smallest fragment containing the complete *mtlK* gene is a 2.1-kb *Sma*I fragment. This *Sma*I fragment was isolated from plasmid pAK62 and cloned into the *Sma*I site of pUC19. Two different plasmids, pAK74 and pAK75, were obtained containing the *Sma*I fragment in opposite orientation. Because the cloned fragment comprises no *Eco*RI or *Hin*dIII restriction site, it could be isolated by an *Eco*RI/*Hin*dIII double digest of pAK74 or pAK75 and cloned into an expression vector in a definite orientation. Plasmids pAK76 and pAK77 were constructed by cloning the fragment in both orientations into the expression vector pKK223-3, pAK80 and pAK81 by cloning the fragment into pRK415 (Fig. 1), a broad-host-range vector that replicates in *R. sphaeroides* (Table 1).

Expression of *mtlK* in *E. coli* and *R. sphaeroides*

E. coli JE85 was transformed with pAK76 and pAK77 and the strains obtained were cultivated in a 2-1 bioreactor in LB medium with 0.1 mM isopropyl-β-D-thiogalacto pyranoside (IPTG) and 75 $\mu g\,ml^{-1}$

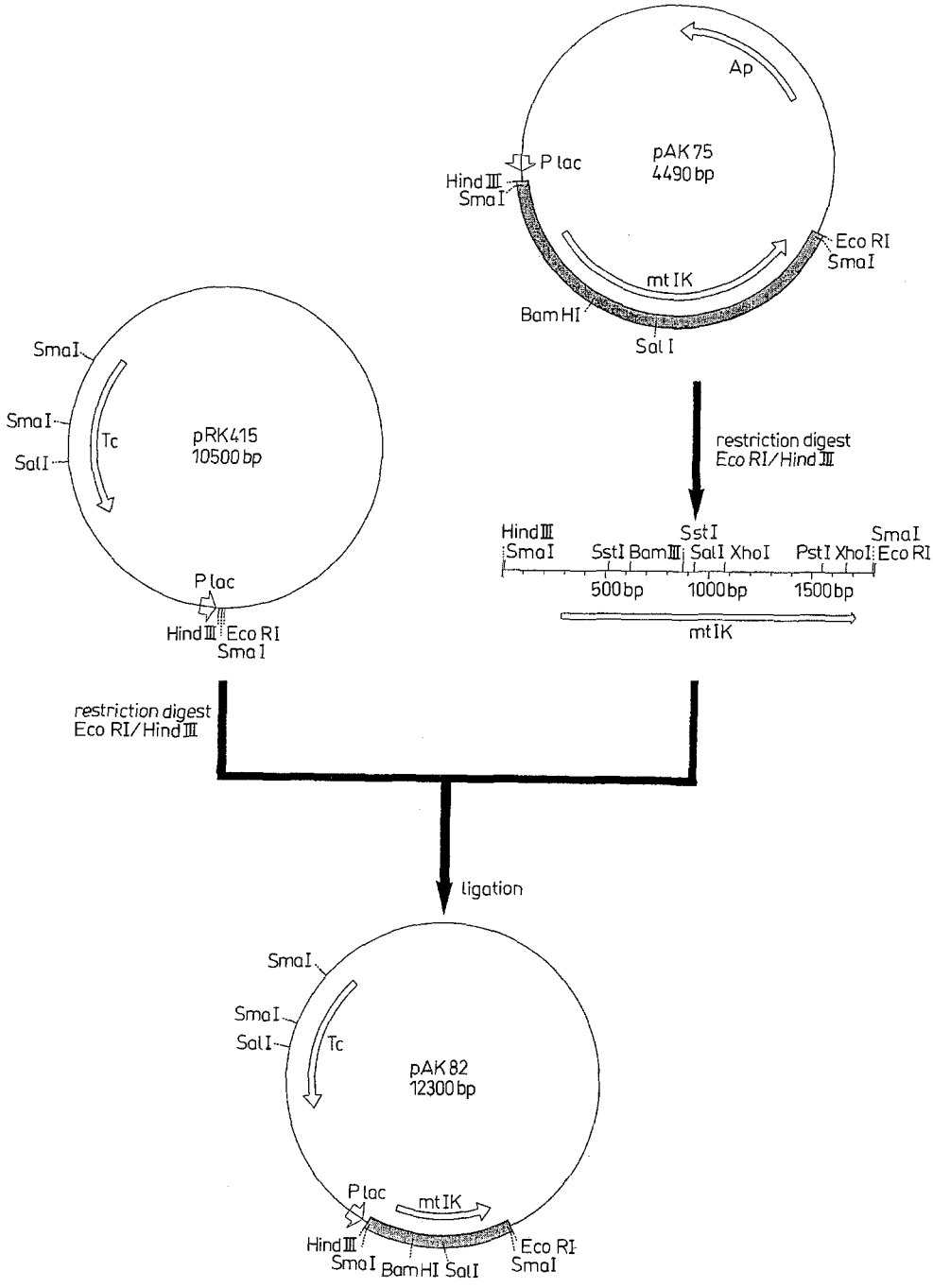

Fig. 1 Construction of the expression vector pAK82 (*Ap* ampicillin gene, *Tc* tetracycline gene, *P lac* lac promoter)

ampicillin. Polyol dehydrogenase activities were determined in crude extracts of the different strains (Table 2). No polyol dehydrogenase activities were detectable when *E. coli* JE85 contained no plasmid or plasmid pAK76, in which *mtlK* is in opposite orientation to the *tac* promotor. When *E. coli* contained the plasmid pAK77 with *mtlK* under control of the *tac* promoter all three polyol dehydrogenase activities provided by the mannitol dehydrogenase could be measured (Table 2). However, the specific activities were even lower than in crude extracts of *R. sphaeroides* Si4. The MDH activity obtained from cultivation of *E. coli* JE85 [pAK77] could not be increased by adding IPTG at different time points during cultivation or in different concentrations. The most likely explanation for the poor MDH production despite the fact that the enzyme is under the control of a strong promotor is that the translation occurs with very low efficiency.

As an alternative to MDH production with *E. coli*, the overexpression of the enzyme in *R. sphaeroides* was investigated. Plasmid pAK82, which contains *mtlK* under control of the *lac* promoter from pRK415, was transferred to *R. sphaeroides* Si4 by triparental mating. The resulting strain exhibited 3.4-fold higher

Table 2. Specific polyol dehydrogenase activities in crude extracts of *E. coli* JE85 [pAK76], *E. coli* JE85 [pAK77], *R. sphaeroides* Si4, and *R. sphaeroides* Si4 [pAK82]. The bacterial strains were grown in a 2-l bioreactor with pH regulation, aeration (3 l min^{-1}) and agitation (400 rpm). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989) with 75 μg ml^{-1} of ampicillin and 0.1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG), *R. sphaeroides* stains at 30°C in mannitol minimal medium, *R. sphaeroides* Si4 [pAK82] in addition with 1 μg ml^{-1} of tetracycline and 0.1 mM IPTG. Cells were harvested in the late logarithmic phase. In crude extracts polyol dehydrogenase activities and protein concentrations were determined (see Materials and methods) (*U* units)

Polyol in enzyme assay	Specific activity U mg^{-1} in the crude extract			
	E. coli JE85 [pAK76]	*E. coli* JE85 [pAK77]	*R. sphaeroides* Si4	*R. sphaeroides* Si4 [pAK82]
D-Mannitol	0.00	0.08	0.14	0.48
D-Arabitol	0.00	0.17	0.32	1.11
D-Glucitol	0.00	0.06	0.28	0.83

Table 3. Production of mannitol dehydrogenase with *R. sphaeroides* by batch and fed-batch cultivation. *R. sphaeroides* Si4 and *R. sphaeroides* Si4 [pAK82] were grown in batch culture as well in fed-batch culture in a 2-l bioreactor with pH regulation, aeration (3 l min^{-1}) and agitation (400 rpm) at 30°C. Mannitol minimal medium was used for all cultivations, complemented with 1 μg ml^{-1} or tetracycline and 0.1 mM IPTG for growth of *R. sphaeroides* Si4 [pAK82]. Cells were harvested at the transition to the stationary growth phase; crude extracts were prepared and polyol dehydrogenase activities were determined

Polyol in enzyme assay	Polyol dehydrogenase yield from the bioreactor (U l^{-1})		
	R. sphaeroides Si4 batch cultivation	*R. sphaeroides* Si4 [pAK82] batch cultivation	*R. sphaeroides* Si4 [pAK82] fed-batch cultivation
D-Mannitol	110	350	918
D-Arabitol	230	812	1946
D-Glucitol	230	606	1465

mannitol dehydrogenase activity than the wild-type strain (Table 2). The increase in the specific activity with the two other polyols D-arabitol and D-glucitol were 3.5-fold and 3.0-fold, respectively. The factor found for the increase in sorbitol dehydrogenase activity is lower in comparison to the factor for D-arabitol and D-mannitol, because *R. sphaeroides* possesses, in addition to MDH, another enzyme with sorbitol dehydrogenase activity (Schneider et al. 1993). Therefore, the sorbitol dehydrogenase activity measured in crude extracts is the sum of both activities. In *R. sphaeroides* Si4 [pAK82] only the expression of MDH is increased, causing a different increase rate for sorbitol dehydrogenase activity than for the other two polyols.

Fed-batch cultivation of *R. sphaeroides* Si4 [pAK82]

By batch cultivation of *R. sphaeroides* Si4 in mannitol minimal medium in a 2-l bioreactor with pH regulation an optical density at 650 nm (OD_{650}) of 7.1 was reached, corresponding to a yield in dry mass of 4.6 g l^{-1}, with concomitant production of 110 U l^{-1} mannitol dehydrogenase (Table 3). The enzyme yield could be improved to 340 U l^{-1} when *R. sphaeroides* Si4 carrying plasmid pAK82 was used for the batch cultivation (Table 3).

In order to reach even higher yields it was investigated whether productivity of mannitol dehydrogenase could be improved by producing higher cell concentrations. With batch cultivation higher cell concentrations could not be reached. D-mannitol concentrations higher than 50 mM did not result in a higher OD_{650} at the end of cultivation and an increase in the mineral salt concentration resulted in precipitation. Therefore, a fed-batch cultivation with *R. sphaeroides* Si4 [pAK82] was performed. The increase in OD_{650} and MDH production during fed-batch cultivation is shown in Fig. 2. The organism was grown until an OD_{650} of 2.0 was reached and then 400 ml medium concentrate was added to the culture over a period of 8 h (see Materials and methods). After the medium concentrate was completely added to the culture, the cells grew for an additional 20 h. After 40 h, an OD_{650} of 27.6 and a dry mass of 16.6 g l^{-1} were reached. The measurement of D-mannitol concentration in the bioreactor during cultivation revealed that D-mannitol was never limiting during cultivation (Fig. 2). The development in MDH production was parallel to the production of cell mass (Fig. 2). At the end of cultivation a mannitol dehydrogenase yield of 918 U l^{-1} was

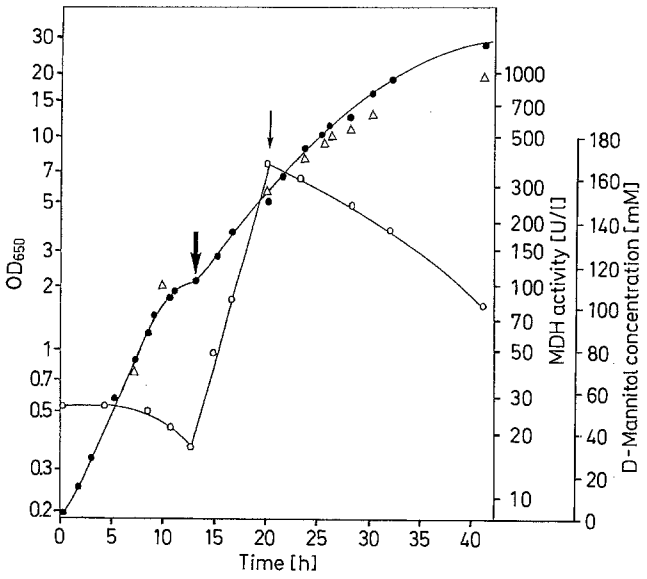

Fig. 2 Fed-batch cultivation of *Rhodobacter sphaeroides* [pAK82]. Cultivation was performed in a 2-l bioreactor at 30°C with pH regulation, aeration at 3 l min^{-1}, and stirring at 400 rpm. Cultivation was started in a volume of 1.5 l mannitol minimal medium, 400 ml medium concentrate was added during cultivation: ↓ = initial addition of medium concentrate, ↓ = end of medium concentrate addition. In samples taken from the bioreactor, D-manitol concentrations were determined by HPLC and crude extracts were prepared for mannitol dehydrogenase determination: ● optical density at 650 nm (OD_{650}); △ mannitol dehydrogenase activity (MDH) in the bioreactor, ○ D-mannitol concentration in the bioreactor (*U* units)

obtained (Table 3). This is an increase in productivity by a factor of 8.3. This yield could be reached after only 40 h of cultivation, unimportantly longer than the time needed for the batch fermentation, which was 24 h.

Discussion

A major condition for the applicability of microbial enzymes for technological purposes is a straightforward and cheap method for enzyme production on a large scale. This requires high enzyme yields from cultivation and an effective method for enzyme purification. Because of possible applications for polyol determination (Scheper et al. 1991) and enzymatic polyol conversion (Schwartz et al. 1994) the production of MDH from *R. sphaeroides* Si4 has been considerably improved by a combination of two methods: optimization of the cultivation process and by constructing a strain that overexpresses the desired enzyme.

Although the MDH gene was cloned into an expression vector, the increase in MDH activity in the overexpressing strain was not more than 3.4-fold. Possible reasons are that plasmid pRK415 is not a high copy number plasmid, it possesses only five to eight copies per chromosome (Priefer 1993). However, MDH production was constant during cultivation, so the enzyme yield could be increased by producing a higher cell density by fed-batch cultivation. In this way a method has been established suitable for producing sufficient amounts of MDH for the different applications of this enzyme.

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