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Application of a KDPG-aldolase gene-dependent addiction system for enhanced production of cyanophycin in *Ralstonia eutropha* strain H16

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Abstract

Two different recombinant plasmids both containing the cyanophycin synthetase gene (*cphA*) of *Synechocystis* sp. strain PCC6308 but differing concerning the resistance marker gene were tested for their suitability to produce high amounts of cyanophycin in recombinant strains of *Ralstonia eutropha*. Various cultivation experiments at the 30-L scale revealed very low cyanophycin contents of the cells ranging from 4.6% to 6.2% (w/w) of cellular dry weight (CDW) only, most probably because most cells had lost the corresponding plasmid during cultivation. To establish a cost effective and high efficient system for production of cyanophycin at larger scales using recombinant strains of *R. eutropha*, we applied two strategies: First, we integrated *cphA* into the dispensable chromosomal L-lactate dehydrogenase gene (*ldh*) of *R. eutropha*. Depending on the cultivation conditions used, relatively low cyanophycin contents between 2.2% and 7.7% (w/w) of CDW were reproducibly detected, which might be due to weak expression or low gene dosage in the single *cphA* copy strain of *R. eutropha*. In a second strategy we constructed a KDPG-aldolase gene (*eda*)-dependent addiction system, which combined features of a multi-copy plasmid with stabilized expression of *cphA*. Flasks experiments revealed that the cells accumulated extraordinarily high amounts of cyanophycin between 26.9% and 40.0% (w/w) of CDW even under cultivation conditions lacking cyanophycin precursor substrates or plasmid stabilizing antibiotics. Cyanophycin contents of up to 40.0% (w/w) of CDW were also obtained at a 30-L scale or a 500-L pilot-plant scale under such non-selective conditions. This demonstrates impressively that the stabilizing effect of the constructed *eda*-dependent addiction system can be used for production of enhanced amounts of cyanophycin at a larger scale in recombinant strains of *R. eutropha*.

Keywords: Addiction system; *cphA*; Cyanophycin; Cyanophycin synthetase; Genomic integration; KDPG-aldolase; Metabolic engineering; Plasmid stability; *Ralstonia eutropha*

1. Introduction

Cyanophycin [multi-L-arginyl-poly(L-aspartic acid)] is a protein-like polymer, which is common to cyanobacteria and was discovered more than 100 years ago (Borzi, 1887). This polymer contains aspartic acid and arginine at an about equimolar ratio, and is arranged as a polyaspartic acid backbone with arginine moieties linked to the β-carboxyl group of each aspartate by its α-amino group (Simon, 1976). Cyanophycin serves as a temporary nitrogen, carbon and energy reserve and usually accumulates as cytoplasmic inclusions during the transition from the exponential to the stationary growth phase (Mackerras et al., 1990). At neutral pH and physiological ionic strength cyanophycin is insoluble. Cyanophycin is of biotechnological interest because a derivative with reduced arginine content can be obtained by partial chemical hydrolysis (Joentgen et al., 1998), which can be used as a biodegradable substitute for polyacrylate in various technical applications (Schwamborn, 1998).

Intracellular synthesis and degradation of cyanophycin are catalyzed by cyanophycin synthetase, which is encoded by cyanophycin synthetase gene (*cphA*) (Ziegler et al., 1998), and cyanophycinase, which is encoded by *cphB* (Richter et al., 1999), respectively. The polymerization

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reaction yields polydisperse cyanophycin with molecular weights ranging from 25,000 to 100,000 Da (Simon, 1976). The activity of cyanophycin synthetase depends on the presence of L-aspartic acid, L-arginine, ATP, Mg^{2+}, K^{+}, a sulfhydryl compound and cyanophycin as a primer (Simon, 1976; Ziegler et al., 1998; Aboulmagd et al., 2000). Cyanophycin synthetase has been purified from various cyanobacteria, like *Anabaena variabilis* (Ziegler et al., 1998), *Synechocystis* sp. strain PCC6308 (Aboulmagd et al., 2001a) and the thermophilic *Synechococcus* sp. strain MA19 (Hai et al., 1999). *CphA* was cloned from *A. variabilis* (Ziegler et al., 1998), *Synechocystis* sp. strains PCC6803 (Ziegler et al., 1998; Oppermann-Sanio et al., 1999) and PCC6308 (Aboulmagd et al., 2000), *Synechococcus* sp. strain MA19 (Hai et al., 2002), and *Thermosynechococcus elongatus* (Berg et al., 2000).

More recently, *cphA* homologous genes were also found in several chemotrophic bacteria, like *Acinetobacter* sp. strain ADP1, *Bordetella* sp., *Clostridium botulinum*, *Desulfitobacterium hafniense*, *Nitrosomonas europaea* and others (Krehenbrink et al., 2002; Ziegler et al., 2002). Heterologous expression of *cphA* from *Acinetobacter* sp. strain ADP1 and *Desulfitobacterium hafniense* in *Escherichia coli* resulted in detectable CphA activity and also cyanophycin accumulation, indicating that CphAs of both organisms were functionally active (Krehenbrink et al., 2002; Ziegler et al., 2002). In addition, cyanophycin accumulation occurs also in the wild type *Acinetobacter* sp. strain ADP1 under phosphate limited culture conditions (Krehenbrink et al., 2002; Elbahloul et al., 2005).

For technical production of cyanophycin, heterologous expression of different cyanobacterial *cphA* genes was demonstrated in recombinant strains of *E. coli* (Frey et al., 2002), *Ralstonia eutropha*, *Corynebacterium glutamicum* and *Pseudomonas putida* (Aboulmagd et al., 2001b). More recently it was shown that production of cyanophycin in recombinant strains of *R. eutropha* and *P. putida* is significantly influenced by the source of *cphA* gene, the accumulation of another storage compound like polyhydroxyalkanoates (PHA) as well as by the concentration of precursor substrates (Voss et al., 2004). Very recently *cphA* from *T. elongatus* strain BP-1 was successfully transferred and expressed in tobacco and potato plants (Neumann et al., 2005).

In this study, we report on further strain optimization for cyanophycin production with recombinant *R. eutropha* using stabilized expression systems for *cphA*, which minimize plasmid instability especially during cultivation at a 30- and 500-L scale. A high rate of plasmid instability is a well known problem of recombinant high-level protein expression and is often correlated with a strong decrease in productivity. To overcome this problem and to establish a stable process for cyanophycin production without the need to add antibiotics, we constructed *R. eutropha* strains either containing a single copy of *cphA* integrated into the genome or using a KDPG-aldolase gene-dependent addiction system.

2. Material and methods

2.1. Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in Luria Bertani (LB) medium (Sambrook et al., 1989). *R. eutropha* was grown either in Nutrient Broth (NB) medium (Difco Laboratories, Detroit, USA) or in mineral salts medium (MSM) according to Schlegel et al. (1961) at a temperature of 30 °C. Sodium gluconate, fructose or sodium lactate were used as carbon sources at concentrations of 1–2% (w/v), and aspartic acid and arginine were added as supplements at concentrations of 0.2% (w/v) in some experiments if indicated in the text. For selection of plasmid carrying strains, antibiotics were added to the media at the following concentrations (μg/ml): ampicillin (75, *E. coli*), kanamycin (50, *E. coli* and 300, *R. eutropha*) and tetracycline (12.5, *E. coli* and 25, *R. eutropha*).

2.2. Preparation of cell extracts

After entering the stationary growth phase, cells were harvested by centrifugation (10 min, 2800*g*, 4 °C), washed once with 50 mM Tris–HCl buffer (pH 8.2) and resuspended with 2 mL buffer per gram of fresh cell mass. Cells of *E. coli* and *R. eutropha* were disintegrated by a three-fold passage through an ice-cooled French press cell at 96 MPa. The supernatants of high-speed centrifugations of the broken cells (1 h, 100,000*g*, 4 °C) were desalted on NAP5 columns (Pharmacia Biotech, Freiburg, Germany) and served as 'soluble cell fractions' for enzyme activity measurements.

2.3. Analysis of ammonium and gluconate

The concentration of ammonium was estimated in cell-free supernatants employing ammonium test bars (Merck, Darmstadt, Germany) or a gas sensitive ammonium electrode (Type 152303000; Mettler Toledo GmbH, Greifensee, Switzerland).

The concentration of gluconate was estimated in cell-free supernatants employing an enzyme test kit for D-gluconic acid (Boehringer Mannheim/R-Biopharm, Darmstadt, Germany) following the instructions of the manufacturer.

2.4. Enzyme assays

The activity of KDPG-aldolase (EC 4.1.2.14) was determined by a spectrophotometric assay in 45 mM triethanolamine hydrochloride (pH 7.6) containing 0.25 mM NADH, 1 mM 2-keto-3-deoxy-6-phosphogluconate (KDPG) and 20 U of L-(+)-lactate dehydrogenase per ml (Blackkolb and Schlegel, 1968).

Cyanophycin synthetase activity was determined by a radiometric assay according to the protocol described previously (Aboulmagd et al., 2000). The activity was