Establishment of Cyanophycin Biosynthesis in *Pichia pastoris* and Optimization by Use of Engineered Cyanophycin Synthetases\textsuperscript{\dag}†

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Two strains of the methylotrophic yeast *Pichia pastoris* were used to establish cyanophycin (multi-L-arginyl-poly-L-aspartic acid [CGP]) synthesis and to explore the applicability of this industrially widely used microorganism for the production of this polypeptide. Therefore, the CGP synthetase gene from the cyanobacterium *Synechocystis* sp. strain PCC 6308 (*cphA*\textsubscript{6308}) was expressed under the control of the alcohol oxidase 1 promoter, yielding CGP contents of up to 10.4\% (wt/wt), with the main fraction consisting of the soluble form of the polymer. To increase the polymer contents and to obtain further insights into the structural or catalytic properties of the enzyme, site-directed mutagenesis was applied to *cphA*\textsubscript{6308} and the mutated gene products were analyzed after expression in *P. pastoris* and *Escherichia coli*, respectively. *CphA*\textsubscript{6308Δ1}, which was truncated by one amino acid at the C terminus; point mutated *CphA*\textsubscript{6308C595S}; and the combined double-mutant *CphA*\textsubscript{6308Δ1C595S} protein were purified. They exhibited up to 2.5-fold higher enzyme activities of 4.95 U/mg, 3.20 U/mg, and 4.17 U/mg, respectively, than wild-type *CphA*\textsubscript{6308} (2.01 U/mg). On the other hand, CphA proteins truncated by two (*CphA*\textsubscript{6308Δ2}) or three (*CphA*\textsubscript{6308Δ3}) amino acids at the C terminus showed similar or reduced CphA enzyme activity in comparison to *CphA*\textsubscript{6308}. In flask experiments, a maximum of 14.3\% (wt/wt) CGP was detected after the expression of *CphA*\textsubscript{6308Δ1} in *P. pastoris*. For stabilization of the expression plasmid, the *his4* gene from *Saccharomyces cerevisiae* was cloned into the expression vector used and the constructs were transferred to histidine auxotrophic *P. pastoris* strain GS115. Parallel fermentations at a one-to-one scale revealed 26°C and 6.0 as the optimal temperature and pH, respectively, for CGP synthesis. After optimization of fermentation parameters, medium composition, and the length of the cultivation period, CGP contents could be increased from 3.2 to 13.0\% (wt/wt) in cells of *P. pastoris* GS115 expressing *CphA*\textsubscript{6308} and up to even 23.3\% (wt/wt) in cells of *P. pastoris* GS115 expressing *CphA*\textsubscript{6308Δ1}.

Since the first isolation of a methylotrophic yeast, *Kloeckera* sp. strain 2201, in 1969 (43), the two methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* have become the most popular methylotrophs in industry and academia (9, 23, 24). The main benefits of these organisms for the production of recombinant proteins are their growth to cell densities as high as 130 g cell dry matter per liter (50, 57) and the availability of strong and tightly regulated promoters that result in a high product yield (13). Viral hepatitis B surface antigen, *S. cerevisiae* mating factor α, and *S. cerevisiae* invertase are only a few examples of compounds produced by recombinant *P. pastoris* (reviewed in reference 9).

A variety of strains were optimized for the expression of recombinant proteins (9). Protease-deficient strains such as strain KM71(H) were generated to circumvent the proteolytic degradation of recombinant proteins (17). Three different phenotypes exist that differ in the ability to utilize methanol (reviewed in reference 37). (i) Mut\textsuperscript{T} strains grow on methanol as the sole carbon and energy source at the wild-type rate. (ii) Mut\textsuperscript{t} strains possess a disrupted alcohol oxidase 1 (*AOX1*) gene and therefore rely on the weaker *AOX2* gene, leading to decreased methanol utilization rates in comparison to those exhibited by Mut\textsuperscript{+} strains. (iii) Mut\textsuperscript{−} strains are not able to utilize methanol as a carbon and energy source; consequently, such strains use the compound as an inducer only and are dependent on the concomitant addition of carbon sources that do not repress the *AOX1* promoter (30, 31). Depending on the required product, any of these phenotypes can be optimal (37). The *AOX1* promoter is totally repressed during growth on, e.g., glycerol, whereas it is strongly expressed after methanol is supplied (11). Therefore, *P. pastoris* fermentations are divided into two phases. (i) During growth on glycerol, high cell densities are reached; (ii) subsequent growth on methanol leads to induction of heterologous protein synthesis, resulting in a high product yield (14). Besides glycerol, several other carbon sources, such as, e.g., glucose, acetate, ethanol, or sorbitol, were used for the production of foreign proteins (30, 31). Several fermentation strategies that allow optimal cell and product yields have been established (8, 25, 28).

Besides the *AOX1* promoter, several other suitable promoters are available (10), e.g., the copper-inducible *CUP1* promoter from *S. cerevisiae* (33, 38), the inducible *ICL1* promoter from the isocitrate lyase gene (8), or the constitutive *GAP* promoter from glyceraldehyde-3-phosphate dehydrogenase (56).

Synthesis of cyanophycin (multi-L-arginyl-poly-L-aspartic acid [CGP]) was only recently established in the yeast *S. cerevisiae*. Recombinant strains harboring *cphA* from *Synechocystis* sp. strain PCC 6308 but otherwise with a wild-type background accumulated CGP up to 6.9\% (wt/wt) (52), whereas recombinant strains with a mutation in arginine metabolism...
accumulated CGP even up to 15.3% (wt/wt) of the cell dry mass (CDM) (54). All of the strains synthesized the polymer in soluble and insoluble forms, which was also observed in transgenic plants (29, 42); the soluble type of CGP was first observed in *Escherichia coli* expressing the *cphA* gene from *Deutsche bakterium hafniense* (59). Several cyanobacterial and heterotrophic CGP synthetase genes were expressed heterologously in the past (16, 26, 29, 52, 59). To unravel structurally or heterotrophic CGP synthetase genes were expressed heterol-

![TABLE 1. Strains and plasmids used in this study](https://example.com/table1.png)

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MATERIALS AND METHODS

**Strains, media, and growth conditions.** All of the bacteria, yeast strains, and plasmids used in this study are listed in Table 1. For the growth conditions and media used, see the supplemental material.

**Cultivation in parallel fermentors.** Cultivation at the 1- to 2-liter scale was performed in parallel fermentors (Biostat B—Twin 2 L MO; Sartorius). One liter of medium was inoculated with 100 ml of a well-grown culture in YPG medium (yeast extract-peptone-dextrose with 2%, wt/vol, glycerol instead of glucose). If not indicated otherwise, the pH was kept at 5.0 and the temperature was set at 30°C. The pH was adjusted automatically by addition of 4 M HCl or 4 M NaOH. When not stated differently, the mineral salts medium described by

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$^a$ Ap', ampicillin resistance; Te', tetracycline resistance; Zeo', zeocin resistance.
Henes and Sonneleitner (28) with 2% (wt/wt) glycerol as the carbon source was used. During fermentation, the ammonium concentration was determined by using a Quantofix kit (10 to 400 mg/liter NH₄⁺; Macherey-Nagel, Düren, Germany); when the ammonium was depleted, 4.5 g/liter (NH₄)₂SO₄ was fed aseptically to the cultures. Glycerol concentrations were determined by high-performance liquid chromatography (HPLC) using a LaChrom Elite apparatus (6). Cell-free culture supernatants were used as samples, and a 20-min program was run. Methanol feeding (1%) was started approximately 24 h after inoculation when glycerol was depleted. Cells were harvested in a Sorvall RC5B centrifuge for 40 min at 8,000 rpm (rotor SLA3000) and washed once with saline (0.9% NaCl) before freezing and lyophilization.

Cloning of cphA genes. For cloning of cphA genes into E. coli-P. pastoris shuttle vectors pPICHOLI-3 and pPICHOLI-C (Table 1), PCR was done with Pfu DNA polymerase (Gibco BRL) according to the manufacturer’s instructions by using oligonucleotides fw-Sall and rw-NotI as sense and reverse primers, respectively (Table 2). Plasmid pET-22a::cphA was used as the template. Subsequently, the PCR products were cloned into the Sall-NotI-treated E. coli-yeast shuttle vectors, yielding pPICHOLI-3::cphA and pPICHOLI-C::cphA, respectively. As significant CGP accumulation was only observed using pPICHOLI-3, further cphA genes (see below) were cloned into this vector only.

Generation of cphA genes with site-directed mutations. Five different cphA genes were generated by PCR using specific oligonucleotides (Table 2). Three genes coding for C-terminally truncated CphA proteins were constructed; cphAΔ31 for CphAΔ31 with one amino acid truncated at the C terminus of the enzyme, cphAΔ32 for CphAΔ32 with two amino acids truncated, and cphAΔ33 for CphAΔ33 with three amino acids truncated. Furthermore, the gene cphAΔ35 was coding for CphAΔ35 with the point mutation Cys95Ser (35, 53) was (i) amplified with the primers used for cphAΔ31 and (ii) used as the template to generate cphAΔ32 and cphAΔ33, respectively. As significant CGP accumulation was only observed using pPICHOLI-3, further cphA genes (see below) were cloned into this vector only.

Cloning of his4. Strain GS115 is histidine auxotrophic and lacks an active his4 gene coding for a multifunctional enzyme containing phosphoribosyl-ATP pyrophosphatase, phosphoribosyl-AMP cyclohydrolase, and histidinol dehydrogenase activities; it catalyzes the 2nd, 3rd, 9th, and 10th steps of histidine biosynthesis. To enable strict stabilization of the pPICHOLI vectors in E. coli-P. pastoris, two different promoters were chosen to compare expression, two different promoters were chosen to compare expression strains GS115 and KM71H and BL21(DE3)pLysS. In strain GS115, the his4 gene was amplified with chromosomal DNA from P. pastoris was shown before (12). The sense primer binds 266 bp upstream of the his4 start codon to amplify the gene with its own promoter region. The 2,666-bp PCR product was subcloned into the cloning vector pET1.2 (Merck). Plasmid pET1.2::his4 was restricted with NotI, and his4 was purified and ligated into the vectors pPICHOLI-3::cphAΔ31 and pPICHOLI-3::cphAΔ32Δ159SS, which were also restricted with NotI and dephosphorylated with FastAP (Fermentas) to avoid religation. The constructs obtained, pPICHOLI-3::cphAΔ31::his4 and pPICHOLI-3::cphAΔ32Δ1::his4, were transferred to P. pastoris GS115. Transformants were selected on minimal medium without histidine and supplemented with 100 µg/ml zeocin.

Purification of CphA. His-tagged CphA proteins were purified under native conditions with His SpinTrap columns as described in the manual provided (GE Healthcare). Binding buffer (pH 7.4) contained 20 mM imidazole; elution buffer (pH 7.4) had an imidazole concentration of 500 mM.

### Results

Determination of the optimal cphA gene and a suitable promoter for its expression in P. pastoris. For heterologous expression in P. pastoris, the cphA gene from Synochocystis sp. strain PCC 6308 was chosen, as it was successfully used for heterologous expression in the yeast S. cerevisiae (52, 54). For expression, two different promoters were chosen to compare them for suitability for efficient CGP synthesis in P. pastoris; cphAΔ35 was cloned into the expression vectors pPICHOLI-C with the copper-inducible CUP1 promoter and pPICHOLI-3 with the methanol-inducible AOX1 promoter. In S. cerevisiae, use of the CUP1 promoter resulted in significant CGP synthesis (52, 54). As expression strains, two strains differing in method...
TABLE 3. Measured specific activities of purified CphA proteins

<table>
<thead>
<tr>
<th>CphA</th>
<th>Mean CphA sp act</th>
<th>CphA sp act (U/mg protein) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>6308</td>
<td>2.01 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>C595S</td>
<td>3.20 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>ΔC595S</td>
<td>4.17 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Δ1</td>
<td>4.05 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Δ2</td>
<td>1.48 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Δ3</td>
<td>0.94 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*The specific activity of cyanophycin synthetase is defined as 1-arginine incorporation rate in mol min⁻¹ mg protein⁻¹. Experiments and enzyme assays were done in triplicate.

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FIG. 1. Analysis by SDS-PAGE of the soluble (A) and insoluble (B) forms of CGP synthesized by different CphA proteins. Fifty milligrams of dry cells was used for CGP isolation, and 20 µl of each fraction was applied to an SDS-polyacrylamide gel (11.5%). Acidic extracts were obtained by resuspension of cell debris in 0.1 M HCl and centrifugation. Proteinase K fractions were obtained by digestion of soluble cell fractions with proteinase K. Cells of transgenic strain GS115, expressing the different cphA genes coding for CphA proteins with truncated C termini (CphA6308Δ1, CphA6308Δ2, CphA6308Δ3) lacking one, two, or three amino acids, respectively, were generated by PCR and cloned into the vector pPICHOLI-3 (see Materials and Methods). Moreover, CphA6308C595S harboring the point mutation Cys595Ser and CphA6308Δ1C595S harboring the same point mutation and possessing the C terminus truncated by one amino acid were amplified by PCR and cloned into pPICHOLI-3. The hybrid vectors generated were transferred to P. pastoris strains GS115 and KM71H, respectively. All CphA proteins were purified by His SpinTrap columns, and their purity was verified by SDS-PAGE and Western blotting using anti-His antibodies indicating that the purified enzymes are CphA proteins. A specific immunoreaction was obtained with a protein band of ca. 100 kDa, corresponding to the theoretical size of 98.23 kDa calculated for six-His-tagged CphA6308 (www.expasy.org) (see Fig. S1 in the supplemental material). The enzyme activities of the purified CphA proteins were determined (Table 3). The specific enzyme activities of the purified CphA proteins obtained showed that CphA6308Δ1 exhibited about twofold higher activity (4.95 U/mg) than wild-type CphA6308 (2.01 U/mg), whereas CphA6308Δ2 (1.48 U/mg) and CphA6308Δ3 (0.94 U/mg) showed lower specific activities than wild-type CphA. Also, CphA6308C595S revealed increased enzyme activity (3.20 U/mg) in comparison to that of wild-type CphA but lower activity in comparison to that of CphA6308Δ1. CphA6308Δ1C595S exhibited slightly higher activity (4.17 U/mg) than CphA6308C595S.

Soluble and insoluble CGP polymers of all strains were analyzed by SDS-PAGE to investigate if the mutated CphA proteins lead to differing molecular weight distributions in comparison to CphA6308 (Fig. 1). For this, 50 µg of dry cells of recombinant P. pastoris strain GS115 expressing the different cphA genes was processed as for CGP isolation. Twenty microliters of the soluble cell fractions treated with proteinase K and 20 µl of the acidic fractions obtained after resuspension of the cell debris in 0.1 M HCl were analyzed by SDS-PAGE. Equal amounts of the respective fractions were applied to the gels to display possible differences in the amounts of CGP synthesized. For all strains, except the strain expressing CphA6308Δ3, no differences in the amount of CGP could be concluded from this analysis. For the latter strain, significant differences in the molecular mass distribution of the polymer in comparison to CGP synthesized by CphA6308 were observed: soluble CGP exhibited a maximal mass of 26 kDa, while the masses of the soluble CGPs synthesized by other CphA proteins ranged from 19 to 40 kDa. Additionally, no insoluble CGP could be detected in cells harboring cphA6308Δ3. The other strains synthesized insoluble CGP with a mass distribution between 24 and 35 kDa; thus, this form of the polymer
1.0 with 1 mM isopropyl-
β-D-thiogalactopyranoside (IPTG) and grown for 12 h. Cultivations were performed three times.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CDM (g/liter)</th>
<th>Mean % CGP content ± SD</th>
<th>Amino acid composition of soluble CGP (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Insoluble</td>
<td>Soluble</td>
</tr>
<tr>
<td><strong>P. pastoris GS115/pPICHOLI-3</strong></td>
<td>6.1</td>
<td>0.9 ± 0.1</td>
<td>9.5 ± 0.5</td>
</tr>
<tr>
<td><strong>P. pastoris KM71H/pPICHOLI-3</strong></td>
<td>6.4</td>
<td>0.4 ± 0.2</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>6308</td>
<td>6.1</td>
<td>2.0 ± 0.5</td>
<td>11.5 ± 0.3</td>
</tr>
<tr>
<td>Δ1C595S</td>
<td>6.0</td>
<td>1.9 ± 0.1</td>
<td>12.4 ± 0.2</td>
</tr>
<tr>
<td>Δ2</td>
<td>6.2</td>
<td>0.8 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>Δ3</td>
<td>6.3</td>
<td>—</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td><strong>P. pastoris KM71H/pPICHOLI-3-cphA</strong></td>
<td>6.3</td>
<td>0.1 ± 0.2</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>6308</td>
<td>5.6</td>
<td>0.4 ± 0.1</td>
<td>11.2 ± 0.1</td>
</tr>
<tr>
<td>Δ1C595S</td>
<td>5.8</td>
<td>1.1 ± 0.1</td>
<td>11.0 ± 0.5</td>
</tr>
<tr>
<td>Δ2</td>
<td>6.0</td>
<td>0.1 ± 0.1</td>
<td>11.2 ± 0.5</td>
</tr>
<tr>
<td>Δ3</td>
<td>4.6</td>
<td>0.1 ± 0.1</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Δ3</td>
<td>4.2</td>
<td>—</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td><strong>E. coli BL21(DE3)/pPICHOLI-3-cphA</strong></td>
<td>6.0</td>
<td>8.2 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>6308</td>
<td>1.0</td>
<td>10.8 ± 0.9</td>
<td>—</td>
</tr>
<tr>
<td>Δ1C595S</td>
<td>0.9</td>
<td>12.4 ± 0.8</td>
<td>—</td>
</tr>
<tr>
<td>Δ1</td>
<td>0.9</td>
<td>16.7 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>Δ2</td>
<td>1.1</td>
<td>7.9 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>Δ3</td>
<td>1.1</td>
<td>4.0 ± 0.1</td>
<td>—</td>
</tr>
</tbody>
</table>

Exhibited lower mass distributions than the soluble form of CGP.

**Growth experiments in flasks**. Cultivation experiments in flasks were done to determine and compare the CGP contents of *P. pastoris* strains GS115 and KM71H harboring vector pPICHOLI-3 with the different cphA genes. The cells were grown in 50 ml BMMY medium (39a) supplemented with zeocin. After 24 h, 1% methanol was fed to strain GS115 and 0.5% methanol was fed to strain KM71H; feeding was repeated at the same concentrations for 4 days. Table 4 shows the cell densities obtained in grams per liter and CGP yields in percent (wt/wt). The amino acid compositions of the soluble and insoluble types of the polymer were determined by HPLC. Insoluble CGP, independent of the Cpha or strain used, consisted of a maximum of 3.5 mol% lysine, besides arginine and aspartate. In contrast, soluble CGP exhibited higher molar fractions of lysine (Table 3), ranging from 4.7 to 6.8 mol% lysine in CGP isolated from transgenic *P. pastoris* GS115 strains and up to 13.5 mol% lysine in CGP isolated from transgenic *P. pastoris* KM71H strains.

The CGP contents varied, depending on the Cpha expressed, and were comparable for strains GS115 and KM71H. CGP accumulated mainly in the soluble form, which accounted for 85 to 98% of the total polymer. Strains expressing wild-type Cpha accumulated CGP to 8.4 to 10.4% (wt/wt) of the CDM, while the contents increased to a maximum of 14.3% (wt/wt) after expression of the mutated enzyme CphaC595S, Cpha6308C595S, or Cpha6308Δ1C595S. After expression of Cpha6308Δ2, CGP contents were comparable to those of strains harboring Cpha6308 and cells expressing Cpha6308Δ3 synthesized only about 50% of the amount that cells expressing wild-type Cpha did (Table 4).

**Stabilization of CGP production by applying histidine addition**. As *P. pastoris* strain GS115 enables strict stabilization of the plasmid due to its histidine auxotrophy, it was used in parallel fermentations in small stirred tank reactors for optimization of the CGP contents of the cells. Therefore, the his4 gene was additionally cloned into the vector pPICHOLI-3-cphA6308 and plasmid stabilities were determined in flask experiments first and then in fermentations (see below). After growth in flasks, 85% or 88% of the recombinant cells grown without zeocin in minimal medium harbored the plasmid, while 98% of the cells expressing the his4 gene retained the plasmid episomal.

**Microscopic analysis of recombinant *P. pastoris* cells**. Cells of recombinant *P. pastoris* GS115 strains were analyzed light microscopically before and after induction to investigate if CGP inclusions are visible (see Fig. S2 in the supplemental material). As cells of the strain harboring Cpha6308Δ1 synthesized higher amounts of the polymer, it was analyzed in parallel. All cells, independent of induction, exhibited light-scattering inclusions.
Expression experiments in *E. coli*. As the constructed vectors are also suitable for expression of *cphA* in *E. coli* BL21(DE3) strains under the control of the T7 promoter, the constructs harboring the different *cphA* genes were transferred to *E. coli* BL21(DE3)pLysS (Table 1) to compare the characteristics of CGP synthesis in recombinant yeast and bacteria. Induced cells were analyzed microscopically, and CGP was isolated to examine if CGP accumulation was similar to that of the *P. pastoris* strains. Unlike yeast, all cells accumulated the polymer exclusively in the insoluble form. Therefore, CGP accumulation could be visualized by light microscopy (see Fig. S2 in the supplemental material). When the strains harboring *cpha*6308, *cpha*6308Δ1, and *cpha*6308Δ3 were compared, a behavior similar to that of the recombinant yeast strains became obvious: Light-scattering inclusions were visible in some cells harboring *cpha*6308, while most cells expressing *cpha*6308Δ1 exhibited inclusions and almost no cells harboring *cpha*6308Δ3 showed inclusions. The microscopic analysis could be corroborated by determination of CGP content: *E. coli* expressing *Cpha6308* accumulated 8.2% (wt/wt) CGP, whereas twice as much polymer was isolated from cells expressing *Cpha6308Δ1* (Table 4).

Optimization of CGP production by parallel fermentation. For optimization of CGP synthesis, different parameters were varied by using up to six stirred tank bioreactors in parallel, each with a maximum medium capacity of 2 liters. If not indicated otherwise, transgenic *P. pastoris* strain GS115 harboring pPICHOLI-3:*cpha*6308/his4 was cultivated in 1 liter of medium at 30°C and a pH of 5.0. One percent (vol/vol) methanol was added after depletion of glycerol, and feeding was repeated when required. CGP was isolated from 0.3 g of dry cells as described in Materials and Methods and analyzed by HPLC. No significant variations in amino acid composition were observed in the CGPs in comparison to the analysis of CGPs isolated from flask cultivation experiments. Interestingly, after all cultivations only soluble CGP could be isolated. All of the cell densities and cell CGP contents obtained are depicted in Fig. 2. First, three different media, i.e., mineral salts media established by (i) Henes and Sonnleitner (28) and (ii) Invitrogen and (iii) the complex BMMY medium recommended by MoBiTec, were compared. Cells of *P. pastoris* strain GS115 harboring pPICHOLI-3:*cpha*6308 were cultivated in the presence of 100 µg/ml zeocin. As shown in Fig. 2A, the highest CGP contents of 4.4% were observed in cells grown in the medium described by Henes and Sonnleitner (28), compared to 3.2% CGP in cells grown in the other media. Therefore, this medium was used for further fermentations.

A subsequent experiment was carried out to determine the optimal length of the cultivation period. For this, cells of *P. pastoris* strain GS115 harboring pPICHOLI-3:*cpha*6308 were cultivated for 11 days and samples were withdrawn from the cultivation vessel daily to determine the CGP contents (Fig. 2B). After 6 days of induction, the maximal polymer yield was observed; thereafter, CGP accumulation remained constant and therefore all further cultivations were performed with an induction period of 6 days.

In the next step, the cell densities and CGP contents of *P. pastoris* strain GS115 possessing or not possessing the episomally encoded his4 gene were compared after growth in the presence or absence of zeocin (Fig. 2C). Higher product yields of 8.2% (wt/wt) were detected in cells encoding a functional his4 gene. Furthermore, explicit higher cell densities were obtained for this strain. Addition of zeocin resulted in slight stabilization of the plasmid in cells not expressing his4 and did not show an effect on the other strains.

Because the temperature and pH of the medium often play a crucial role in obtaining high product yields, temperatures ranging from 22°C to 32°C and pH values ranging from 3.0 to 7.0 were used (Fig. 2D and E). The parameters were set after the first induction with methanol. As shown in Fig. 2D, the highest CGP contents of 10.4% (wt/wt) were detected in cells cultivated at 26°C, while cells grown at 30°C accumulated 9.0% CGP; the lowest contents of 4.7% CGP were observed in cells grown at 22°C. Additionally, the CDMs obtained were higher after cultivation at 26°C (13.4 g/liter) than after cultivation at 30°C (12.5 g/liter). In further cultivations, the temperature was therefore set at 26°C during the induction phase. Through variation of the pH, further increases in CGP content were attempted; the lowest CGP amounts were observed after cultivation at pH 4.0, while the highest CGP content of 12.1% (wt/wt) was detected in cells grown at pH 6.0 or 7.0. As the highest cell density was obtained after growth at pH 6.0, this pH was set in all further cultivations.

To further increase the CGP contents of the cells, *P. pastoris* GS115 harboring *cpha*6308Δ1 was cultivated in comparison to the same strain harboring wild-type *cpha*6308. Additionally, to increase cell densities, cells harboring *cpha*6308Δ1 were fed twice with 2% glycerol before induction with methanol. The cell densities and CGP contents obtained are depicted in Fig. 2F. Cells expressing *cpha*6308Δ1 accumulated the polyamide up to 23.3% (wt/wt), compared to the 11.9% (wt/wt) CGP accumulated by cells harboring *cpha*6308. Furthermore, the cell density reached 60 g/liter (CDM) after glycerol-fed batch culture before induction with methanol, compared to the 17.3 and 17.9 g/liter observed for cells grown without additional glycerol feeding.

**DISCUSSION**

This report describes the first synthesis of CGP in a methylo trophic yeast. Besides *P. pastoris, S. cerevisiae*, accumulating CGP up to 15.3% (wt/wt) of its CDM, is the only yeast used for synthesis of the polyamide. As reported before, methylo trophic yeasts offer advantages over *S. cerevisiae* for the production of some proteins (22). This study indicated that *P. pastoris* represents a suitable candidate for synthesis of the polymer. A CGP content of 23.3% (wt/wt) is so far the highest content ever reported in eukaryotes. However, to make production of CGP a realistic industrial process, several parameters need to be optimized to enhance CGP accumulation. Therefore, different approaches might be useful. A first step was taken during this study through the generation and application of *Cpha6308Δ1*, which exhibited 2.5-fold higher specific activity than *Cpha6308*. Furthermore, metabolically engineered *P. pastoris* strains might result in increased product yields, as demonstrated previously for recombinant *S. cerevisiae* or bacteria (19, 54). Also, optimization of fermentation strategies, as shown for *Acinetobacter baylyi* and recombinant strains of *Pseudomonas putida* or *Ralstonia eutropha* (16, 18), might result in a significant increase in CGP accumulation. This study demonstrated that
variation of only a few simple parameters can result in a significant increase in product yield (Fig. 2). However, the maximum achievable CGP content seems not to be reached by far, which becomes clear after comparison of the CGP contents of \( P. \) pastoris and \( E. \) coli, respectively. While expression of CphA\_6308\_\Delta 1 compared to CphA\_6308 resulted in an improved CGP accumulation of 30% in \( P. \) pastoris, \( E. \) coli synthesize twice as much CGP (Table 4). From this it was concluded that cells of \( P. \) pastoris lack specific substrates necessary for synthesis of the polymer. Another aspect for further optimization of CGP synthesis could be a detailed analysis of recombinant Mut\(^+\) or Mut\(^-\) strains. Here, supply of mixed carbon sources might be beneficial to obtain high cell densities and a high product yield (15, 30, 31). As in \( S. \) cerevisiae, it was shown in \( P. \) pastoris that selection of the promoter controlling the expression of cphA is significant; while high CGP contents were observed in strains of \( S. \) cerevisiae expressing cphA under the control of the CUP1 promoter but not by using the GAL1 promoter (52), significant CGP contents were only observed in \( P. \) pastoris using the strong AOX1 promoter. The type of CGP synthesized by \( P. \) pastoris, concerning its solubility behavior and its molecular mass distribution, is comparable to that pro-

FIG. 2. Cell densities and CGP contents of cells of transgenic \( P. \) pastoris strains. Cultivations were done in 2-liter fermentors; up to six reactors were run in parallel. If not indicated otherwise, strain GS115 harboring pPICOLI-3::cphA\_6308\_his4 was cultivated at a pH of 5.0 and a temperature of 30°C. Aeration was kept at 3.0 liters/min with agitation by a stirrer at 200 to 800 rpm. For induction of CphA synthesis, 1% (vol/vol) methanol was added to strain GS115 and 0.5% (vol/vol) was added to strain KM71H when required and after depletion of glycerol. Experiments were done in duplicate, and standard deviations of CGP contents and CDMs were below 1.4%. (A) Comparison of different media for the cultivation of strain GS115 harboring pPICOLI-3::cphA\_6308. (B) CGP contents observed in cells of strain GS115 harboring pPICOLI-3::cphA\_6308 after different cultivation times. Samples were taken daily during fermentation, and CGP was isolated from 80 mg of dry cells. Induction started on the second day. (C) Comparison of vector pPICOLI-3::cphA\_6308 harboring or not harboring the his4 gene not supplemented or supplemented with 100 \( \mu \)g/ml zeocin. (D) Comparison of different temperatures set after induction with methanol. (E) Comparison of different pH values set after induction with methanol with the temperature set to 26°C. (F) Comparison of strain GS115 harboring pPICOLI-3::cphA\_6308\_\Delta 1\_his4 or pPICOLI-3::cphA\_6308\_\Delta 1\_his4 without glycerol feeding and with glycerol feeding.
duced in *S. cerevisiae*; in both yeasts, mostly the soluble type of the polymer is accumulated in flask cultures (52, 54) and no insoluble CGP is detected after cultivation in fermentors (54a). Additionally, complete stabilization of the episomal plasmid encoding *cphA* was achieved during the present study through provision of a gene (*his4*) essential to the organism. Stabilization of *cphA*-encoding plasmids dependent on the addition of antibiotics provides problems during the cultivation of recombinant bacteria. The strategy of construction of an addiction system, as also used here, represents an effective method (35, 55).

An interesting aspect analyzed during this study was the comparison of CGP synthesis in yeast and in *E. coli* expressing identical *cphA* genes. The assumption that synthesis of soluble CGP is not dependent on the host or on the *cphA* source (21) could be partially confuted. Here, it was shown that synthesis of soluble CGP is dependent on the host organism, not on the CphA source. However, this cannot explain the occurrence of soluble CGP in *E. coli* observed by Ziegler et al. (59).

Nowadays, an interesting aspect is the amino acid composition of the synthesized polymer, as synthesis of dipeptides derived from CGP could find applications in pharmacy (45), and the production of a variety of these compounds with different compositions should be considered. Therefore, the comparably high fractions of lysine incorporated into CGP by *P. pastoris* make this organism a possible candidate for the production of lysine-rich CGP and an interesting and special candidate for industrial production of the polymer in comparison to other organisms. In addition, yeasts are especially promising hosts in this regard as they synthesize mainly the soluble form of the polymer, which exhibits a broader composition range in comparison to insoluble CGP (54). To further increase incorporated fractions of lysine, engineering of strains would be necessary, i.e., through directed changes in lysine metabolism, for example, by generating lysine-overproducing mutants (20). Furthermore, *P. pastoris* might represent a suitable candidate for the production of further CGP variants, as described previously in *S. cerevisiae* (54). Therefore, corresponding mutants defective in arginine metabolism, already described by Nett et al. in 2005 (41), should be used and analyzed. Production of CGP variants is of special interest for the synthesis of structurally different β dipeptides employed as pharmaceutical agents (46).

The conferred increased specific activities of CphA<sub>6308Δ1</sub>, CphA<sub>6308ΔC595S</sub>, and CphA<sub>6308Δ1C595S</sub>, in comparison to that of wild-type CphA<sub>6308</sub>, were corroborated by increased CGP contents observed in recombinant strains of *P. pastoris* and *E. coli*. Residue C595 is part of the putative ATP-binding region in CphA; however, a mutation of this residue did not lead to loss of activity, indicating that C595 is not directly involved in the binding of ATP. After the mutation of residues K261 and K497, which are parts of ATP-binding motifs, complete loss of enzyme activity occurred (3, 4, 53). Furthermore, residue C595 seems not to be involved in the formation of a disulfide bond, as in this case a loss of activity would be expected due to misfolding of the enzyme. Increased CphA activity resulting from C-terminal truncation was previously observed by applying CphA from *N. ellipsosporum* NE1 consisting of 901 amino acids. (26, 27). Hai et al. (26) postulated that a CphA protein composed of 872 amino acids, corresponding to CphA<sub>6308Δ2</sub>, is optimal concerning high enzyme activity. However, in this study it was shown that truncation to a length of 873 amino acids led to the highest enzyme activities. The amino acid composition was not affected by the use of the different CphA proteins, leading to the assumption that the mutated or deleted residues do not play a role in substrate binding during catalysis, although the postulated amino acid substrate binding region includes the deleted residues (3, 53). It can be postulated that the last three C-terminal residues are not directly involved in the formation of the active site of CphA. Thus, detection and characterization of the putative substrate binding sites require further intensive research, which might open new ways for expansion of the substrate specificity of CphA. In conclusion, generation of CphA proteins with increased enzyme specific activities provides an important tool for increasing the CGP yields of various recombinant bacteria, yeast, or higher eukaryotes.

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