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Pullulanase from the hyperthermophilic bacterium *Thermotoga maritima*: purification by β-cyclodextrin affinity chromatography

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Abstract

This is the first report about the isolation of a type I pullulanase from a hyperthermophilic bacterium, *Thermotoga maritima* strain MSB8. Purification of the enzyme from a cleared cell-free extract was achieved by anion-exchange chromatography and β-cyclodextrin affinity chromatography. Using this convenient two-step method we have purified the pullulanase 406-fold with a 26% yield. The purified enzyme displayed maximum pullulan hydrolysis at pH 5.9 and 90°C (15-min assay) and was remarkably resistant against thermoinactivation, having a half-life at 90°C of about 3.5 h. To our knowledge, the *T. maritima* pullulanase is the most thermostable type I pullulanase known to date. The affinity-based purification protocol described here may be useful for the efficient isolation of other pullulanases. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Purification; *Thermotoga maritima*; Enzymes; Pullulanase

1. Introduction

Pullulanases are defined as enzymes that hydrolytically cleave pullulan, an α-glucan polysaccharide, which can be regarded as a chain of maltotriose units linked by α-1,6-glycosidic bonds. Pullulan serves as a model substrate for amylolytic enzymes, especially those specific for α-1,6-bonds. Based on the glycosidic bond type and the constellation of adjacent glycosidic bonds, the repeated unit of pullulan contains three different kinds of linkages to be cleaved, i.e., (i) α-1,6-bonds with the neighbouring bonds both being α-1,4, (ii) α-1,4-bonds with the neighbouring bonds being an α-1,6-bond on the non-reducing side and an α-1,4-bond on the reducing side, respectively, and (iii) α-1,4-bonds with the neighbouring bonds being an α-1,4-bond on the non-reducing side and an α-1,6-bond on the reducing side, respectively. These three kinds of bonds are cleaved by enzymes called pullulanases (EC 3.2.1.41), isopullulanases (EC 3.2.1.57), and neopullulanases (EC 3.2.1.135), respectively. The pullulan hydrolysis products formed by these enzymes are the trisaccharides maltotriose, isopanose (6-*O*-α-maltosylglucose), and panose (6^2-*O*-α-glucosylmaltose), respectively. The pullulanases can be subdivided into two types, i.e., pullulanase type I and type II. Both cleave the α-1,6-bonds in pullulan, yielding maltotriose, but pullulanases type II in contrast to type I can additionally hydrolyse α-1,4-glycosidic linkages

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in other α-glucans like amylose or amylopectin and therefore sometimes are also called amylopullulanases or α-amylase-pullulanases.

In combination with other amylolytic enzymes (α-amylase, β-amylase, glucoamylase), pullulanases are used in the starch processing industry for the production of sugar syrups [1,2]. In these processes, they act as debranching enzymes to improve the saccharification of starch. For this purpose, thermoresistant and thermoactive pullulanase are needed. Thermostable type I pullulanases (but not type II pullulanases=amylopullulanases) also may be useful for the production of long-chain dextrins from starch or amylopectin. Several pullulan-hydrolysing enzymes have been found in extreme thermophiles and hyperthermophiles (those with an optimum growth temperature of 80°C or higher), e.g., from *Thermoanaerobacterium saccharolyticum, Thermoanaerobacterium thermosulfurigenes* (previously *Clostridium thermosulfurogenes*), *Thermoanaerobacter* sp., *Clostridium thermohydrosulfuricum*, *Pyrococcus furiosus*, *Pyrococcus woesei*, or *Thermococcus litoralis* but these in general are type II pullulanases and thus cleave α-1,4-bonds from starch in addition to α-1,6-bonds from pullulan [2–9]. Only two enzymes with type I pullulanase specificity were reported from thermophiles, i.e., from the aerobe, *Bacillus flavocaldarius* KP 1228 [10], and the anaerobe, *Fervidobacterium pennavorans* Ven5 [11], but none of these enzymes have been described from a hyperthermophile.

The hyperthermophilic bacterium, *Thermotoga maritima*, is characterised by a growth range between 55°C and 90°C with an optimum at 80°C [12], making it one of the most thermophilic bacteria known to date. This organism degrades and fermentatively utilises various organic compounds, including starch and its α-glucan polysaccharide constituents amylose and amylopectin. We have identified several different enzyme activities putatively involved in starch utilisation by *T. maritima* MSB8, among them a pullulanase ([13]; unpublished data), and have isolated the corresponding chromosomal genes [13–19]. Previous work had indicated that the amylolytic enzymes of *T. maritima* are only expressed at a very low level ([17,18,20]; unpublished data). Since, in addition, the growth yields reached with strictly anaerobic hyperthermophiles are usually very low, it was necessary to find an efficient purification procedure for the isolation of the pullulanase of *T. maritima*. We now report an efficient and convenient two-step method for the isolation of this highly thermostable enzyme from cells of *T. maritima* strain MSB8.

2. Experimental

2.1. Growth of T. maritima and preparation of crude extract

T. maritima strain MSB8 (DSM 3109), the type strain of *T. maritima* [12], was grown anaerobically at 80°C in 500 ml marine broth (Difco 2216, Detroit, MI, USA) supplemented with 0.5% soluble starch (Merck, Darmstadt, Germany), 0.0001% resazurin, and 0.5% sodium sulfide. This culture was used to inoculate a total of 50 l medium containing 0.5% peptone, 0.1% yeast extract, 0.25% soluble starch, 3% NaCl, 0.5% Na_2S, 0.0001% resazurin dissolved in tap water and preheated to 80°C. After about 18 h growth at 80°C, the cells were harvested, washed twice with diluted (1:10) McIlvaine buffer, pH 6.0, and suspended in 50 ml of the same buffer. The cells were disintegrated by a twofold passage of the suspension through a French pressure cell (American Instrument Company, Silver Spring, USA) at 6.9 MPa. The crude extract was cleared by centrifugation at 20 000 *g* at 4°C for 40 min followed by ultracentrifugation at 140 000 *g* at 8°C for 3 h. The cleared crude extract thus obtained had a volume of 50 ml and contained about 540 mg total protein.

2.2. Purification of the T. maritima pullulanase

All chromatographic procedures were performed at room temperature, using a fast protein liquid chromatography (FPLC) system (Pharmacia, Freiburg, Germany). In a first step, the *T. maritima* proteins were subjected to anion-exchange chromatography on Q Sepharose HP (Pharmacia). The anion-exchange matrix (25 ml bed volume) was packed into a XK 16/20 column (200 mm×16 mm) (Pharmacia) and equilibrated with 20 m*M* Tris–HCl, pH 7.5. Loading of the column with cleared crude extract was carried out at a flow-rate of 2.5 ml

min^{-1}. After sample application, the column was washed with one bed volume of equilibration buffer. Elution took place with a linear 0–700 m*M* sodium chloride gradient in the same buffer, and 2.5 ml fractions were collected throughout. The pullulanase activity eluted at a sodium chloride concentration of about 300 m*M* (Fig. 1). This step was carried out in two batches, applying 25 ml cleared extract (about 270 mg protein) to the column each time. The anion-exchange chromatography step serves the following purposes: (i) separation from DNA, RNA, lipids and other high- and low-molecular-mass substances present in the crude extract; (ii) partial separation of the enzyme from other proteins; (iii) elimination of other hydrolytic activities (cyclodextrinases and some α-amylases can hydrolyse β-cyclodextrin and therefore would destroy the column-bound ligand during the next step, i.e., β-cyclodextrin affinity chromatography); (iv) separation of the pullulanase from other proteins with affinity to β-cyclodextrin.

The second purification step was done with affinity chromatography using β-cyclodextrin, an efficient inhibitor of the *T. maritima* pullulanase (data not shown), as the affinity ligand. For this purpose, β-cyclodextrin was covalently coupled to activated Mini-Leak High (Kem-En-Tec, Copenhagen, Denmark), an agarose-based gel matrix modified with vinyl groups. A 2.25-ml volume of this material was washed and suspended in 10 ml distilled water. After the addition of 0.2 g β-cyclodextrin and 0.04 g sodium hydroxide (the pH should be 10–12), the coupling reaction was allowed to proceed for 24 h at 45°C with weak agitation. The gel matrix was packed into a XK16/10 column (100 mm×16 mm) (Pharmacia) and washed (about 10 bed volumes each) with distilled water, 0.1 *M* sodium acetate–0.5 *M* sodium chloride, pH 5.0, distilled water, and finally equilibrated with 20 m*M* Tris–HCl, pH 6.0–20 m*M* sodium chloride–0.01% sodium azide. Before use, the column was washed with five bed volumes of 20 m*M* Tris–HCl, pH 6.0–20 m*M* sodium chloride containing 0.5% β-cyclodextrin (elution buffer) and then equilibrated with 20 m*M* Tris–HCl, pH 6.0–20 m*M* sodium chloride (equilibration buffer) before application of the sample dissolved in equilibration buffer at a flow-rate of 0.5 ml min^{-1}. Non-bound proteins were removed by washing with equilibration buffer. Elution of bound enzyme was done with elution buffer at the same

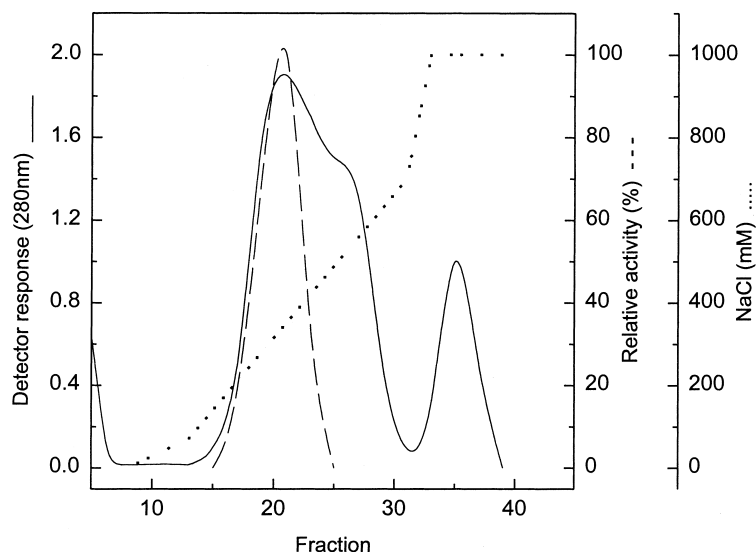

Fig. 1. Anion-exchange chromatography on a Q-Sepharose HP column. The column was equilibrated with 20 m*M* Tris–HCl, pH 7.5. Loading of the column (200 mm×16 mm, bed volume 25 ml) with cleared crude extract was carried out at a flow-rate of 2.5 ml min^{-1}. Elution took place with a linear 0–700 m*M* sodium chloride gradient in the same buffer. 2.5-ml fractions were collected and aliquots were checked for thermostable pullulanase activity.

flow-rate (Fig. 2). Fractions of 0.5 ml were collected and samples thereof were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with subsequent zymogram staining for pullulanase activity. The pooled pullulanase fractions were concentrated with centrifugation-aided ultrafiltration (Centricon 10 000, Amicon) and dialysed against 20 m*M* Tris–HCl, pH 6.0 in order to remove the pullulanase inhibitor β-cyclodextrin.

An alternative purification protocol using affinity chromatography with a β-cyclodextrin-ligated resin made with a different coupling chemistry, i.e., by coupling of the ligand to epoxy-activated Sepharose 6B (Pharmacia) yielded similar results as those just described (results not shown).

2.3. Enzyme assays and analytical methods

Unless mentioned otherwise, the following standard pullulanase assay based on the enzymatic liberation of reducing groups from pullulan was used. Standard assay mixtures (total volume 500 μl) contained 100 μl McIlvaine buffer (0.1 *M* citric acid titrated with 0.2 *M* Na_2HPO_4) pH 6.0 and 250 μl of a 1% pullulan (ICN, M_r 200 000) solution. After pre-equilibration for 5 min at 75°C the reaction was started by addition of enzyme. After 15 min at 75°C, the reaction mixture was cooled on ice and mixed with 0.75 ml dinitrosalicylic acid (DNSA) reagent [21]. Incubation of the tubes in boiling water for 15 min led to the development of a brown colour whose intensity (determined at 575 nm) was proportional to the amount of reducing groups present. One unit of pullulanase liberates 1 μmol of reducing groups (as maltotriose equivalents) per minute. For the determination of enzyme activity versus pH (at 75°C) or temperature (at pH 6.0) profiles, the assay mixtures (500 μl) contained 100 μl McIlvaine buffer, 250 μl of a 1% pullulan solution, and purified enzyme (final concentration 0.2–0.7 μg ml^{-1}). Thermostability data were obtained by pre-incubating pullulanase samples (about 3 μg ml^{-1}) in McIlvaine buffer, pH 6.0 and then measuring residual activity under the standard assay conditions described above.

Protein concentrations were determined by the method of Bradford [22] with bovine serum albumin as a standard. Discontinuous SDS–PAGE was done according to the method of Laemmli [23]. For the detection of pullulanase activity in SDS–PAGE gels, gels containing 0.42 mg ml^{-1} of remazol brilliant blue (RBB) pullulan were used. After electrophoretic protein separation, the gels were washed for 15 min at 40°C in a mixture of McIlvaine buffer, pH 6.0–isopropanol–water (1:1:2, v/v/v) to remove the SDS. Then, the gels were transferred to McIlvaine buffer, pH 6.0 without isopropanol and incubated at 75°C until white bands of pullulanase activity against a light blue background were visible.

Thin-layer chromatography (TLC) of mono- and oligosaccharides was done on 0.2 mm silica-gel coated aluminium sheets (type 60, Merck, Darmstadt, Germany) as described before [17], using 1 *M* DL-lactic acid–isopropanol–acetone (2:4:4, v/v/v) as the solvent system. Commercially available maltooligosaccharides and isomaltooligosaccharides were used as standards. Carbohydrate spots were made visible by spraying the chromatograms with diphenylamine-aniline reagent [1% (w/v)

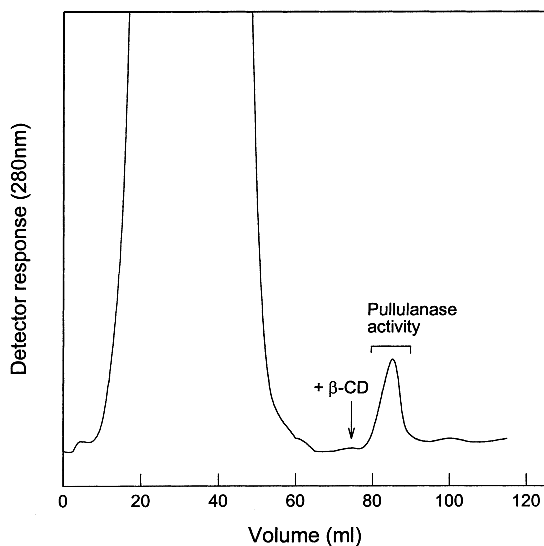

Fig. 2. Affinity chromatography using a β-cyclodextrin-coupled Mini-Leak High column. The column (100 mm×16 mm, bed volume 1.8 ml) was equilibrated with 20 m*M* Tris–HCl, pH 6.0–20 m*M* sodium chloride (equilibration buffer) before application of the sample at a flow-rate of 0.5 ml min^{-1}. After washing through non-bound proteins, elution of bound enzyme was done at the same flow-rate with 20 m*M* Tris–HCl, pH 6.0–20 m*M* sodium chloride–0.5% β-cyclodextrin. Fractions of 0.5 ml were collected and samples thereof were checked for pullulanase activity by SDS–PAGE and zymogram staining.

diphenylamin and 1% (v/v) anilin in acetone, mixed with 0.1 volume of 85% phosphoric acid just before use] and incubating the plates at 140°C for 12 min.

3. Results and discussion

An extremely thermostable pullulanase was purified from a crude extract of starch-grown *T. maritima* MSB8 cells. The purification protocol reported here led to an about 406-fold purified enzyme preparation with a reasonably good yield (about 26%) (Table 1). The enzyme was homogeneous as judged by SDS–PAGE analysis (Fig. 3).

The purified enzyme efficiently hydrolysed pullulan while amylose, a linear α-1,4-glucan, was not hydrolysed significantly as judged by measuring the increase of reducing groups and by thin layer chromatographic analysis. Therefore it is clear that in contrast to most pullulan-cleaving enzymes from extreme thermophiles and hyperthermophiles the *T. maritima* enzyme is not an amylopullulanase (type II pullulanase). Thin layer chromatographic and high-performance liquid chromatography (HPLC) analysis (data not shown) revealed maltotriose as the product of pullulan hydrolysis which indicates the specificity of the enzyme towards α-1,6-glycosidic bonds. In conclusion, the *T. maritima* MSB8 pullulanase can be classified as a type I pullulanase. To our knowledge the enzyme reported here is the first example of a type I pullulanase to be described from a hyperthermophile.

The pullulanase purification method described in this work makes use of an affinity purification step that is based on the fact that the enzyme binds to (and thereby is inhibited by) β-cyclodextrin. Other pullulanases of type I as well as type II have been shown to be inhibited by cyclodextrins, for example, pullulanases from *Klebsiella oxytoca* (type I), *Thermoanaerobacter* strain B6A (type II), *Clostridium thermohydrosulfuricum* (type II), *Pyrococcus woesei* (type II), *Pyrococcus furiosus* (type II), or *Thermococcus litoralis* (type II) [3,6–8,24], and cyclodextrin chromatography was used to isolate type II pullulanase [3,5]. However, to our knowledge, affinity chromatography with cyclodextrin as the ligand has not previously been used to purify a type I

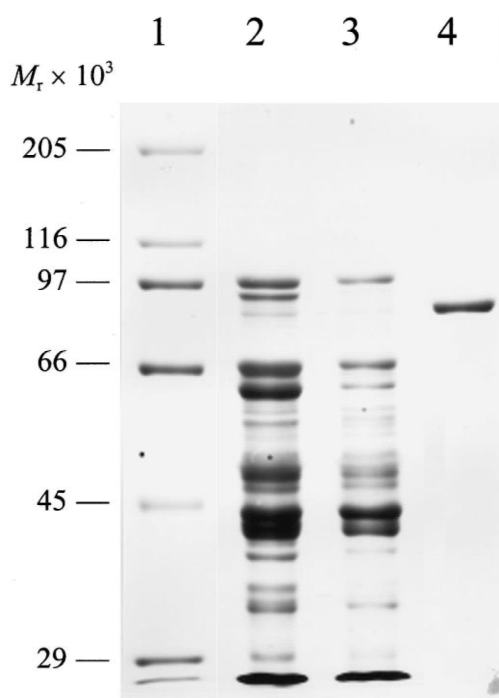

Fig. 3. SDS–PAGE gel (10%) of pullulanase purification steps. Lane 1, molecular mass markers; lane 2, cleared crude extract of *Thermotoga maritima* MSB8 (11 μg); lane 3, pooled fractions with pullulanase activity after Q Sepharose HP chromatography (5 μg); lane 4, purified pullulanase in pooled fractions after β-cyclodextrin-coupled Mini-Leak High chromatography (1 μg). The sizes of the molecular mass markers are indicated at the left margin.

Table 1
Summary of the purification of *Thermotoga maritima* MSB8 pullulanase

Purification step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification factor (-fold)	Yield (%)
Crude extract	536	23	0.043	1	100
Q Sepharose HP pool	126	20	0.16	3.7	87
β-Cyclodextrin affinity chromatography pool	0.09	6	65	406	26

pullulanase. We have now demonstrated that a chromatography matrix customised by covalent attachment of β-cyclodextrin moieties can be used for this purpose. Elution of pullulanase enzyme adsorbed to this affinity matrix can be achieved by addition of dissolved β-cyclodextrin to the mobile phase. In the case of pullulanase purification from *T. maritima* crude extract the affinity chromatography step resulted in a nearly 110-fold enrichment of the enzyme. Affinity chromatography is perhaps the most elegant and efficient technique available for enzyme purification, because it makes use of the highly selective interaction between the protein and its ligand, which mostly is tight but reversible. We believe that β-cyclodextrin affinity chromatography may be useful for the purification of a broad variety of type I and type II pullulanases.

The size of the purified *T. maritima* pullulanase as determined by SDS–PAGE analysis was about 89 000 which is slightly smaller than the size calculated from the primary structure (94 100). The effect of the pH value on pullulanase activity was determined in a 15 min assay in 50 m*M* sodium phosphate–citric acid buffer between pH 4.0 and pH 8.0. More than 50% of the maximum activity was measured between pH 4.5 and pH 7.5, with an optimum at about pH 5.9. The effect of increasing temperature on the activity was determined in a 15 min assay at pH 6.0. Maximum pullulan hydrolysis under these conditions was observed at 90°C. The effect of long-term incubation at elevated temperatures on the pullulanase was measured in McIlvaine buffer at pH 6.0 at a very low enzyme concentration of 3 μg ml^{-1} without the addition of any additional stabilising compounds. At 75°C the enzyme was remarkably stable, showing no significant loss of activity over a period of at least 6 h. At 90°C, which is 10°C higher than the optimum growth temperature of *T. maritima*, significant inactivation of the pullulanase occurred (half-life about 3.5 h) (Fig. 4). It is noteworthy that the *T. maritima* enzyme is the most thermoactive and the most thermostable type I pullulanase known. The enzyme may be an interesting candidate for application in the starch processing industry. High-level expression in a heterologous mesophilic host for a more detailed enzyme characterisation is currently in progress. The affinity purification method described here should also be helpful for the efficient purification of the recombinant enzyme.

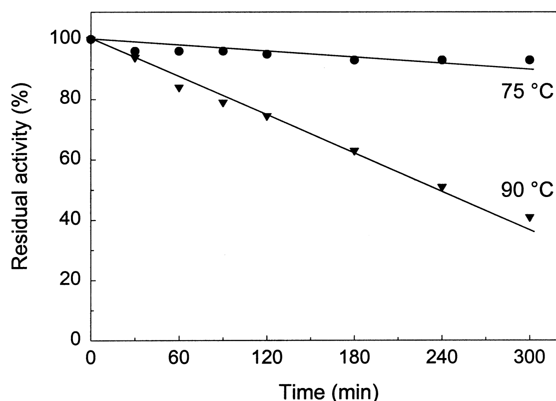

Fig. 4. Pullulanase inactivation kinetics at 75°C and 90°C. Purified enzyme at a concentration of about 3 μg ml^{-1} was incubated in McIlvaine buffer, pH 6.0. The residual activity present after various periods of incubation was measured under the standard assay conditions.

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References

[1] J.F. Kennedy, V.M. Cabalda, C.A. White, Trends Biotechnol. 6 (1988) 184.
[2] B.C. Saha, J.G. Zeikus, Trends Biotechnol. 7 (1989) 234.
[3] S.H. Brown, R.M. Kelly, Appl. Environ. Microbiol. 59 (1993) 2614.
[4] H. Melasniemi, Biochem. J. 250 (1988) 813.
[5] M.V. Ramesh, S.M. Podkovyrov, S.E. Lowe, J.G. Zeikus, Appl. Environ. Microbiol. 60 (1994) 94.
[6] A. Rüdiger, P.L. Jorgensen, G. Antranikian, Appl. Environ. Microbiol. 61 (1995) 567.
[7] B.C. Saha, S.P. Mathupala, J.G. Zeikus, Biochem. J. 252 (1988) 343.
[8] B.C. Saha, R. Lamed, C.-Y. Lee, S.P. Mathupala, J.G. Zeikus, Appl. Environ. Microbiol. 56 (1990) 881.
[9] A. Spreinat, G. Antranikian, Appl. Microbiol. Biotechnol. 33 (1990) 511.
[10] Y. Suzuki, K. Hatagaki, H. Oda, Appl. Microbiol. Biotechnol. 34 (1991) 707.
[11] R. Koch, F. Canganella, H. Hippe, K.D. Jahnke, G. Antranikian, Appl. Environ. Microbiol. 63 (1997) 1088.

[12] R. Huber, T.A. Langworthy, H. König, M. Thomm, C.R. Woese, U.B. Sleytr, K.O. Stetter, Arch. Microbiol. 144 (1986) 324.
[13] M. Bibel, C. Brettl, U. Gosslar, G. Kriegshäuser, W. Liebl, FEMS Microbiol. Lett. 158 (1998) 9.
[14] A. Steinbüchel (Ed.), Biochemical Principles and Mechanisms of Biosynthesis and Biodegradation of Polymers, Wiley–VCH, 1999, p. 239.
[15] W. Huber, W. Liebl, Biocatalysis 11 (1994) 105.
[16] P. Heinrich, W. Huber, W. Liebl, System. Appl. Microbiol. 17 (1994) 297.
[17] W. Liebl, R. Feil, J. Gabelsberger, J. Kellermann, K.H. Schleifer, Eur. J. Biochem. 207 (1992) 81.
[18] W. Liebl, I. Stemplinger, P. Ruile, J. Bacteriol. 179 (1997) 941.
[19] H. Meissner, W. Liebl, Eur. J. Biochem. 250 (1998) 1050.
[20] J. Schumann, A. Wrba, R. Jaenicke, K.O. Stetter, FEBS Lett. 282 (1991) 122.
[21] P. Bernfeld, Methods Enzymol. 1 (1955) 149.
[22] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[23] U.K. Laemmli, Nature 277 (1970) 680.
[24] H. Iwamoto, M. Ohmori, M. Ohno, J. Hirose, H. Keitaro, H. Fukada, K. Takahashi, H. Hashimoto, S. Sakai, J. Biochem. 113 (1993) 93.