Biocatalysis and Agricultural Biotechnology 17 (2019) 339–346

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Optimization of conditions and partial characterization of cyanophycin synthetase from a thermophilic cyanobacterium *Chlorogloeopsis fritschii*

Jeevan Jyoti[a], J.I.S. Khattar[a,*], A. Gulati[b], D.P. Singh[a]

[a] Department of Botany, Punjabi University, Patiala 147002, India
[b] CSIR-IHBT, Palampur, India

ARTICLE INFO

Keywords:
Cyanobacteria
Cyanophycin
Cyanophycin synthetase
Optimization
Thermal stability

ABSTRACT

Cyanophycin granule polypeptide (CGP), a polymer of aspartic acid and arginine, synthesized by cyanophycin synthetase can be converted to polyaspartate which has many industrial applications. Cyanophycin and cyanophycin synthetase from a thermophilic cyanobacterium *Chlorogloeopsis fritschii* have been studied expecting enzyme from this organism to be thermostable. The organism exhibited best growth in Chu-10 medium at 45 °C. Maximum amount of cyanophycin was observed on 21 d when organism entered into stationary phase. Optimum conditions for cyanophycin synthetase were 30–40 °C and pH 8.0–9.5. The enzyme showed high specificity to aspartic acid and arginine but synthesized polyaspartate when arginine was omitted from the assay mixture. The enzyme activity doubled when Zn^{2+} were used as cofactor in place of Mg^{2+}. The enzyme exhibited good thermal stability as it showed 66% activity when treated with 45 °C for one hour. Since cyanophycin synthetase formed an industrially valuable molecule polyaspartic acid and showed thermal stability, it has potential applications which needs to be further investigated.

1. Introduction

Bio-sourced macromolecules are an attractive, sustainable and environment friendly compatible feedstock for material synthesis (Khlystov et al., 2017). Biopolymers are abundant, and can be replenished efficiently and quickly than petroleum based polymers (Parikka, 2004). Microorganisms have already been successfully demonstrated for the production of chemicals, fuels etc. Similar efforts can address environment and sustainable concerns in polymer materials as well (Naik et al., 2010; Rehm, 2010). Cyanophycin, also referred to as cyanophycin granule polypeptide (CGP), a biopolymer synthesized by cyanobacteria and some non- photosynthetic bacteria is an interesting molecule with industrial applications (Sherman et al., 2000; Wingard et al., 2002). Cyanophycin (multi-L-arginyl-poly[L-aspartic acid]) is a protein-like, branched, non-ribosomally synthesized polypeptide consisting of equimolar amounts of aspartic and arginine arranged as a polyaspartic acid back-bone, to which arginine residues are linked to the β-carboxyl group of each aspartate by its α-amino group (Oppermann-Sanio and Steinbuchel, 2002; Krehenbrink and Steinbuchel, 2004). This polymer in cyanobacteria is present in membraneless granules deposited in the cytoplasm and in the so- called polar plugs of heterocysts (Simon, 1987; Krehenbrink and Steinbuchel, 2004). CGP serves as a dynamic reservoir of nitrogen, carbon and energy in cyanobacteria (Hai et al., 2002). It accumulates usually during the transition from the exponential to the stationary growth phase (Berg et al., 2000), under stress conditions (Liotenberg, 1996) and in specific cell forms such as akinetes and heterocysts (Sarma et al., 2004; Frommeyer et al., 2016) and disappears when balanced growth resumes (Mackerras et al., 1990; Liotenberg et al., 1996).

CGP in cyanobacteria is synthesized via an ATP-dependent step by step reaction catalyzed by a single enzyme, cyanophycin synthetase (Berg et al., 2000). Cyanophycin synthetase requires L- aspartic acid (Asp), L-arginine (Arg), ATP, Mg^{2+} and a primer (low molecular mass cyanophycin) and catalyzes the elongation of cyanophycin primer (Arai and Kino, 2008). Cyanophycin has shown its special and unique solubility behavior. It is insoluble under neutral pH condition, but is soluble under acidic pH condition due to its structural feature and is therefore easily purified (Frey et al., 2002).

CGP is an interesting material as a biodegradable source of polyaspartic acid. Purified cyanophycin can be chemically converted to polypeptides with reduced arginine content, which might be used like polyaspartic acid as a biodegradable substitute for synthetic polyacrylate in various chemical processes (Schwamborn, 1998; Mooibroek et al., 2007). Technical applications for the production of polyaspartate from cyanophycin exist (Joentgen et al., 2001; Zotz et al., 2001; Erickson et al., 2001). Polyaspartic acid has potential application areas

* Corresponding author.
E-mail address: jisk@pbi.ac.in (J.I.S. Khattar).

https://doi.org/10.1016/j.bcab.2018.12.011
Received 25 November 2018; Received in revised form 7 December 2018; Accepted 10 December 2018
Available online 11 December 2018
1878-8181/

as an additive in paper, paint, building or oil industry (Rehm, 2009). This polymer has also recently attracted the attention of scientific community as a biodegradable replacement for petrochemical based industrial products. Bio-sourced macromolecules such as cyanophycin are an attractive source for alternative, sustainable plastics (Khlystov et al., 2017). Several examples of biosourced polymers used as consumer plastics already exist, and certain other high-production plastics can be feasibly replaced by those derived from biomass (Joentgen et al., 2001; Ragauskas et al., 2006; Rehm, 2010; Shen, 2010).

Polyaspartic acid, backbone of cyanophycin, has potential market value in waste water treatment industry since this can serve as an environment friendly substitute for polyacrylic acid currently being used as a water softener (Schwamborn, 1998). CGP can also serve as a source for dipeptides and amino acids in food, feed and pharmaceutical industries. It has broad spectrum of nutritional or therapeutic applications (Sallam et al., 2010). It is desired that industrial enzymes are thermostable. Studies on cyanophycin synthetase mainly focused on mesophilic cyanobacteria and two thermophilic cyanobacteria namely, *Synechococcus* sp. and *Thermosynechococcus elongatus* (Hai et al., 1999, 2002; Arai and Kino, 2008). The present work was aimed to characterize cyanophycin synthetase from another thermophilic cyanobacterium *Chlorogloeopsis fritschii* in terms of thermal stability.

2. Material and methods

2.1. Organism and culture conditions

The cyanobacterial strain *Chlorogloeopsis fritschii* was isolated from Gauri Kund hot water spring (30° 43′ 50.67″ N, 79° 03′ 59.13″ E) which is situated at an altitude of 3505 m above sea level, on the way to Kedarnath, 28 km from Ukhimath, Uttarkashi district of Uttarkhand, India (Singh et al., 2018). The organism was grown in slightly modified Chu-10 medium (Safferman and Morris, 1964) in which $CaNO_3$ was replaced by equivalent concentration of $CaCl_2$, supplemented with 10 mM KNO_3 and pH of the medium was adjusted to 7.8 (see Sections 2.2.2 and 3.1.1). The stock and experimental cultures were incubated in a BOD incubator at 45 °C ± 2 °C and illuminated for 14 h daily with a light intensity of 44.5 μmol photon flux intensity $(m^2)^{-1}$ s^{-1} at the surface of culture vessels. Exponentially growing stock cultures (5–8 days old) were used throughout the study.

2.2. Optimization of condition for growth of the organism

2.2.1. Selection of medium

Appropriate culture medium for the organism was selected by growing it at 40 °C in six different growth media such as Chu-10 (Safferman and Morris, 1964), Hoagland medium (HM) (Hoagland and Arnon, 1950), Bold Basal medium (BBM) (Bischoff and Bold, 1963), BG11 medium (BG11) (Rippka et al., 1979), Pavensoli Hassel medium (PHM) (Borowitzka, 1988) and Allen & Arnon medium (A&A) (Allen and Arnon, 1955). The composition of the individual medium is given in Table 1. The growth of the organism was studied for 12 days in terms of increase in absorbance and biomass dry weight of the cultures and the medium supporting maximum growth was chosen for further study.

2.2.2. Optimization of nitrate concentration

Chu-10 medium supported maximum growth in the above experiment. Concentration of nitrate in the above medium was optimized by replacing calcium nitrate with equal amount of calcium chloride and varied concentrations of nitrate (0.5–10 mM) were supplemented and growth of the organism was studied as described above. This medium is defined as modified Chu-10 medium.

2.2.3. Optimization of temperature

To optimize temperature for growth of the organism it was grown in Chu-10 medium (medium selected from the above experiment) at different temperature (35 °C, 40 °C, 45 °C, 50 °C and 55 °C) in BOD incubators.

Table 1
Composition of the media used in the present study.

Nutrient	Chu-10	BG-11	BBM	HM	A&A	PHM
$CaCl_2.2H_2O$	–	0.03[a]	0.025[a]	–	0.055[a]	
$CaSO_4$ (Sat. soln)						20 mL
$MgSO_4.7H_2O$	0.025[a]	0.075[a]	0.075[a]	–	0.246[a]	0.2[a]
Na_2CO_3	0.020[a]	0.02[a]	–	–	–	–
$Na_2SiO_3.5H_2O$	0.044[a]	–	–	–		–
K_2HPO_4	0.010[a]	0.04[a]	0.075[a]	0.136[a]	0.348[a]	0.2[a]
Citric acid	0.035[a]	0.006[a]	–	–		–
Ferric citrate	0.035[a]	0.006[a]	–	–		–
KNO_3	–	0.02[a]	–	0.202[a]	1.10[a]	1.0[a]
EDTA	–	0.001[a]	0.050[a]	–	0.001[a]	–
$NaNO_3$	–	1.5[a]	0.25[a]	–		–
$Ca(NO_3)_2$	0.232[a]	–	–	0.236[a]		
KH_2PO_4	–	–	0.175[a]	–		–
$NH_4(NO_3)_2$	–	–	–	–		–
KOH	–	–	0.031[a]	–		–
NaCl	–	–	0.025[a]	–	0.232[a]	–
$B(H_3BO_3)$	0.5[b]	2.86[b]	0.00[b]	2.86[b]	0.5[b]	0.612[b]
$Zn(ZnSO_4.7H_2O)$	0.05[b]	0.22[b]	0.008[b]	0.22[b]	0.05[b]	0.04[b]
$Mn(MnCl_2.4H_2O)$	0.5[b]	1.81[b]	0.44[b]	1.81[b]	0.5[b]	0.04[b]
$Cu(CuSO_4.5H_2O)$	0.02[b]	0.079[b]	1.57[b]	0.08[b]	0.02[b]	0.06[b]
$Mo(MoO_3)$	0.01[b]	0.049[b]	0.71[b]	0.02[b]	0.01[b]	–
$(NH_4)_6Mo_7O_{24}.4H_2O$						0.06[b]
$Co(CoCl_3)$	0.04[b]	0.39[b]	–	–	0.04[b]	–
$FeSO_4.7H_2O$	–	–	0.0049[b]	–	–	–
$Co(NO_3)_26H_2O$	–	–	–	–	–	0.0494[b]

[a] g L^{-1}.
[b] mg L^{-1}.

2.3. Growth

Exponentially growing cultures were washed twice by centrifugation at 5000 g for 10 min and inoculated in 150 mL medium contained in 250 mL Erlenmeyer flasks to attain 0.1 absorbance at 750 nm. Immediately a known volume of cultures was withdrawn to determine biomass dry weight. The cultures were incubated in incubators at 45 °C unless otherwise stated. At regular interval of 3 days, 20 mL of cultures were withdrawn, washed by centrifugation, and made the same volume with distilled water and then absorbance and biomass dry weight was noted. The cells of the organism were observed under light microscope for the presence of CGP granules.

2.4. Quantification of cyanophycin

Twenty millilitres of experimental cultures were withdrawn, washed with double distilled water and centrifuged at 5000 *g* for 10 min. Biomass pellet was suspended in phosphate buffer (0.06 M; pH 7.2) and disintegrated by sonication for isolation of cyanophycin granules. The extract obtained was centrifuged at 20, 000 g at 4 °C for 15 min. The supernatant was stored for protein estimation and was also used as enzyme extract for cyanophycin synthetase activity. The pellet containing broken cell walls, membranes and cyanophycin granules was used for the determination of cyanophycin. Cyanophycin granules were hydrolyzed with 1.0 N HCl at 30 °C for 1 h. Amount of arginine in CGP hydrolysate was determined as described earlier (Sarma and Khattar, 1986). Since CGP is a polymer of aspartic acid and arginine in equimolar ratio, the amount of arginine determined was doubled to get the amount of CGP.

2.5. Determination of cyanophycin synthetase activity and characterization of CGP formed

The supernatant saved from above was used as enzyme extract to

determine cyanophycin synthetase activity. Enzyme activity was measured by slightly modifying the method of Arai and Kino (2008) by replacing Tris-HCl buffer with imidazole buffer. The assay mixture contained in a final volume of 300 µL:12.5 mM each of ATP, $MgSO_4.7H_2O$, aspartic acid and arginine in 100 mM imidazole-HCl buffer (pH 8.0) and 100 µL enzyme extract. The assay mixture was incubated at desired temperature. At desired time, the assay mixture was centrifuged at 20,000 g for 15 min. The pellet was used for the characterization of CGP formed. The amount of Pi released by the activity of cyanophycin synthetase was determined in the supernatant obtained above by using Pi determination kit (Emerck, India). Specific activity of cyanophycin synthetase is expressed as nmol of Pi released mg^{-1} protein h^{-1}.

The CGP formed by the activity of cyanophycin synthetase in the above experiment was characterized by HPLC by slightly modifying the method of Hai et al. (1999). CGP was hydrolyzed with 1.0 N HCl at 30 °C for 1.0 h. Hydrolyzed cyanophycin was lyophilized and dissolved in starting eluent (74 mM sodium acetate in 52% methanol, pH 7.0). The above solution was clarified by centrifugation at 10,000 *g* for 5 min. To 460 µL of this solution, 200 µL 0.5 M sodium borate (pH 9.5) and 100 µL OPA reagent (100 mg ortho-phthaldialdehyde, 9 mL methanol, 1 mL 0.5 M sodium borate (pH 9.5), 100 µL 2-mercaptoethanol) were added. After exactly 200 s, 60 µL of 0.75 N HCl was added. To 100 µL of this mixture, 400 µL starting eluent was added. Ten microlitre of this solution was injected with Waters 2707 auto sampler into Waters Sun Fire C_{18} column (5.0 µm × 4.6 mm × 250 mm) fitted with Water HPLC system. OPA amino acids were eluted with 30% methanol at a flow rate of 1.0 mL min^{-1} and were detected with PDA detector at 280 nm. Ampower2 software was used to analyze the spectra.

2.6. Optimization of enzyme assay conditions

2.6.1. Temperature

Optimum temperature for cyanophycin synthetase was determined by incubating the assay mixture for 8 h at different temperature i.e., 20–50 °C with an increment of 5 °C and the enzyme activity was determined.

2.6.2. pH

To determine the optimum pH for cyanophycin synthetase, enzyme activity was assayed in the reaction mixture having 100 mM imidazole-HCl buffer with different pH i.e. 7.5–11.0 with an increment of 0.5.

2.7. Characterization of cyanophycin synthetase

2.7.1. Substrate specificity

Asp and Arg are the main substrates for cyanophycin synthetase. The specificity of this enzyme for other amino acids such as L-glutamic acid (Glu), L-histidine (His), L-Lysine (Lys) and L-Glycine (Gly) was studied as described and optimized above. These amino acids were used one by one by replacing arginine unless otherwise stated.

2.7.2. Cofactors for cyanophycin synthetase

Mg^{2+} in assay mixture was replaced with Mn^{2+}, Fe^{2+}, Zn^{2+}, K^{+}, Cd^{2+} or Ca^{2+} to check whether these acted as cofactors or not for the enzyme.

2.7.3. Source of energy

ATP was replaced by GTP, CTP or TTP, to check whether these can act as source of energy.

2.7.4. Thermal stability of the enzyme

The enzyme extract was incubated at different temperature (30–60 °C) for 1 h and then its activity was determined as described in Section 2.5.

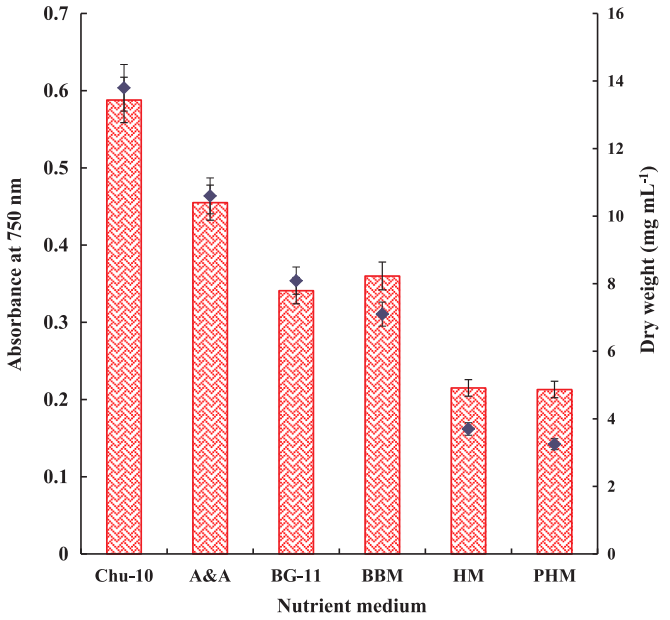

Fig. 1. Growth of *Chlorogloeopsis fritschii* in different media on 12 day at 40 °C. Bars: absorbance; diamonds: dry weight.

2.8. Protein content

Protein content of the enzyme extract was determined following Lowry et al. (1951).

The data are average of three independent experiments each with three replicates ± S.D.

3. Results

3.1. Optimization of conditions for growth

3.1.1. Selection of medium

Among the six growth media tested, Chu-10 medium supported maximum growth of the organism. The absorbance and biomass dry weight of *Chlorogloeopsis fritschii* in this medium increased from 0.1 to 0.47 and from 2.7 to 10.6 mg mL^{-1}, respectively, on 12 d (Fig. 1). Modified Chu-10 medium supplemented with 10 mM potassium nitrate supported maximum growth of the organism (data not shown). Thus this medium was used for rest of the study.

3.1.2. Temperature

Maximum growth of the organisms was achieved when grown at 45 °C. At lower and higher temperature than this, growth of the organism was decreased. The absorbance and biomass dry weight of cultures of *Chlorogloeopsis fritschii* at 45 °C was 0.59 and 13.3 mg mL^{-1}, respectively (Fig. 2).

3.2. Growth and cyanophycin content

The organism exhibited linear growth up to 18 d and then entered into stationary phase of growth (Fig. 3). Observation of cells under the microscope revealed appearance of CGP granules on 12 d and maximum number of CGP granules were observed on 21 d. Amount of CGP in the cells on 12 d was 14 pmol which increased to 152 pmol mg^{-1} dry weight on 21`d. As maximum amount of CGP was observed on 21 d, thus activity of cyanophycin synthetase was studied by taking cultures of 21 d.

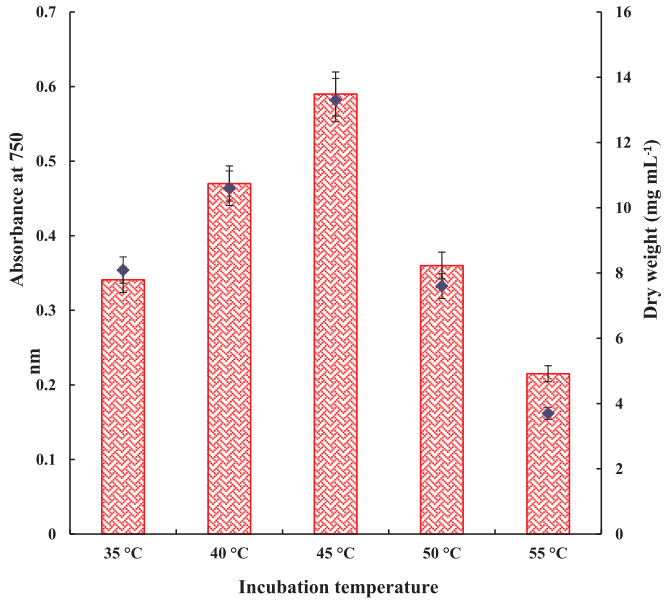

Fig. 2. Growth of *Chlorogloeopsis fritschii* at different temperature on 12 day in modified Chu-10 medium. Bars: absorbance; diamonds: dry weight.

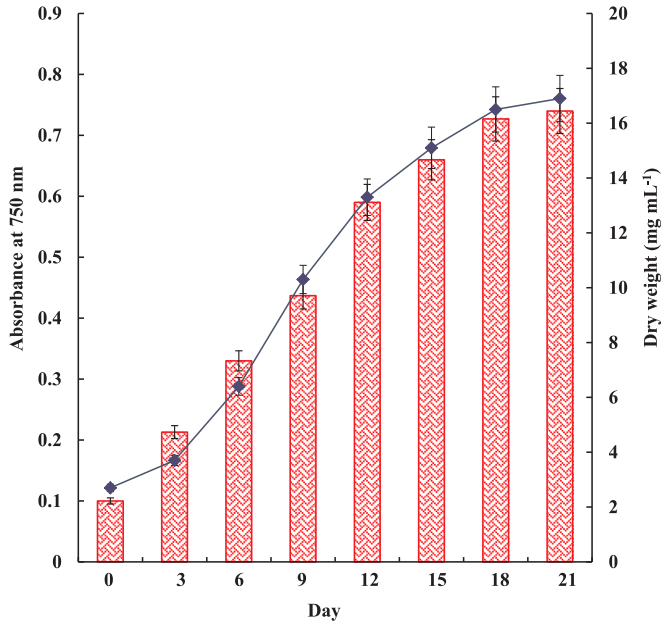

Fig. 3. Growth of *Chlorogloeopsis fritschii* in modified Chu-10 medium at 45 °C. Line: Absorbance; Bars: Biomass dry weight.

3.3. Cyanophycin synthetase

3.3.1. Optimization of assay conditions

3.3.1.1. Temperature. Optimum temperature for cyanophycin synthetase activity was determined by incubating the assay mixture at different temperatures (20–50 °C). *Chlorogloeopsis fritschii* exhibited maximum cyanophycin synthetase activity at 30 and 40 °C. A decrease in enzyme activity was observed at temperature beyond 40 °C (Fig. 4).

3.3.1.2. pH. When cyanophycin synthetase activity was determined at pH 7.5–11.0, it was observed that enzyme activity was almost same in the pH range of 8.0–9.5. Below and above this range of pH, activity of the enzyme decreased (Fig. 5).

Thus for further experiments 30 °C and pH 8.0 were selected.

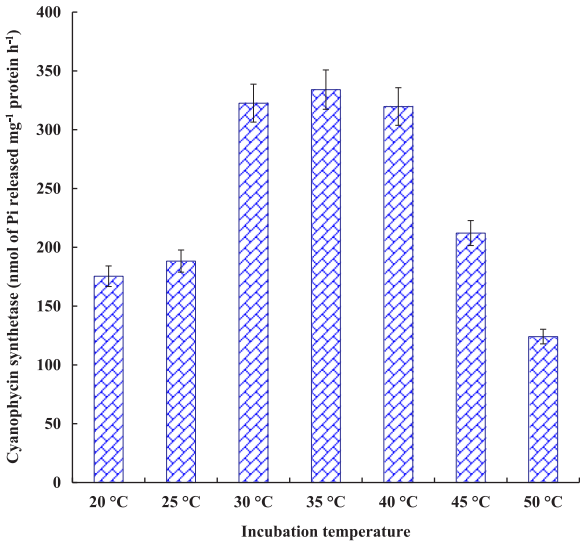

Fig. 4. Effect of temperature on cyanophycin synthetase activity of *Chlorogloeopsis fritschii* Assay conditions :100 mM Imidazole-HCl buffer; pH −8.0, Incubation time: 8 h.

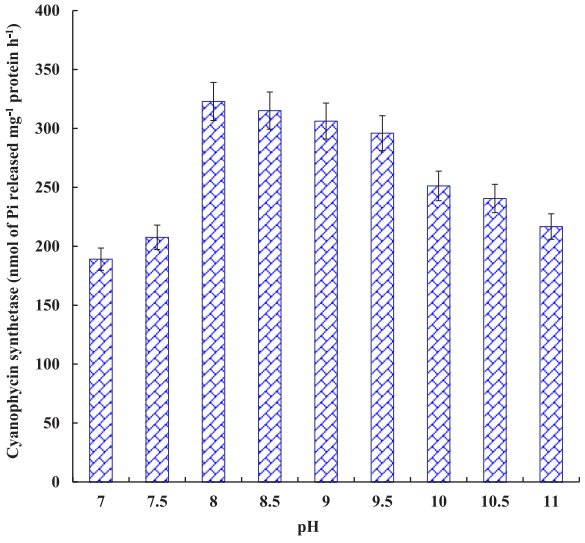

Fig. 5. Effect of pH on cyanophycin synthetase activity of *Chlorogloeopsis fritschii* Assay conditions :100 mM Imidazole-HCl buffer; pH −8.0, Incubation time: 8 h, temperature: 30 °C.

3.4. Characterization of cyanophycin synthetase

3.4.1. Substrate specificity

When aspartic acid and arginine were used as substrate, enzyme activity was 322.9 nmol of Pi released mg^{-1} protein h^{-1}. But when arginine was omitted from the assay mixture and only aspartic acid was used as substrate, the enzyme activity sharply decreased to 131.43 nmol of Pi released mg^{-1} protein h^{-1} (Fig. 6). No enzyme activity was observed when only arginine was used as substrate. When arginine in the assay mixture was replaced with Glu/Gly/Lys/His, enzyme activity was significantly less than control condition but comparable to when only aspartic acid was used as a substrate (Fig. 6).

3.4.2. Cofactors for cyanophycin synthetase

No enzyme activity was observed, when Mg^{2+} were omitted from the assay mixture. It confirmed that Mg^{2+} acted as cofactor for this enzyme. Mg^{2+} was replaced with Mn^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, K^{+}, and Cd^{2+}. When Mg^{2+} was replaced with Mn^{2+}, Zn^{2+} or Fe^{2+}, enzyme

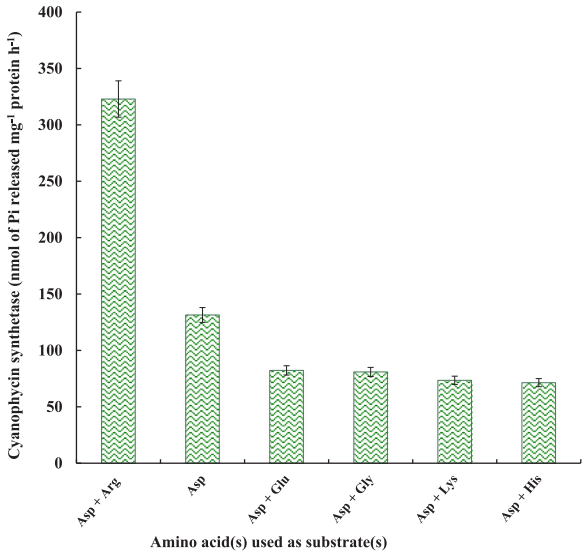

Fig. 6. Cyanophycin synthetase activity when arginine was replaced with different amino acids. Assay conditions: 100 mM Imidazole-HCl buffer; pH −8.0, Incubation time: 8 h, temperature: 30 °C.

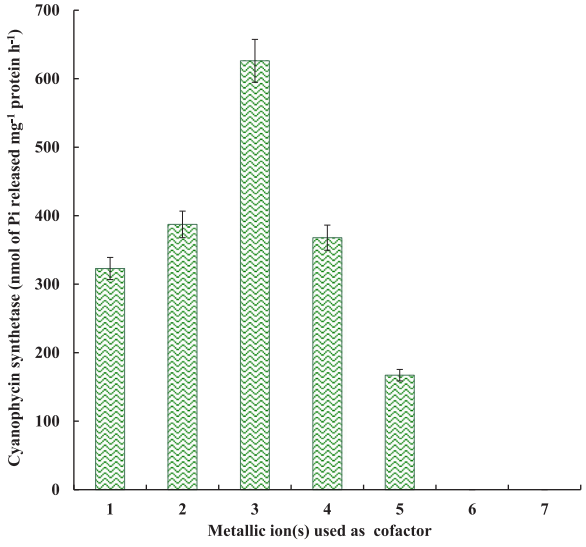

Fig. 7. Cyanophycin synthetase activity when Mg^{2+} was replaced with different metallic ions. 1:Mg^{2+}, 2:Mn^{2+}, 3:Zn^{2+}, 4:Fe^{2+}, 5:Cd^{2+}, 6:K^{+} and 7:Ca^{2+} Assay conditions: 100 mM Imidazole-HCl buffer; pH −8.0, Incubation time:8 h, temperature:30 °C.

activity was 387.13, 626.13 and 367.92 nmol of Pi released mg^{-1} protein h^{-1}, respectively (Fig. 7). It was evident that enzyme activity was almost double when Mg^{2+} was replaced with Zn^{2+}. No enzyme activity was observed when Ca^{2+}, K^{+} were used as cofactors.

3.4.3. Source of energy

For cyanophycin synthetase activity, ATP is a source of energy. No enzyme activity was observed when ATP was omitted from the assay mixture as well as when ATP in assay mixture was replaced with TTP, CTP or GTP.

3.4.4. Thermal stability

When the enzyme was treated with different temperature for one hour, there was not much decrease in activity up to 40 °C. However, enzyme activity decreased by 34% and 65% when the enzyme was incubated at 45 and 50 °C, respectively (Fig. 8).

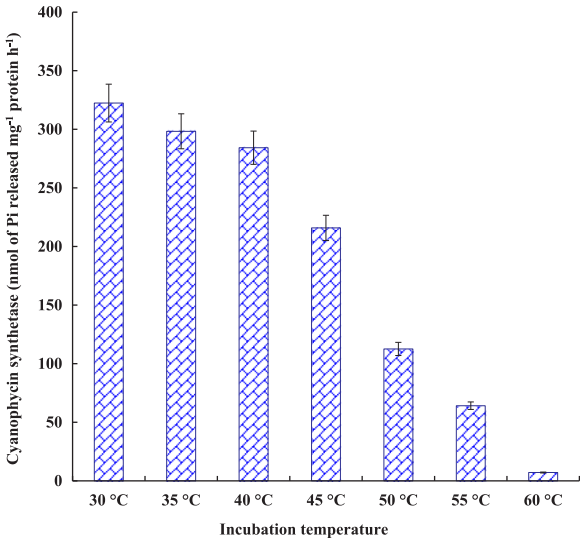

Fig. 8. Thermal stability of cyanophycin synthetase. The enzyme was treated with different temperature for one hour and used as enzyme extract. Assay conditions: 100 mM Imidazole-HCl buffer; pH −8.0, Incubation time:8 h, temperature:30 °C.

3.5. Characterization of CGP formed in vitro by cyanophycin synthetase

CGP formed in the standard assay mixture revealed presence of aspartic acid and arginine in 1:1.16 ratio through HPLC analysis (Fig. 9). Cyanophycin synthetase activity was observed, though to lesser extent, when only aspartic acid was used as substrate in the assay mixture (see Fig. 6). To confirm whether polyaspartate is being synthesized by the enzyme, HPLC analysis of cyanophycin formed was done. Only single peak corresponding to aspartic acid was observed indicating enzyme is able to synthesize polyaspartate (Fig. 9).

4. Discussion

The organism employed during the present study, *Chlorogloeopis fritschii*, is an isolate from a hot water spring. The microorganisms naturally growing at high temperature are source of valuable thermostable molecules (Abed et al., 2009). Cyanophycin and the enzyme cyanophycin synthetase are industrially important molecules (Schwamborn, 1998; Joentgen et al., 2001; Hai et al., 2006). Of the six media tested for the growth of the organism, Chu-10 medium proved to the best medium. Optimum temperature for the growth of the cyanobacterium was observed to be 45 °C. The growth of cyanobacteria is affected by physical and nutrient factors (Raven, 1988; Rai et al., 2014) as each cyanobacterium has specific nutritional and environmental requirements. For the optimum growth of the organism it is very essential to optimize nutritional conditions.

Cyanophycin is temporary nitrogen reserve of the most of the cyanobacteria and is accumulated in the cells during late stationary phase or in specialized cells such as akinetes and heterocysts (Berg et al., 2000; Hai et al., 2002; Frommeyer et al., 2016). The test organism exhibited growth up to 18 days and then entered into stationary phase. Low amounts of cyanophycin were accumulated during active vegetative growth while highest amounts of cyanophycin were accumulated when the organism entered into stationary phase. Cyanophycin synthetase is responsible for the non-ribosomal synthesis of CGP (Berg et al., 2000; Arai and Kino, 2008). This enzyme has potential industrial application for the synthesis of CGP which can be converted to polyaspartate, technology for which exists (Mooibroek et al., 2007). Polyaspartate can substitute polyacrylate for various applications (Schwamborn, 1998). In the present study, cyanophycin synthetase exhibited maximum activity at 30 and 40 °C. At temperature above this,

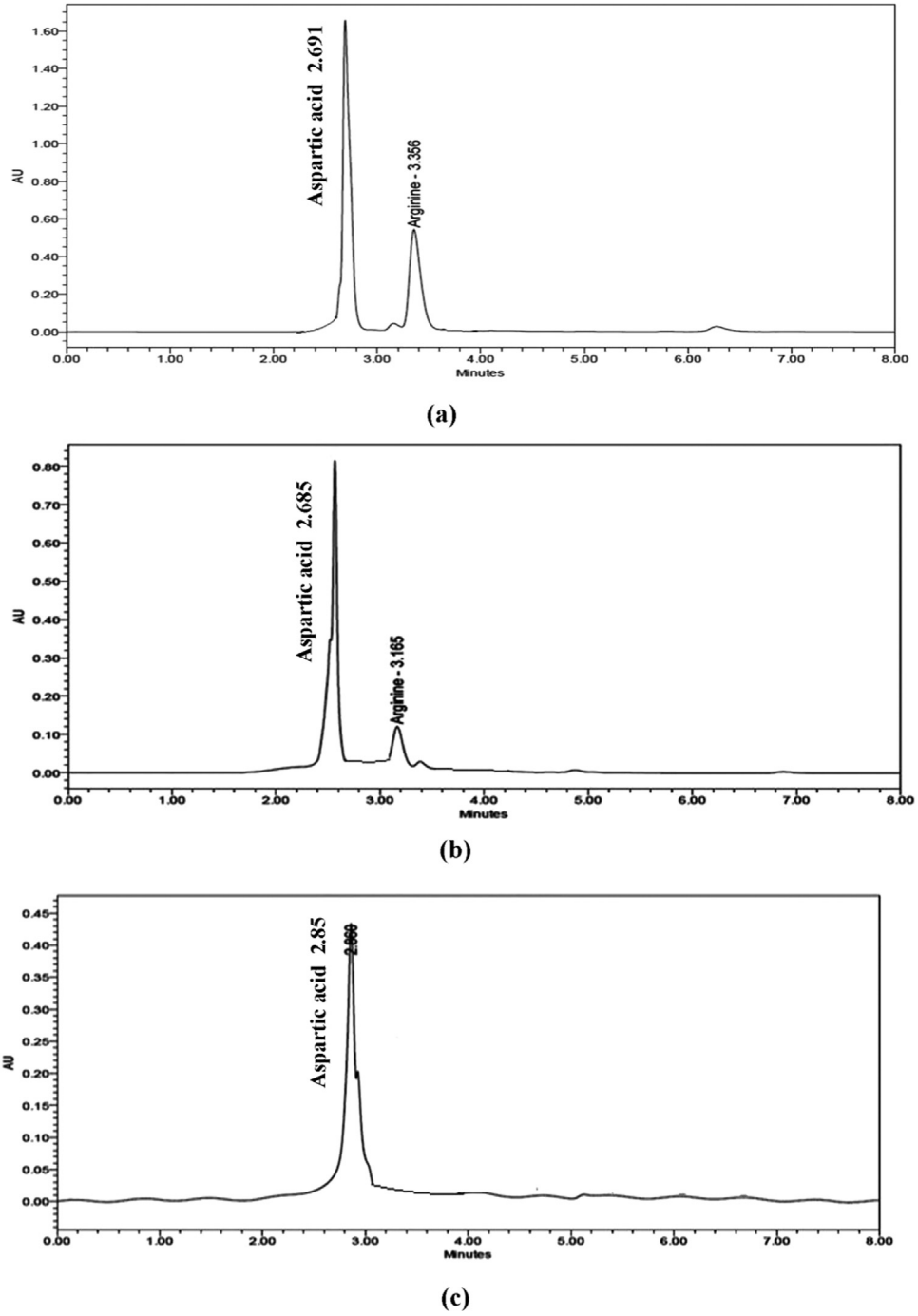

Fig. 9. HPLC Chromatogram of OPA derivatives (a) standard of aspartic acid and arginine, (b) cyanophycin hydrolysate when aspartic acid and arginine were substrate (c) when only aspartic acid was substrate.

the enzyme activity slightly decreased but nearly 25% activity was observed even at 50 °C. Optimum pH for cyanophycin synthetase was observed in the range of 8.0–9.5. Aboulmagd et al. (2001) also observed pH 8.2 as optimum pH for cyanophycin synthetase of *Synechocystis* sp. PCC 6308. On the other hand, pH 8.2 and 9.0 were observed to be the optimum pH for cyanophycin synthetase of *Synechocystis* sp. strain PCC 6308 and *Thermosynecoccocus elongatus,* respectively (Aboulmagd et al., 2001; Arai and Kino, 2008). Wide range of pH optima. for this enzyme is advantageous.

When aspartic acid and arginine were used as substrates for the enzyme, specific activity of cyanophycin synthetase was 323 nmol of Pi released mg^{-1} protein h^{-1} which decreased to 131 nmol of Pi released mg^{-1} protein h^{-1} when only aspartic acid was used. This indicated that enzyme is able to synthesize polyaspartate though at lower rate. This was also confirmed by HPLC analysis of CGP formed under above conditions. Polyaspartate is an important molecule which is generally produced by chemical degradation of CGP. This is perhaps the first report that cyanophycin synthetase of this organism has the potential to synthesize polyaspartate which needs further investigation. Aboulmagd et al. (2001) recorded only 2.4% cyanophycin synthetase activity in the absence of arginine, which is much lower than the presently observed activity (nearly 30% of the control). When arginine was replaced with Glutamic acid/Glycine/Lysine/Histidine, enzyme activity was comparable to activity when only aspartic acid was used as substrate. It thus

indicated that cyanophycin synthetase of this organism has high substrate specificity (Aboulmagd et al., 2001; Hai et al., 2002; Krehenbrink and Steinbuchel, 2004; Arai and Kino, 2008). Magnesium ions are reported as cofactors of cyanophycin synthetase. We observed that besides Mg^{2+}, metal ions Zn^{2+}, Mn^{2+} and Fe^{2+} also acted as cofactors, but Zn^{2+} proved to be the most effective cofactor as enzyme activity almost doubled. Ziegler et al. (1998) also confirmed Mg^{2+} requirement for cyanophycin synthetase while Arai and Kino (2008) while studying cyanophycin synthetase of *Thermosynecoccocus elongatus* observed that Mg^{2+} can be substituted by Mn^{2+}. For industrial purpose thermal stability of enzymes is an important factor. Since presently studied cyanobacterium is a thermophilic one, it was expected that enzyme(s) of this organism is/are thermostable. Thus thermal stability of cyanophycin synthetase was studied by incubating the enzyme at different temperatures. There was not much loss of enzyme activity when the enzyme was incubated at 40 °C. The enzyme exhibited 66% and 35% activity when incubated at 45 and 50 °C, respectively indicating significant thermal stability. Aboulmagd et al. (2001) showed that cyanophycin synthetase of *Synechocysits* sp. when incubated at 50 °C for 30 min exhibited maximum activity, but sharp decrease in activity was observed when incubation time was increased. Arai and Kino (2008) observed 80% thermal stability of the enzyme of *Thermosynechococcus elongatus* after 60 min incubation at 50 °C.

5. Conclusions

Thermophilic cyanobacterium *Chlorogloeopsis fritschii* grows best in Chu-10 medium at 45 °C. Optimum temperature for cyanophycin synthetase were observed to be 30–40 °C and pH range of 8.0–9.5. The organism is able to synthesize industrially important molecule polyaspartate when only aspartic acid is used as substrate. The enzyme showed 66% activity when treated with 45 °C for one hour. The results indicate that the enzyme has potential commercial applications which need further investigation.

Acknowledgements

The authors are thankful to Head of the Department and Coordinator SAP-I of University Grants Commission and FIST of Department of Science & Technology, New Delhi for laboratory facilities. This work was funded by Science and Engineering Research Board, New Delhi, India vide research scheme No. SR/SO/PS-74/2012 and is highly acknowledged.

References

Abed, R.M.M., Dobrestov, S., Sudosh, K., 2009. Applications of cyanobacteria in biotechnology. J. Appl. Microbiol. 106, 1–12.
Aboulmagd, E., Oppermann-Sanio, F.B., Steinbuchel, A., 2001. Purification of *Synechocystis* sp. strain PCC 6308 cyanophycin synthetase and its characterization with respect to substrate and primer specificity. Appl. Environ. Microbiol. 67, 2176–2182.
Allen, M.B., Arnon, D.I., 1955. Studies on nitrogen fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. 30, 366–372.
Arai, T., Kino, K., 2008. A cyanophycin synthetase from *Thermosynechococcus elongatus* BP-1 catalyzes primer-independent cyanophycin synthesis. Appl. Microbiol. Biotechnol. 81, 69–78.
Berg, H., Ziegler, K., Piotukh, K., Baien, K., Lockau, W., Volkmer-Engert, R., 2000. Biosynthesis of cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartic acid (cyanophycin) Mechanism of the cyanophycin synthetase reaction with studied synthetic primers. Eur. J. Biochem. 267, 5561–5570.
Bischoff, H.W., Bold, H.C., 1963. Phycological studies. IV. Some soil algae from Enchanted Rock and related algal species. - University of Texas Publications 6318, 1-95.
Borowitzka, M.A., 1988. Algal growth media and sources of algal cultures. In: Borowitzka, M.A., Borowitzka, L.J. (Eds.), Micro-algal Biotechnology. Cambridge University Press, Cambridge, pp. 456–465.
Erickson, N.A., Kolodny, N.H., Allen, M.M., 2001. A rapid and sensitive method for the analysis of cyanophycin. Biochim. Biophys. Acta 1526, 5–9.
Frey, K.M., Oppermann-Sanio, F.B., Schmidt, H., Steinbüchel, A., 2002. Technical-scale production of cyanophycin with recombinant strains of *Escherichia coli*. Appl. Environ. Microbiol. 68, 3377–3384.
Frommeyer, M., Wiefel, L., Steinbuchel, A., 2016. Features of the biologically relevant polyamide family 'cyanophycins' and their biosynthesis in prokaryotes and eukaryotes. Crit. Rev. Biotechnol. 36, 153–164.
Hai, T., Frey, K.M., Steinbuchel, A., 2006. Engineered cyanophycin synthetase (CphA) from *Nostoc ellipsosporum* confers enhanced CphA activity and cyanophycin accumulation to *E. coli*. Appl. Environ. Microbiol. 72, 7652–7660.
Hai, T., Oppermann-Sanio, F.B., Steinbuchel, A., 1999. Purification and characterization of cyanophycin and cyanophycin synthetase from the thermophilic *Synechococcus* sp. MA19. FEMS Microbiol. Lett. 181, 229–236.
Hai, T., Oppermann-Sanio, F.B., Steinbuchel, A., 2002. Molecular characterization of a thermostable cyanophycin synthetase from the thermophilic cyanobacterium *Synechococcus* sp. Strain MA19 and in vitro synthesis of cyanophycin and related polyamides. Appl. Environ. Microbiol. 68, 93–101.
Hoagland, D.R., Arnon, D.I., 1950. The Water-culture Method for Growing Plants without Soil. University of California, College of agriculture, Agricultural Experiment Station, Berkely, California.
Joentgen, W., Groth, T., Steinbüchel, A., Hai, T., Oppermann, F.B., 2001. Polyaspartic Acid Homopolymers and Copolymers: Biotechnical Production and Use thereof. US patent 6, 180, 752 B1.
Khlystov, N.A., Chan, W.Y., Kunjapur, A.M., Shi, W.C., Prather, K.U., Olsen, B.D., 2017. Material properties of the cyanobacterial reserve polymer multi-L-arginyl-poly-L-aspartate (cyanophycin). Polymer 109, 238–245.
Krehenbrink, M., Steinbuchel, A., 2004. Partial purification and characterization of a non-cyanobacterial cyanophycin synthetase from *Acinetobacter calcoaceticus* strain ADP1 with regard to substrate specificity, substrate affinity and binding to cyanophycin. Microbiol 150, 2599–2608.
Liotenberg, S., Campbell, D., Rippka, R., Houmard, J., De Marsac, N.T., 1996. Effect of the nitrogen source on phycobiliprotein synthesis and cell reserves in a chromatically adapting filamentous cyanobacterium. Microbiol 142, 611–622.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurements with Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Mackerras, A.H., De Chazal, N.M., Smith, G.D., 1990. Transient accumulations of cyanophycin in *Anabaena cylindrica* and *Synechocystis* 6308. J. Gen. Microbiol. 136, 2057–2065.
Mooibroek, H., Oosterhuis, N., Giuseppin, M., Toonen, M., Franssen, H., Scott, E., Sanders, J., Steinbuchel, A., 2007. Assessment of Technological options and economical feasibility for cyanophycin biopolymer and high-value amino acid production. Appl. Microbiol. Biotechnol. 77 (2), 257–267.
Naik, S., Goud, V.V., Rout, P.K., Dalai, P.K., 2010. Production of first and second generation biofuels, a comprehensive review. Renew. Sustain Energy Rev. 14, 578–597.
Oppermann-Sanio, F.B., Steinbuchel, A., 2002. Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. Naturwissenschaften 89, 11–22.
Parikka, M., 2004. Global biomas fuel resources. Biomass Bioenergy 27, 613–620.
Raven, J.A., 1988. Limits to growth. In: Borowitzka, M.A., Borowitzka, L.J. (Eds.), Micro-algal Biotechnology. Cambridge University Press, Cambridge, pp. 331–356.
Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., 2006. The path forward for biofuels and biomaterials. Science 311 (5760), 484–489.
Rai, S.V., Rajashekhar, M., 2014. Effect of pH, salinity and temperature on the growth of six species of marine phytoplankton. J. Algal Biomass Utln 5, 55–59.
Rehm, B., 2009. Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives. Caister Academic Press, Massey University, New Zealand.
Rehm, B., 2010. Bacterial polymers: biosynthesis, modifications and applications. Nat. Rev. Microbiol. 8, 578–592.
Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stainer, R.Y., 1979. Generic assignment, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1–61.
Safferman, R.S., Morris, M.E., 1964. Growth characteristics of the blue green algal virus LPP-1. J. Bacteriol. 88, 771–775.
Sallam, A., Steinbuchel, A., 2010. Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. Appl. Microbiol. Biotechnol. 87 (3), 815–828.
Sarma, T.A., Ahuja, G., Khattar, J.I.S., 2004. Nutrient stress causes akinete differentiation in cyanobacteria with concomitant increase in nitrogen reserve substances. Folia Microbiol. 49, 557–562.
Sarma, T.A., Khattar, J.I.S., 1986. Accumulation of cyanophycin and glycogen during sporulation in the blue-green alga *Anabaena torulosa*. Biochem. Physiol. Pflanz. 181, 155–164.
Schwamborn, M., 1998. Chemical synthesis of polyaspartates: a biodegradable alternative to currently used polycarboxylate homo and copolymers. Polym. Degrad. Stabil. 59, 39–45.
Shen, L., Worrell, E., Patel, M., 2010. Present and future development in plastics from biomass. Biofuels, Bioprod. Bioref. 4 (1), 25–40.
Sherman, D.A.K., Nelson, L.D., Steele, C.M., 2000. Do messages about health risks threaten the self? Increasing the acceptance of threatening health messages via self-affirmation. Personal. Social. Psychol. Bull. 26, 1046–1058.
Simon, R.D., 1987. Inclusion bodies in the cyanobacteria: cyanophycin, polyphosphate, polyhedral bodies. In: Fay, P., van Baalen, C. (Eds.), The Cyanobacteria. Elsevier Science Publishers B.V, Amsterdam, the Netherlands, pp. 199–225.
Singh, Y., Gulati, A., Singh, D.P., Khattar, J.I.S., 2018. Cyanobacterial community structure in hot water springs of Indian north-western himalayas: a morphological, molecular and ecological approach. Algal Res. 29, 179–192.
Wingard, L.L., Miller, S.R., Sellker, J.M., Stenn, E., Allen, M.M., Wood, A.M., 2002.

Cyanophycin production in a phycoerythrin-containing marine *Synechococcus* strain of unusual phylogenetic affinity. Appl. Environ. Microbiol. 68, 1772–1777.
Ziegler, K., Diener, A., Herpin, C., Richter, R., Deutzmann, R., Lockau, W., 1998. Molecular characterization of cyanophycin synthetase, the enzyme catalyzing the biosynthesis of cyanobacterial reserve material multi-L-arginyl-poly-L-aspartate (cyanophycin). Eur. J. Biochem. 254, 154–159.
Zotz, R.J., Schenk, S., Kuhn, A., Schlunken, S., Krone, V., Bruns, W., Genth, S., Schuler, G., 2001. Safety and efficacy of LK565 - a new polymer ultrasound contrast agent. Z. fur Kardiol. 90, 419–426.