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Bankar, Sandip B.; Nimbalkar, Pranhita R.; Chavan, Prakash V.; Singhal, Rekha S.

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Published in:
Role of Materials Science in Food Bioengineering

DOI:
[10.1016/B978-0-12-811448-3.00012-7](https://doi.org/10.1016/B978-0-12-811448-3.00012-7)

Published: 02/04/2018

Document Version
Peer reviewed version

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Please cite the original version:
Bankar, S. B., Nimbalkar, P. R., Chavan, P. V., & Singhal, R. S. (2018). Microbial Polyamino Acids: An Overview for Commercial Attention. In *Role of Materials Science in Food Bioengineering* (pp. 381-412). Academic Press. <https://doi.org/10.1016/B978-0-12-811448-3.00012-7>

Microbial polyamino acids: an overview for commercial attention

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Abstract

Although, polyamino acids are structurally similar to proteins, they are not proteins and do not have a specific sequence. Polyamino acids are polymerized from single amino acid that has molecular mass and polydispersity as similar as polysaccharide. Polyamino acid biosynthesis is considered to be an interesting example of biopolymer synthesis that is being produced by fermentation process. Furthermore, polyamino acids have wide range of applications from food additives and biomedical agents to biodegradable and renewable resources. The materials produced from polyamino acids are environment friendly, biodegradable and independent of oil-based resources. Three common natural polyamino acids studied extensively in the literature are, poly- ϵ -lysine(ϵ -PL), poly- γ -glutamic acid (γ -PGA), and cyanophycin.

The present chapter covers a wide range discussion on importance of polyamino acids including structure, biosynthesis and biodegradation of naturally occurring ϵ -PL, γ -PGA and cyanophycin. Fermentation and biosynthetic pathway study, along with downstream processing and characterization of these polyamino acids are detailed extensively in the current chapter. Besides, large-scale production and challenges associated with it are also discussed. Multifarious applications of polyamino acids in the food as well as pharmaceutical industries have been summarized comprehensively. Finally, various challenges and opportunities in well-designed trials that are needed to improve the current knowledge on polyamino acids are conjectured.

Keywords

Biopolymer, Polyamino acids, Poly- ϵ -lysine, Poly- γ -glutamic acid, Cyanophycin

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1. Introduction

In recent times, natural polymers are being developed as eco-friendly alternatives to non-degradable synthetic polymers which are based on petroleum refining. Among the many synthetic polymers, polyethylene, polyvinyl chloride, and polystyrene plays crucial role in chemical industries. These polymers cause environmental damage due to their non-degradability for several hundred years

(Kavitha et al., 2010). The primary goal of natural polymer based research is the development of a system that can mimic the structure and function of native non-degradable synthetic polymers so that they can be replaced from extensive use for environment safety. Natural polymers offer numerous advantages over synthetic polymers such as complete degradation, increased soil fertility, low accumulation of bulky plastic materials in the environment, and reduction in the cost of waste management (Gupta and Nayak, 2015). Natural polymers can be obtained from three kinds of renewable resources: (a) plant origin polymers such as starch (Smith and Zeeman, 2006), soy protein (Su et al., 2010), and cellulose (Cai et al., 2012); (b) animal origin polymers such as chitosan (Dash et al., 2011), keratin (Bertini et al., 2013), silk (Xu et al., 2012); and (c) from microbial fermentation such as poly- ϵ -lysine (ϵ -PL) (Bankar and Singhal, 2013), poly- γ -glutamic acid (γ -PGA) (Bajaj and Singhal, 2011), cyanophycin (Mooibroek et al., 2007) and poly hydroxy alkanoates (PHA) (Panchal et al., 2013).

1.1. Proteins vs. poly (amino acid)s

The poly (amino acid)s have very similar nature to that with proteins, but they are not proteins and do not have a specific sequence. There are several differences between poly (amino acid) s and proteins (Bankar and Singhal, 2013) such as:

(1) Proteins are composed of many amino acids unlike polyamino acid, which comprised of a single amino acid, at least in the backbone.

(2) DNA directs the amino acids in a specific sequence to form a protein. Amino acids are joined covalently between α -carboxyl group and α -amino group of the amino acid by peptide bonds to yield proteins (including enzymes, venom and hormones). Hence, protein synthesis is in a template-dependent mode, including complex ribosome transcription and translation mechanism. Instead, polyamino acids are biosynthesized differently with enzyme catalysis in some microorganisms. Therefore, the inhibitors of translation such as chloramphenicol do not affect the biosynthesis of polyamino acids. The enzymes catalyzing biosynthesis of polyamino acids are grouped into the carbon-amino binding enzymes.

(3) Proteins exhibit an exact polymer length, whereas poly amino acids show remarkable dispersal in molecular weight with poly dispersity as wide as that of a polysaccharide.

(4) Proteins use α -amino and γ -carboxylic groups (α -amide linkages) to link variety of amino acids. On contrary, β - and γ -carboxylic and ϵ -amino groups are used for amide bonds in poly amino acids (Candela and Fouet, 2006).

Poly amino acids can be produced in large amounts by fermentation technology. Moreover, materials produced from poly amino acids are environment friendly, biodegradable and independent of oil-based resources. For example, poly- γ -glutamic acid (γ -PGA) is regularly being used in the traditional Japanese food, *natto*, made from soy beans and fermented by *Bacillus* strains. γ -PGA contains glutamic acid residues connected between the α -amino group and the γ -carboxyl group (Liu et al., 2010). In 1977, another polyamino acid namely poly- ϵ -lysine (ϵ -PL) was found in the culture filtrate of a Japanese soil sample. Structurally, ϵ -PL has L-lysine molecules connected between the α -carboxyl group and the ϵ -amino group (Chen et al., 2016).

1.2. Polyamino acids

Polyamides are composed of large number of polymer compounds linked by amide bonds. The polyamides are divided into the homopolyamides, consisting of one type of monomers, and the copolyamides, which are composed of different kinds of constituents. The majority of polyamides are co-polymeric proteins. A small group of polyamides are referred to as polyamino acids, in which multiple amino acids of the same type are connected through amide bonds. They exhibit excellent biocompatibility and other properties that are important for a biomaterial to be used in various industries, especially in the biomedical and biopharmaceutical fields (Shi et al., 2015).

Small polyamino acids are important in organic synthesis mainly because: (i) they are efficient catalysts for promoting highly enantio-selective epoxidations; (ii) they have interesting laboratory as well as large-scale applications; and (iii) they exhibit enzyme-like synthetic properties which suggests them to possibly have a role in the prebiotic origin of life (Carrea et al., 2005).

2. Types of polyamino acids

There are three important polyamino acids available in the nature: i) poly- γ -glutamic acid (γ -PGA), ii) poly- ϵ -lysine (ϵ -PL), and iii) cyanophycin (Table 1). The chemical structures of these polyamino acids are shown in Figure 1. γ -PGA consists of D- and L-glutamic acid units linked by amide linkages (Bajaj and

Singhal, 2011) while ϵ -PL contains lysine monomer linkage (Bankar et al., 2014b). Different from these two polyamino acids, cyanophycin contain α -aspartic residues with pendent arginine residues linked to the β -carboxyl group (Feng et al., 2007).

1a. poly- ϵ - lysine(ϵ -PL)

1b. poly- γ -glutamic acid (γ -PGA)

1c. Cyanophycin

Figure 1. Chemical structure of, (1a) poly- ϵ - lysine (ϵ -PL), (1b) poly- γ -glutamic acid (γ -PGA) and (1c) cyanophycin

Table 1. Physicochemical properties and stability of polyamino acids

Polyamino acid	ϵ -PL (ε-Polylysine)	γ -PGA (γ-Polyglutamic acid)	Cyanophycin
Amino acid	Lysine	Glutamic acid	Aspartic acid, Arginine
Microorganism	<i>Streptomyces</i> sp.	<i>Bacillus</i> sp.	<i>Cyanobacterial</i> sp.
Linkage	α -Carboxyl group and ϵ -amino group	α -Amino group and γ -carboxyl group	α -Amino- α -carboxy and β -carboxylic groups
Nature of the polymer	Cationic polymer	Anionic polymer	Anionic/cationic
Location	Extracellular	Extracellular	Intracellular
Backbone	Single amino acid	Single amino acid	Two amino acids
Secondary structures	Random coil, α -helix, or β -sheet conformations		Radiating structure
Biodegradable	Yes	Yes	Yes
Important Physiological function	Antimicrobial	Sequestration of toxic metal ions and source of glutamate during starvation	Intracellular nitrogen reserve
Stability	pH, temperature	pH, temperature, chemical	pH, temperature

2.1. ϵ -Polylysine (ϵ -PL)

ϵ -PL is an unusual cationic polymer comprising of 25 to 30 residues of L -lysine with ϵ -amino group and α -carboxyl group linkage (Figure 1a) (Shukla et al., 2012). ϵ -PL differs from α -PL, which is a homopolymer of lysine that has peptide linkages between α -carboxyl and ϵ -amino groups of lysine (Bankar and Singhal, 2013). ϵ -PL is being used as a food preservative in Japan, South Korea, United States of America and several other countries, because of its safety, heat stability and wide

antimicrobial activity spectrum. Moreover, ϵ -PL is approved by Japanese Ministry of Health, Labor and Welfare as a food preservative since late 1980s. It is also certified as generally recognized as safe (GRAS) in the United States from January 2004.

Various ϵ -PL producers have been reported in the literatures (Bankar and Singhal, 2013). However, *Streptomyces* sp. are widely used for its commercial applications. Besides *Streptomyces*, several other microorganisms have also been investigated recently by researchers to produce ϵ -PL. Nishikawa and Ogawa (2002) effectively isolated various ϵ -PL producers belonging to the genera *Kitasatospora* and *Epichloe*. An ergot fungus, *Epichloe* sp. MN-9, producing ϵ -PL with 24–29 residues, is the first ϵ -PL producer reported in eukaryotes. Bankar and Singhal (2010) have attempted to use *Streptomyces noursei* as a better alternative for *S. albulus*, which is as yet very rarely reported in the literature. Various problems arise during the production of ϵ -PL from *S. albulus*, the major being the degradation of secreted ϵ -PL in the culture broth itself. This is because of self-protecting mechanism of organism producing ϵ -PL degrading enzymes (Bankar et al., 2014a; Bankar and Singhal, 2010, 2011a).

2.2. Poly- γ -glutamic acid (γ -PGA)

γ -PGA consists of D- and L-glutamic acid amide linkages between α -amino and γ -carboxylic acid groups (Figure 1b). It is naturally occurring water-soluble anionic and biodegradable polymer. It is also edible, and non-toxic towards human and environment. Therefore, γ -PGA is utilized in various industrial applications viz. thickener, cryoprotectant, drug carrier, bitterness reliving agent, biological adhesive, sustained release material, curable biodegradable fibers, biopolymer flocculants, highly water absorbable hydrogels, and heavy metal absorbers (Shih and Van, 2001; Shih et al., 2001; Zhao et al., 2013). A Japanese traditional food, 'natto' (fermented soybeans), is a blend of γ -PGA and fructan obtained by *Bacillus natto* (Bajaj and Singhal, 2011).

γ -PGA is largely produced by *Bacillus* sp. such as *B. licheniformis*, *B. subtilis*, *B. megaterium*, *B. pumilis*, *B. mojavensis* and *B. amyloliquefaciens* (Bajaj and Singhal, 2011). Besides, two halophilic eubacteria, *Sporosarcinahalophila*, *Planococcus halophile* and a halophilic archaeobacterium, *Natrialbaegyptiaca* sp. have also been found to produce γ -PGA. In addition to prokaryotes, γ -PGA is

detected in significant amount in the nematocysts of Cnidaria (Hezayen et al., 2001).

2.3. Cyanophycin (CGP)

Cyanophycin (also referred to as cyanophycin granule polypeptide (CGP)) a bacterial polyamino acid was discovered in 1887, during microscopic studies of cyanobacteria and was later found in all groups of cyanobacteria (Oppermann-Sanio and Steinbüchel 2002).

The cyanophycin structure is related to poly(aspartic acid). However, unlike synthetic polyaspartic acid, it has a comb-like polymer with α -amino- α -carboxy-linked L-aspartic acid residues representing poly(α -L-aspartic acid) backbone and L-arginine residues bound to the β -carboxylic groups of aspartic acids (Obst and Steinbüchel 2004)(Figure 1c). Cyanophycin is highly polydisperse and exhibits a molecular weight range from 25–100 kDa corresponding to a polymerization degree of 90–400 (Oppermann-Sanio and Steinbüchel 2002).

Cyanophycin is a transiently accumulated storage compound, which is synthesized under conditions of low temperature or low light intensity. Its accumulation can be artificially enhanced by addition of chloramphenicol as an inhibitor of ribosomal protein biosynthesis. Cyanophycin is produced by most cyanobacteria as a temporary nitrogen reserve material during the transition of cells from the exponential phase to the stationary phase and subsequently to death phase. It is insoluble at neutral pH and physiological ionic strength (Obst and Steinbüchel 2004). Moreover, cyanophycin is present inside the producing cells, unlike γ -PGA and ϵ -PL. It is accumulated in cytoplasm of cyanobacteria as membrane less granules during early stationary growth phase. Cyanophycin can be chemically converted into a polymer with reduced arginine, which might be used like polyaspartic acid as a biodegradable substitute for synthetic polyacrylate in various technical processes (Mooibroek et al., 2007).

Cyanophycin plays an important role in the conservation of nitrogen, carbon, and energy. It is produced by non-ribosomal synthesis by cyanophycin synthetase (CphA) in the presence of chloramphenicol (Oppermann-Sanio and Steinbüchel 2002). Krehenbrink et al. (2002) and Ziegler et al. (2002) showed that cyanophycin are produced even in heterotrophic bacteria like *Acinetobacter* sp. and *Desulfitobacterium hafniense* and therefore confirmed the wide distribution of this biopolymer and its function in nature as a general storage compound.

3. Biosynthesis of polyamino acids

Various antimicrobial compounds composed of amino acids and having peptide linkages in the structure are produced by actinomycetes. These compounds have diverse primary structures. The peptide linkages therein are formed independently of ribosomes. Unlike proteins, non-ribosomal peptides (NRPs) are synthesized independent of messenger RNA and often have cyclic and/or branched structures. Besides, they also contain non-proteinogenic amino acids that carry modifications like N-methyl and N-formyl groups (Fischbach and Walsh, 2006). The synthesis of naturally occurring ϵ -PL, γ -PGA and cyanophycin has independent synthesis template and resistance against translation inhibitors (e.g. chloramphenicol) with NRP synthesis.

3.1. Biosynthetic pathway of ϵ -PL

In bacteria, lysine is synthesized through diaminopimelate (DAP) pathway. DAP is formed via aspartate (Asp) produced by combination of oxaloacetate (OXA) in tricarboxylic acid (TCA) cycle and ammonium ion of a nitrogen source of fermentation medium via aspartate pathway. Lysine is a direct monomeric precursor during the biosynthesis of ϵ -PL. Furthermore; citrate facilitates the conversion of OXA to Asp by inhibition of cycle-forming reaction. In contrast, the addition of succinate inhibits reactions from fumarate to L-malate as well as from OXA to Asp (Bankar and Singhal 2011a). The inhibition of the first reaction is lethal to TCA cycle, which plays a key role in cellular metabolism. The first step of biosynthesis of L-lysine is adenylated at its own carboxyl groups with an ATP-PPi exchange reaction. The active site of a sulfhydryl group of an enzyme form active aminoacylthioester intermediates, leading to condensation of activated L-lysine monomer (Figure 2). This is the characteristic feature of enzymes of non-ribosomal peptide synthetases (NRPSase) (Finking and Marahiel, 2004).

Figure 2. Biosynthetic pathway of ε-PL from L-aspartic acid (aspartate pathway) (Bankar and Singhal, 2013)

ε-PL produced by several *Streptomyces* strains varies in length from 10 to 35 residues, depending on the producing strain. *S. albulus* No. 346 produces and excretes L-lysine polymer with 25-35 residues. NRPSase have been suggested as the regulatory mechanism for the elongation reaction or excretion system in the biosynthesis of ε-PL (Bankar and Singhal, 2013).

3.2. Biosynthesis of γ-PGA

Various researchers have studied metabolic pathway, and information on the enzymes related to synthesis and polymerization of γ-PGA (Ashiuchi et al., 2001; Bajaj et al., 2009; Bajaj and Singhal 2009a, 2009b; Kunioka, 1997). γ-PGA is synthesized by ribosome-independent way unlike ε-PL. Multienzyme systems are frequently operated on the basis of thio-template mechanism which is highly

studied ribosome-independent mechanism. The thio-template mechanisms usually are highly stereo selective for amino acid precursors to produce comparatively small peptides in which amino acid arrangements are strictly regulated. γ -PGA has extremely unique mechanism of synthesis when compared with other peptides (Ashiuchi et al., 2001).

γ -PGA biosynthesis in bacteria is carried out in two steps. Firstly, the synthesis of L - and D -glutamic acid takes place and secondly, the D - and L -glutamic acid units are combined together to form γ -PGA. Figure 3 shows the schematic pathway for γ -PGA synthesis in *B. subtilis* IF03335.

Figure 3. Biosynthetic pathway of γ -PGA in *Bacillus subtilis* IF03335: 1) Glutamate dehydrogenase; 2) glutamate 2-oxoglutarate aminotransferase; 3) glutamine synthetase; 4) L-glutamic acid: pyruvic acid aminotransferase; 5) alanine racemase; 6) D-glutamic acid: pyruvic acid aminotransferase; 7) γ -PGA polymerase (Kunioka, M., 1997)

Step 1: Synthesis of L- and D-glutamic acid

It is presumed that L-glutamic acid is produced from citric acid through isocitric acid and α -ketoglutaric acid in the TCA cycle, and γ -PGA is polymerized from glutamic acid. A large amount of γ -PGA is thus produced from citric acid and

ammonium sulfate (Kunioka, 1997). Two different pathways for formation of L-glutamic acid are reported.

(a) In the absence of glutamine - the glutamate dehydrogenase (GDH) pathway is used, in which L-glutamic acid is synthesized from α -ketoglutaric acid and ammonium sulfate, with the synthesis being catalyzed by GDH.

(b) In the presence of L-glutamine - another pathway involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT) are activated. It involves formation of L-glutamic acid from α -ketoglutaric acid and L-glutamine that is catalyzed by GOGAT, and regeneration of glutamine from L-glutamic acid and ammonium sulfate, which is catalyzed by GS.

D-glutamic acid is produced from L-glutamic acid through indirect conversion (Hara et al., 1982) in three steps as follows:

(i) L-alanine is formed by L-amino acid aminotransferase that catalyzes the transamination between pyruvic acid and L-glutamic acid.

(ii) L-alanine so formed is then converted to D-alanine by alanine racemase.

(iii) D-amino acid aminotransferase (DAT) catalyzes the transamination between α -ketoglutaric acid and D-alanine to produce D-glutamic acid and pyruvic acid.

Although DAT has been assumed to produce D-glutamic acid in *Bacillus*, its activity has not been detected in the cell extract of *B. subtilis* IFO 3336. In this strain, L-glutamic acid is directly converted to D-glutamic acid by using glutamic acid racemase (Ashiuchi et al., 1998). In *B. subtilis* (chungkookjang), the conversion of L-glutamic acid to D-glutamic acid takes place in the presence of DAT and glutamic acid racemase.

Step 2: Synthesis of γ -PGA

D- and L-glutamic acid is converted to γ -PGA by the γ -PGA synthetase complex. The membranous γ -PGA synthetase complex has been studied and the reaction mechanism for γ -PGA synthesis is proposed in *B. licheniformis* and *B. Subtilis* (Ashiuchi et al., 2001).

3.3. Biosynthesis of cyanophycin

Cyanophycin is not produced in the ribosomes and is most frequently found as

insoluble inclusions in cyanobacteria, oxygenic photosynthetic prokaryotes that are capable to acclimatize in many environments (Oppermann-Sanio and Steinbüchel, 2002). Survival of cyanobacteria under stressful conditions such as intense light, or starvation for carbon dioxide, sulfur, or phosphorus, results in accumulation of cyanophycin granules in stationary phase. Under these conditions, the polyamide is thought to serve as a nitrogen reserve (Stubbe et al., 2005). A single protein, cyanophycin synthetase, catalyzes the *de novo* synthesis of cyanophycin. Incubation of Mg^{2+} ATP with aspartate and arginine results in the formation of product.

The amino acid substrates are activated by phosphorylation of carboxyl groups and ATP is converted to ADP and phosphate, without production of AMP. The cyanophycin synthesizing enzyme of *Anabaena cylindrica*, are identified and characterized as a non-ribosomal peptide synthetase, named as cyanophycine synthetase (CphA). Interestingly, CphA is the only enzyme responsible for biosynthesis of cyanophycin. It has ATP-dependent carboxylate-amine/thiol ligases in the N-terminal/central region and ATP-dependent substrate ligases in the central/C-terminal region (Feng et al., 2007). The catalytic reaction cycle includes the addition of one dipeptide unit to C-terminus of the cyanophycin primer in four successive steps. Firstly, the transfer of γ -phosphoryl group of ATP activates the carboxylic group of the polyaspartic acid backbone. This results in the binding of the amino group of aspartic acid at the C-terminus of the cyanophycin-primer to form a peptide bond. Subsequently, a second molecule of ATP hydrolyses which in turn links arginine to the primer through isopeptide bond (Berg et al., 2000). Low amounts of cyanophycin consisting of at least three Asp-Arg dipeptides are required as a primer for maximal enzyme activity.

CphA from *Anabaena cylindrica* is dependent on the presence of both the monomers, aspartic acid and arginine. Besides, ATP, Mg^{2+} , K^+ , a sulfhydryl reagent and small amounts of cyanophycin as primer are also important in CphA biosynthesis. CphA obtained from microbial sources consist of identical subunits of 90–130 kDa, which possibly assemble two homodimers under physiological conditions. It also has been proved that synthetase can prolong the pre-existing cyanophycin primers in presence of both the amino acid substrates. The K_m value of this enzyme showed it to have high affinity towards L-arginine, L-aspartic acid and ATP (Oppermann-Sanio and Steinbüchel, 2002).

4. Production of Polyamino acids

4.1. Production of ϵ -PL

ϵ -PL can be synthesized by both chemical polymerization or by microbial fermentative production processes. Linear ϵ -PL by polymerization can be obtained by applying 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as an activating agent for the polycondensation of L -lysine in an aqueous medium.

ϵ -PL as an extracellular material is industrially being produced in an aerobic fermentation system with *S. albulus* (Table 2) (Shukla and Mishra, 2012). During the first fermentation study with a wild strain of the bacterium and under optimum culture conditions, the accumulated concentration of ϵ -PL in the culture medium was 0.5 g/l. Citrate has been found to facilitate the production of ϵ -PL more than other organic acids of TCA cycle (Bankar and Singhal, 2011a; Saimura et al., 2008). Studies on kinetic properties of ϵ -PL with *S. albulus* showed that a decrease in pH during the fermentation process is essential for accumulation of ϵ -PL (Saimura et al., 2008).

Table 2. Comparative bioprocess parameters for polyamino acids

Polyamino acid	ϵ -PL (ε-Polylysine)	γ -PGA (γ-Polyglutamic acid)	Cyanophycin
Microorganisms	<i>Streptomyces</i> sp. <i>Kitasatospora</i> sp.	<i>Bacillus</i> sp.	<i>Cyanobacterial</i> sp.
Nutrients	Glucose, KH_2PO_4 , K_2HPO_4 , and $(\text{NH}_4)_2\text{SO}_4$ + ferrous ion, ammonium sulphate, citric acid, aspartic acid	Glutamic acid, glycerol, citric acid, NH_4Cl , sucrose, $(\text{NH}_4)_2\text{SO}_4$, casein hydrolysate, KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, K_2HPO_4 ,	Ammonium compounds, urea, glycine, aspartic acid, and arginine, transcriptional inhibitors, low sulphur and phosphorous medium
Key processes	Batch, fed batch and continuous	Batch, fed batch and continuous	

	fermentation, pH control strategy	fermentation, solid state fermentation	
Maximum production	48.3 g/l (Kahar et al., 2001)	83.20 g/l (Jung et al., 2006)	266 g/l (Aboulmagd et al., 2000)
Large scale production	Yes	Yes	Yes

Two-step cultivation method was carried out for *S. albulus* in which the strain was first grown for 24 h in a culture medium containing glycerol/glucose as carbon source with yeast extract (growth phase), and then replacement of the medium by glucose, citric acid and $(\text{NH}_4)_2\text{SO}_4$ (production phase) (Bankar and Singhal, 2013). Eventually, fed batch cultivation can be adopted for ϵ -PL production with two distinct phases as growth phase and production phase. In the first phase, cells are grown at optimum for culture growth at pH 6.8. This is followed by second phase wherein the pH is adjusted to 4.0 by addition of glucose. Depletion of glucose leads to increase in pH of culture broth that degrades produced ϵ -PL. Hence, fed batch culture with pH control strategy could successfully enhance the yield of ϵ -PL from 5.7g/l to 48.3g/l (Kahar et al., 2001). The acidic pH shock treatment also enhances ϵ -PL production in a controlled bioreactor. An integrated pH-shock strategy contains pre-acid-shock adaption at pH 5.0 and acidic pH shock at 3.0 to positively regulate the metabolic activities. The restoring of pH to 4.0 provides optimal condition for improved ϵ -PL production (Ren et al., 2015).

Airlift bioreactor (ABR) has shown the production of ϵ -PL to be similar to that with jar fermenter with an added advantage of significantly lower power consumption (0.3kW/m^3 in ABR vs. 8.0kW/m^3 in jar fermenter) (Kahar et. al., 2002). In some ϵ -PL producing strains, the production of ϵ -PL is unstable and depends on cell density, which may cause problem such as high viscosity and low oxygen transfer efficiency. Bankar and Singhal (2011b) reported that aeration and agitation in the fermentation broth has a marked effect on ϵ -PL production, glycerol (substrate) utilization and cell mass formation. In addition, the ϵ -PL fermentative production was found to be growth-associated production. The maximum yields obtained, were at agitation speed of 300 rpm and aeration rate of 2.0 vvm. Moreover, the ϵ -PL production can also be enhanced by addition of oxygen-vectors to the culture broth of *Streptomyces albulus* (Xu et al., 2015). Metabolic precursors such as amino

acids, TCA cycle intermediates and cofactors were found to notably improve the ϵ -PL production (Bankar and Singhal, 2011a). Zhang et al. (2010) further investigated the production of ϵ -PL on immobilized cells of *Kitasatospora* sp. MY 5-36 on bagasse, macroporous silica gel, synthetic sponge, *loofah* sponge and found *loofah* sponge to give highest production of ϵ -PL in shake flask culture. Consequently, the use of inexpensive and abundantly available raw materials is highly important for economic production of ϵ -PL. Therefore, Xia et al. (2014) used cane molasses and hydrolysate of *Streptomyces* cells as a substrate for ϵ -PL production by *S. albulus* for green, economic and effective process (Shukla and Mishra, 2013).

As explained earlier, the pH of production medium is most important parameter during biosynthesis of ϵ -PL. The pH of culture broth decreases from its initial value of 6.8 to 3.0 after 24 h of fermentation regardless of the culture medium, and remains constant thereafter (Chheda and Vernekar, 2014; Zong et al., 2010). The decrease in pH is likely due to the rapid consumption of glucose (50 g/l to nearly 10 g/l) within 24 h, after which it slows down and remains steady around 7 g/l even after 96 h of cultivation (Kahar et al., 2001). Glucose consumption increases the cell growth during fermentation with rapid decrease in cell growth and results in cell lysis and subsequent cell death. The rapid cell death is probably caused by extremely low pH in the culture broth at a time when maximum cell growth is achieved.

4.2. Production of γ -PGA

Many *Bacillus* strains have been studied for the production of γ -PGA (Table 2). The fermentation conditions have been optimized to study the nutritional requirements of bacteria for cell growth and improved γ -PGA production conditions. Moreover, the variation in D/L-repeat unit compositions was also optimized (Lin et al., 2013). The nutritional requirements for γ -PGA production are dependent on the strain used during fermentation. On the basis of nutrient requirement, the γ -PGA producing bacteria are classified in to two groups as 'glutamic acid dependent bacteria' and 'glutamic acid independent bacteria'. Furthermore, several other factors such as carbon and nitrogen sources, ionic strength, aeration, agitation and medium pH also have noteworthy effect on productivity and characteristics of γ -PGA (Jeong et al., 2010).

4.2.1. Production of γ -PGA by glutamic acid-dependent bacteria

Bacteria such as *B. anthracis*, *B. subtilis* sp. and *B. licheniformis* sp. require glutamic acid in the production medium for γ -PGA production (Bajaj et al., 2009; Bajaj and Singhal, 2009a). The γ -PGA production will be hindered in these bacteria in the absence of glutamic acid. Exogenous D-glutamate is considered to be the preferred substrate for γ -PGA synthesis (Ashiuchi et al., 1998). However, because of strict stereo specificity of glutamate transporter, the cells take up very little D-glutamate during the biosynthesis. Hence, exogenous L-glutamate is apparently more effective to enhance the γ -PGA production than D-glutamate. L-glutamic acid interacts with other medium components during fermentation of γ -PGA. Hence its concentration needs to be optimized. Moreover, L-glutamic acid is a comparatively expensive and directly affects the final production cost of γ -PGA. Therefore, L-glutamic acid conversion rate into γ -PGA is critical to determine its effective concentration in fermentation medium (Bajaj and Singhal 2011).

Citric acid is another important component of γ -PGA production. Cromwick and Gross (1995) found that L-glutamic acid and citric acid act as precursors for production of γ -PGA from *B. licheniformis* ATCC 9945A. Furthermore, L-glutamic acid is produced from citric acid through isocitric acid and α -ketoglutaric acid in the TCA cycle, which subsequently polymerizes γ -PGA. Effect of citric acid on the γ -PGA production is also influenced by other medium components and/or the fermentation conditions. The addition of sufficient ammonium ions is necessary for efficient conversion of citric acid to glutamic acid. Likewise, Bajaj et al. (2009) also found a significant interaction between citric acid and ammonium sulfate during γ -PGA fermentation by using *B. licheniformis* NCIM 2324.

Improvement of γ -PGA biosynthesis

For the improvement of γ -PGA production, three approaches have been used. (i) use of precursors as intermediates in γ -PGA biosynthesis, (ii) enhancing cell membrane permeability for higher consumption of extracellular glucose and L-glutamic acid, and secretion of γ -PGA (iii) redistribution of the metabolic flux.

(i) Use of precursors as intermediates in the γ -PGA biosynthesis

TCA cycle plays critical role in the biosynthesis of γ -PGA in bacteria. Hence, various TCA cycle intermediates such as citric acid, succinic acid, fumaric acid, L-malic acid have been evaluated for γ -PGA production (Xu et al., 2005). Among these, citric acid is considered as the favorable precursor for enhanced production of γ -PGA. Bajaj and Singhal (2009b) studied the effect of different TCA cycle

intermediates such as α -ketoglutaric acid, succinic acid, malic acid and pyruvic acid on γ -PGA production by *B. licheniformis*. They showed that α -ketoglutaric acid supported maximum γ -PGA production. The yield of γ -PGA can further be increased, when a combination of both glutamine and α -ketoglutaric acid is added to the medium. Addition of L -glutamine and α -ketoglutaric acid to the medium also increases the molecular weight of γ -PGA.

(ii) Improving the permeability of cell membrane

γ -PGA is an extracellular polymer produced from the intracellular glutamic acid and with a membranous synthesis mechanism. However, the extracellular substrates must be transported into cells to synthesize the polymer (Wu et al., 2006). Addition of glycerol, Tween-80 or DMSO (Wu et al., 2008) facilitates the uptake of extracellular substrates and secretion of γ -PGA by improving the permeability of cell membrane for γ -PGA, and thereby improves the yield.

(iii) Redistribution of metabolic flux

Wu et al. (2008) have suggested an increase in the formation of glutamic acid from glucose with Tween-80 or DMSO in a medium. Tween-80 and DMSO stimulates the conversion of glucose to glutamate, which resulted in higher γ -PGA formation. The glycerol also increases the flux from 2-oxoglutarate to glutamic acid and lower to the succinyl CoA, which ultimately enhances the γ -PGA formation. Zhu et al. (2013) showed that acetoin and 2,3-butanediol are produced as byproducts during γ -PGA production. It was observed that the biosynthesis of acetoin was induced at low pH and inadequate oxygen in the broth, which directly inhibits the cell growth and subsequently γ -PGA biosynthesis. Hence redistribution of metabolic flux by adjusting the pH, temperature and dissolved oxygen concentration is highly important to further improve γ -PGA production (Zeng et al., 2014; Zhang et al., 2012).

4.2.2. Production of γ -PGA by L -glutamic acid-independent bacteria

Bacterial strains such as *B. subtilis* TAM-4, *B. licheniformis* S173 and *B. licheniformis* A35 do not require glutamic acid in the medium for γ -PGA production (Kambourova et al., 2001). Recently, *B. amyloliquefaciens* LL3, a new glutamic acid independent strain was identified to produce γ -PGA with L -glutamate monomer content of 98.47% and molecular weight of 470801 daltons (Cao et al., 2011).

The change in broth viscosity during the fermentation has a severe impact on γ -PGA batch cultivation. It severely affects mass and heat transfer which directly impedes the γ -PGA production at shake flask level. Besides, γ -PGA has chelating characteristics, which might affect nutrient and trace element availability, resulting in substrate limitations. Hence, continuous cultivation experiments as well as pulse experiments can effectively control and promote the γ -PGA production (Wilming et al., 2013). Additionally, the immobilized and fed batch bioreactors also offer improved productivity and yields for industrial applications (Xu et al., 2014).

4.2.3. Production of γ -PGA in solid-state fermentation

The main problem during submerged fermentation of γ -PGA is significant increase in culture broth viscosity, which results in uncontrollable foaming. Moreover, there are also limitations of the volumetric oxygen mass transfer, which leads to insufficient cell growth and a decreased yield of γ -PGA. Hence, solid state fermentation (SSF) is an important mode of fermentation involving solids, in absence (or near absence) of free water. SSF offers an alternative to solve many problems encountered in submerged fermentation for production of γ -PGA and also has reduced energy requirements (Pandey, 2003).

B. subtilis CCTCC 202048, *B. subtilis* B6-1, *B. subtilis* ME714 and *B. licheniformis* NCIM 2324 are reported to grow on solid substrates like soybean cake powder, soybean meal, wheat bran, dairy manure, swine manure and sweet potato residues etc. for γ -PGA production (Wang et al., 2014; Yao et al., 2012). Factors such as composition of substrates, initial moisture content, incubation temperature, fermentation time, and additional nutrients such as mineral salts, carbon sources and nitrogen sources are important parameters to be considered during the process. Maximum γ -PGA production of 83.61 g/kg of dry substrate was obtained in the mixed substrates of soybean cake powder and wheat bran supplemented with glutamic acid, citric acid and ammonium nitrate with initial moisture content as 65% (Yong et al., 2011). The higher yields and low cost nature of optimal SSF medium can also offer the possibility of economic large-scale production of γ -PGA.

The enhanced γ -PGA productivity is highly necessary for its successful commercial application. Therefore many researchers have attempted to optimize γ -PGA production conditions for industrial scale. Batch, fed batch and continuous

fermentation strategies have all been evaluated for their feasibility in large-scale γ -PGA production (Wilming et al., 2013; Xu et al., 2014; Yoon et al., 2000).

4.3. Production of Cyanophycin

As detailed earlier, cyanophycin is used as a temporary nitrogen reserve in the cells. Cyanophycin contains five nitrogen atoms for every building block of polymer, and it is insoluble at internal pH and ionic strength in the cells (Burnat et al., 2014). When the cyanobacteria are cultured in medium containing low ammonium concentration, it begins to synthesize cyanophycin before ammonium gets exhausted in response to decrease in the concentration of extracellular nitrogen (Sherman et al., 2000).

A systematic study of fermentation parameters influencing cyanophycin synthesis by a recombinant *Escherichia coli* showed that high aeration conditions (1.0 L/min air flow and 400 rpm stirrer speed) produce high cyanophycin in fermentation broth. Apart from several other carbon sources, glycerol is considered as high yielding substrate with simplified fermentation scheme (Solaiman et al., 2012). Many other strategies have also been reported in the literature to enhance synthesis and accumulation of cyanophycin such as deprivation of sulfur or phosphorus, and addition of translatable or transcriptional inhibitors. Cyanophycin accumulation increases on addition of different nitrogen-containing compounds into the medium, viz. ammonium, urea, glycine, aspartic acid, and arginine. The accumulation of cyanophycin mostly depends on the cellular arginine level (Watzet et al., 2015). Moreover, cyanophycin content also increases on cultivation under reduced light and temperature (Feng et al., 2007). Various renewable medium components such as soybean meal, corn-derived zein hydrolysate and potato wastes can also be used for efficient and economic cyanophycin production (Zhang et al., 2013).

Large scale production of cyanophycin has also been attempted by few researchers (Steinle and Steinbüchel, 2010). Maximum cyanophycin content of 28% (w/w) was obtained in 6% (v/v) protamylasse medium, utilizing a residual compound of starch production at an initial pH of 7.0 within 24 h (Frommeyer et al., 2014). The effective use of antibiotics as a supplementation material for plasmid harboring in genetically modified microorganisms also improved the cyanophycin production (exceeding 40% g/g) (Frommeyer et al., 2014). Recently, another form of cyanophycin which is soluble at neutral pH unlike insoluble form,

has also been studied. The soluble cyanophycin contains high lysine monomers, which is assumed to be the reason for solubility of this polymer (Wiefel and Steinbüchel, 2014).

cphA genes of many cyanobacteria have been identified, cloned, and heterologously expressed (Kroll et al., 2011). It enables the ability of cyanobacteria to produce comparably larger amounts of cyanophycin (up to 50% of biomass) in very short time (1–2 days) when compared with wild type cyanobacteria (about 4 weeks). The cyanophycin production has been demonstrated at 10 L to 500 L scale (Solaiman et al., 2011). Improvement of fermentation conditions, feeding regimen, and the use of genetically engineered bacteria can improve the fermentative production of cyanophycin.

Attempts have also been made to synthesize cyanophycin in plants such as in tobacco and potato plants. The cultivation of bacteria for efficient cyanophycin production needs the supply of substrate, energy, a sterile environment and disposal of waste material, which causes high costs and energy demand. Therefore, the production in plants, supported only by fertilizer and solar energy, is highly attractive. In addition, the production costs can be further reduced if cyanophycin is produced as a by-product in plants that are already cultivated to build other industrial products like starch. In this case, cyanophycin can be isolated from the left over and should therefore be a cheap alternative. Besides, cyanophycin biosynthesis in plants is CO₂ neutral and environmentally safe, which have a huge potential to become a substitute for fossil hydrocarbon. cphA gene was expressed constitutively in the plants to produce approximately 1.14 and 0.24% dry weight cyanophycin in the cytosol of tobacco and potato leaves, respectively (Mooibroek et al., 2007; Neumann et al., 2005).

5. Biodegradation of polyamino acids

Several researchers have reported on the biological degradation of polyamino acids in natural environments. The production and secretion of biopolymers by microorganisms serves the purpose of protection against unfavorable environmental conditions or the establishment of extracellular energy reserves. In order to metabolize these energy reserves efficiently, the secreted biopolymer has to be broken down into smaller fragments. This implies the presence of extracellular enzymes capable of degrading biopolymers. However, the presence of degrading enzyme is unfavorable for industrial production of polyamino acids.

Hence, these enzymes have been purified and characterized for its further applications (Kreyenschulte et al., 2014).

The ϵ -PL degrading enzymes are produced during ϵ -PL production, which hinders the commercial production of this highly important polyamino acid. ϵ -PL degrading enzymes of *S. albulus* are tightly bound to the cell membrane and are solubilized by sodium thiocyanate in presence of Zn^{2+} (Kito et al., 2002). The ϵ -PL degradation is of exotype and enzyme releases N-terminal of L -lysine residues one after another. This enzyme is classified as aminopeptidase containing Zn^{2+} to hydrolyze L -Lysyl-p-nitroanilides, L -arginyl-p-nitroanilides and L -leucyl-p-nitroanilides (Kito et al., 2002). An increase in pH above 6.0 during the fermentation degrades the produced ϵ -PL in the broth itself. Hence it was concluded that the ϵ -PL degrading activity is highest at pH 7.0. Interestingly, microbial strains, which have the ability to produce ϵ -PL, also exhibit considerable ϵ -PL degrading aminopeptidase activity. On the other hand, microbial strains, which show lower ϵ -PL degrading activity, are not known to produce ϵ -PL. This correlation reveals that ϵ -PL producing microorganisms generally possess membrane bound ϵ -PL degrading enzyme. Hence, ϵ -PL degrading enzymes of ϵ -PL producers play a self-protection role against producing microorganism. Therefore, ϵ -PL degrading enzymes are also found in ϵ -PL tolerant microorganisms (Bankar and Singhal, 2013). ϵ -PL tolerant bacteria does not produce ϵ -PL, and hence their adventitious possession of proteases which catalyzes the ϵ -PL degradation enables them to grow at a high concentration of ϵ -PL (Hiraki, 2000).

All γ -PGA producing strain can also metabolize γ -PGA as carbon or nitrogen source in the natural habitats of the microorganisms. Depolymerase (hydrolase) are known to breakdown γ -PGA, which decreases broth viscosity of *B. licheniformis* during late stationary phase. Richard and Margaritis (2006) also observed γ -PGA concentration to decrease in batch production by *B. subtilis* IFO 3335 during the late stationary phase due to excretion of poly glutamyl hydrolase into the broth. Endo- γ -glutamyl peptidase and exo- γ -glutamyl peptidase are mainly associated with in vivo degradation of γ -PGA. γ -PGA can be depolymerized and consumed by the microorganisms when other carbon and nitrogen sources are depleted. Various bacteria are able to hydrolyze γ -PGA that possesses various enzymes with largely different substrate specificities and different activities resulting in the formation of specific degradation products. In addition, proteases such as trypsin, pronase E, or cathepsin B also hydrolyzes γ -PGA during extended incubation

periods (30 days) to some extent (55-75% degradation). The large diversity of γ -PGA and the high number of γ -PGA degrading enzymes presumes the existence of more degrading enzymes in nature which have remained as yet unidentified (Obst and Steinbüchel, 2004). The degrading enzymes are active between 30-40°C at pH range of 5-8. At optimal pH (5) and temperature (30°C) the molecular weight of γ -PGA was found to be decreased from 1000 – 20 kDa. The polydispersity decrease is a function of depolymerization time. Furthermore, enzymatic degradation of γ -PGA is a milder technique for reduction in molecular weight than physical and chemical methods. With enzymatic degradation, the molecular weight of γ -PGA can be reduced with better control and narrow polydispersity. It also does not disturb the chemical constitution of a polymer (Yao et al., 2009).

The literature on decomposition of cyanophycin is still in scarce. However, microbes that can use cyanophycin as the only carbon and energy source has been isolated recently (Obst et al., 2005). Cyanophycin can be depolymerized by intracellular cyanophycinase which has been isolated from *Synechocystis* sp. (Liu et al., 2016). However, the final product is a dipeptide consisting of L-arginine and L-aspartic acid instead of amino acid monomer. For complete release of the amino acids, the dipeptides have to be cleaved by another enzyme. Surprisingly, the enzyme that decomposes dipeptide to single monomer is still unknown. Arginine and aspartic acid are presumably catabolized on a special arginine catabolic route, combining the arginase pathway and the urea cycle, which makes fixed nitrogen available to the cell during cyanophycin mobilization (Obst et al., 2005). Obst et al. (2002) suggested some bacteria to possess cyanophycin hydrolyzing enzymes. *Streptomyces* is one of the bacteria that grow on cyanophycin as the sole carbon and energy source. Cyanophycin depolymerase (CphE_{Bm}) is the first enzyme involved in cyanophycin metabolism of a Gram-positive bacterium that has been investigated in detail. CphE_{Bm} has been identified as a serine-type hydrolase by inhibitor experiments. Furthermore, the degradation rate for cyanophycinase was determined by Obst et al. (2004). The K_m value of CphE_{Bm} for cyanophycin produced in recombinant *E. coli* was found to be 2.2 μ M.

6. Purification and characterization

The commercial importance of polyamino acids demands not only a search for newer and better yielding microbial strains, but also economically viable bioprocesses for its large-scale production. Optimization of fermentation parameters alone is not sufficient to ensure a high yield of biopolymers. The next

crucial step after the completion of successful fermentation is the downstream processing. The cost of recovery of microbial polymers is a significant part of total production cost of given product.

6.1. Purification and characterization of ϵ -PL

At the end of cultivation, the whole broth is centrifuged to remove the cells and supernatant is adjusted to pH 8.5. Ultrafiltration for fractionation of ϵ -PL of different molecular weight has been used. The fractions of ϵ -PL with molecular weight above 2 kDa take up a β -turn structure, while the fractions with molecular weight below 2 kDa take up a random coil conformation. Accordingly, fractions of ϵ -PL with molecular weight above 2 kDa have significant antibacterial activity, while the fractions with molecular weight below 2 kDa show restricted antibacterial activity (Jia et al., 2010). Further, the supernatant can be purified by ion exchange chromatography using an Amberlite IRC-50 column. Subsequently, the elution can be made with 0.1N hydrochloric acid, and the eluate can be neutralized with 0.1N sodium hydroxide to pH 6.5. Activated charcoal is generally used to decolorize the sample. The samples can then be evaporated under vacuum for storage in small volume. The 6 M HCl hydrolyzate of the purified material obtained from *S. noursei* NRRL 5126 solely consisted of lysine. Thin layer chromatography of the hydrolyzate performed on a cellulose thin layer plate and visualized with 0.2% ninhydrin ferric reagent indicated a single spot with R_f value identical to that of pure lysine (Bankar et al., 2014b). Pure ϵ -PL can also be obtained as white powder by repeated precipitation with methanol and ethanol/diethyl ether (2:1) mixture. It can be further purified to homogeneity as a single fraction by column chromatography on CM-cellulose and gel filtration on Sephadex column under a constant flow rate and monitoring the UV absorbance at 220 nm (Bankar and Singhal, 2013; Wibowo et al., 2013). The product homogeneity and structure elucidation has been done by IR, proton (^1H)-NMR, carbon (^{13}C)-NMR and molecular weight measurement.

The purified ϵ -PL can be tested with Dragendorff's, ninhydrin and Cl_2 -KI- starch reagents. Further characterization can be done by amino acid and molecular weight analysis by gel filtration on a Sephadex column (Nishikawa and Ogawa, 2002). The determination of definite molecular size of ϵ -PL is difficult due to non-availability of authentic standard polymers. However, molecular weight markers such as bradykinin (MW 1060), cytochrome c (MW 12,300), myoglobin (MW 17,800) and α -chymotrypsinogen A (MW 25,000) can effectively be used to

determine molecular weight of ϵ -PL by using gel filtration chromatography. The molecular weight of ϵ -PL varies from 3 to 6 kDa depending on the number of lysine monomers in the chain.

ϵ -PL is a homopolymer that contains peptide linkage between α -carboxyl group and ϵ -amino group of L-lysine. Therefore, the environment of ϵ -methylene at N-terminal and α -methine at C-terminal are different from those in the intermediate part of L-lysine units. These differences can be used to determine the degrees of polymerization and the number average molecular weights of ϵ -PL. Jia et al. (2010) have measured the ^1H NMR spectra of ϵ -PL with different molecular weight cut-offs. Chemical shift of ϵ -PL in various solvents concluded that the chemical shifts of corresponding hydrogens in ϵ -PL were all in similar regions, reflecting similar conformations that ϵ -PL with different molecular weights adopt in aqueous solution state.

6.2. Purification and characterization of γ -PGA

Most *Bacillus* sp. produces γ -PGA outside the cells. Therefore extracellular separation and purification techniques can be used for isolation of γ -PGA. It generally involves three steps: (i) the removal of cells by centrifugation or filtration, (ii) precipitation of the product from cell-free medium by ethanol, methanol, or 1-propanol, and (iii) the dialysis of low molecular weight impurities below a nominal molecular weight limit (Bajaj and Singhal, 2011).

The cells capsulated with γ -PGA possess negative charges near neutral pH because of the ionization of carboxyl group. Due to negative surface charges, these cells have high stability in culture broth which makes the sedimentation of cells difficult during separation process. The high stability of capsulated cells and high viscosity of culture broth presents major problems in separation of cells and γ -PGA from culture broth. Therefore it is necessary to decrease the stability of capsulated cells and viscosity of cultured broth for efficient separation of γ -PGA. Do et al. (2001) found the lowering of medium pH to reduce the negative surface charges of the cells causing them to aggregate together and easily settle down. Hence, the energy demand for separation of cells from culture broth by centrifugation has been reduced to 17 % after acidification.

Another most widely used method for the recovery of γ -PGA from cell free broth is alcohol precipitation. The quantity of alcohol required for γ -PGA precipitation decreases with an increase in γ -PGA concentration. Hence, it is advantageous to

concentrate cell-free culture broth containing γ -PGA during recovery process. Do et al. (2001) further showed the volume of ethanol required for precipitation could be reduced by 25% by concentrating 20 g/l of γ -PGA solution to 60 g/l at pH 5 by ultrafiltration. Alcohol precipitation has also been used to recover γ -PGA after SSF. The sufficient amount of water can mixed with fermented matter at room temperature for 1 h for further filtration. The filtrate can then be used for precipitation of γ -PGA by using ethanol.

The purified γ -PGA is generally characterized by amino acid analysis, thin layer chromatography (TLC), proton (H^1) and carbon (C^{13})-NMR for product homogeneity, and gel filtration for molecular weight and polydispersity (Shih and Van, 2001). The γ -PGA is considered to be pure when only glutamic acid is detected in TLC analysis. The average molecular weight of γ -PGA is in a range between 10^5 and 10^6 and polydispersities are observed between 2 and 5 (Shih and Van, 2001).

6.2.1. Control of the molecular weight of bacterial γ -PGA

The molecular weight and poly dispersity are very important features of microbial γ -PGA production. The most common method for determination of molecular mass of γ -PGA is, gel permeation chromatography (GPC). Molecular weight of γ -PGA is dependent on various factors such producing bacterial strains, media components, culture conditions and other unknown reasons. *Bacillus* sp. generally produces relatively high molecular weight γ -PGA. These high molecular weight polymers are useful as a viscosifying agent. However, they are not applicable to other uses, because it is too viscous and unmanageable and difficult to modify by chemical reagents. Therefore, development of approaches to control the molecular weights is not only of fundamental interest but also of practical importance for commercial development. Several methods such as alkaline hydrolysis, ultrasonic degradation, microbial or enzymatic degradation, and alteration of medium composition have been used to obtain microbial γ -PGA with various molecular weights. Richard and Margaritis (2006) studied *in situ* depolymerization of γ -PGA in cell-free fermentation broth of *B. subtilis*. They found that the dispersity of γ -PGA was decreased as a function of hydrolysis time.

6.2.2. NMR and Fourier transform-IR spectroscopy of γ -PGA

Generally, 1H - and ^{13}C -NMR spectroscopies can be performed to study the homogeneity and degree of esterification of γ -PGA (Borbely et al., 1994). The

Fourier transform-IR (FTIR) spectroscopy also produces IR spectra of γ -PGA with peaks corresponding to specific bonds in the compound.

6.2.3. Conformation of γ -PGA

γ -PGA is resistant to proteases, which cleave only α - amino bonds (Richard and Margaritis, 2001). The natural γ -PGA consists of D - and L -glutamic acids mixture. However, γ -PGA from *B. anthracis* produces optically pure D -enantiomorph polymer. The structure of poly-(γ - D -glutamic acid) in solution showed the unionized polymer to take α -helical conformation, while the ionized polymer behaves as a random coil. The solution temperature, pH, polymer concentration and ionic strength are important factors that can affect the orientation of biopolymer functional groups, the apparent (effective) charge and, consequently, the overall conformation and local structure of biopolymer. The conformational change of γ -PGA affects its metal binding properties, and subsequently, the environmental mobility and toxicity of the metals (He et al., 2000).

6.2.4. Stereochemistry of γ -PGA

B. subtilis, *B. licheniformis* and *B. megaterium* produce stereochemically different γ -PGA (D -isomer contents > 50 %). Conversely, *B. anthracis* and *B. halodurans* produce homogeneous (only D -isomer and L -isomer, respectively) γ -PGA.

6.3. Purification and characterization of cyanophycin

Cyanophycin granules can be purified by differences in molecular size, density and solubility properties. Cell disruption is followed by centrifugation for 10 min at 20000 g. Repeated washing and re-suspending of the cells during centrifugation results in pellet formation whose lower white layer consists almost entirely the cyanophycin granules. This polypeptide is insoluble in organic solvents. Hence, cyanophycin can also be purified by organic solvent precipitation method (Feng et al., 2007).

Circular dichroism (CD) and laser Raman spectroscopy results has shown cyanophycin to exhibit a defined secondary structure (β -sheets) in acidic solutions. However, this structure is not seen under alkaline conditions. Standard method for quantifying the amount of cyanophycin in cells is HPLC. However, more rapid and sensitive method for the quantification of polymer from crude preparations on the basis of 1H NMR has also been described (Erickson et al.,

2001).

SDS-PAGE of purified cyanophycin indicates a polydisperse polymer with molecular masses ranging from 20 kDa to more than 130 kDa. In order to determine the amino acid composition of the isolated cyanophycin, complete hydrolysis and HPLC analysis are the central techniques. Aspartic acid and arginine have been detected at almost equimolar amounts (1:0.9) as constituents of the polymer. Other amino acids comprise less than 0.7% and the isolated material contained less than 0.01% carbohydrates, 0.1% nucleic acids and 0.5% of other substances like N-acetyl-glucosamine (Hai et al., 1999).

6.3.1. Purification of cyanophycin synthetase

Very limited information is available on the purification and biochemical characterization of cyanophycin synthetases. The supernatant containing the soluble cell fraction, obtained after centrifugation, is used for gel filtration column (Superdex 200 prep equilibrated with buffer). Protein can be eluted with a constant flow rate and fractions containing a high enzyme activity are combined and applied onto an anion exchange column. The protein elution can be achieved by NaCl gradient at a constant flow rate. The fraction with highest enzyme activity after gel filtration represents the final product of purification (Hai et al., 1999).

7. Applications of polyamides

7.1. Applications of ϵ -PL

ϵ -PL is considered as very effective food additive to protect the stored food materials (Hiraki, 2000), although it can exhibit a bitter taste at higher concentration. However, its high antimicrobial activity requires low concentration for food preservation which resolves the problem of unpleasant taste. The safety of ϵ -PL as food additive has also been experimentally verified. ϵ -PL together with glycine, vinegar, ethanol and thiamine lauryl sulfonate is very effective for the food preservation. The use of ϵ -PL in variety of food products such as boiled rice purchased from supermarkets and drug stores is very common in Japan (Bankar and Singhal, 2013; Pandey and Kumar, 2014). ϵ -PL is available in a wide range of molecular weights. Being a polypeptide, cells can degrade ϵ -PL effortlessly. Hence, it has been used as a delivery vehicle for small drugs. The polycationic ϵ -PL ionically interacts with polyanions such as DNA. Besides, ϵ -PL has several medical applications which include neurological and immunological functions, free from

toxicity in reproduction and embryonic and fetal development. In addition, the ϵ -amino group is nucleophile (above pH 8.0) and can easily react with numerous reagents to form stable bond and covalently attach ligands to the molecule (Means and Feeney, 1990).

The macromolecule gels with high water-binding capacity are extensively used in agriculture, food and medical industries. Amidation between the ester site of propylene glycol alginate and gelatin with ϵ -PL forms hydrogels with high water-binding capacity, high gel strength and high swelling rate (Kuraray Co., 2001). Han et al. (2010) synthesized new sulfated oligosaccharide cluster consisted of ϵ -PL core scaffold as a new anti-HIV dendrimer. The anti-HIV activity of ϵ -PL dendritic sulfated cellobiose is as high as that of the currently clinically used AIDS drugs. The other specific applications of ϵ -PL are summarized in Figure 4.

Figure 4. Applications of ϵ -PL

7.2. Applications of γ -PGA

Being a natural biopolymer, γ -PGA has numerous applications in diversified fields such as in cement industry, sensor manufacturing, vaccine adjuvant, immobilization matrix and as a carrier for microencapsulation.

γ -PGA is commercially produced as a debittering agent in salt substitute products containing potassium chloride. γ -PGA is biodegradable and biocompatible with human tissue and hence can be used as a drug carrier in medicines. It is also being used as biological adhesive for tissue adhesion and closing air and liquid leaks during surgery. Furthermore, γ -PGA has reactive carboxyl groups that can serve as point of attachment for pharmaceutical agents. By the regulation of γ -PGA biodegradation, the therapeutic agent can be released more rapidly or more slowly in the body. The hydrating properties of γ -PGA can effectively be used in several other applications, such as in preparation of facial creams. γ -PGA can also be used in the food sector as a stabilizer or a texture enhancer. Addition of γ -PGA increases the bioavailability of calcium by improving the solubility and the intestinal absorption. Thus, γ -PGA may be an interesting therapeutic tool in the treatment of osteoporosis. Furthermore, γ -PGA can also be used in wastewater treatment as a bio-sorbent for the removal of heavy metals like Cu^{2+} , Ni^{2+} , Al^{3+} and Mn^{2+} . It can also be used as a biological flocculant, contrast agent and cryoprotectant or biodegradable plastic (Bajaj and Singhal 2011). Several researchers have reported many other applications of γ -PGA in the literature (Bajaj and Singhal 2011; Kreyenschulte et al., 2014; Ogunleye et al., 2015), which are compiled in Figure 5.

Figure 5. Applications of γ -PGA

7.3. Applications of cyanophycin

Chemical structure and composition of cyanophycin offer diverse opportunities for its applications in various sectors. Although, cyanophycin synthesis is efficient due to advanced production engineering techniques at large scale, its applications, as a complete polymer are currently unknown. The profitability of industrial scale production of cyanophycin in cyanobacteria is limited by a low polymer content and slow growth of the bacteria

Cyanophycin can be used as water softener, as a biodegradable polyacrylate substitute after removal of the arginyl residues or as a dispersant. Cyanophycin accumulates during the transition of the cells from the exponential to the stationary phase as a temporary nitrogen reserve. The other applications of cyanophycin are similar to that of γ -PGA including medicine, cosmetics, environment protection and agriculture. However, because of the low cell yield, low cyanophycin, long cultivation time and highly sophisticated fermentation processing (Hai et al. 2000), cyanobacteria are unsuitable sources for cyanophycin with respect to cost effectiveness. Hence, its use at large scale is still limited. This has turned the attention to derivatives of cyanophycin in recent times.

7.3.1. Polymeric derivatives of cyanophycin

Cyanophycin is a highly bioavailable source for arginine, lysine, aspartate and perhaps all other amino acids. Hence, its use in drug development and discovery are always highlighted (Sallam and Steinbuchel, 2010; Santos et al. 2012). Since aspartic acid is a part of cyanophycin backbone, copolymers of polyaspartic acid (PASP) with reduced arginine content or even PASP homopolymer (water soluble and biodegradable polymer) can theoretically be obtained from cyanophycin by mild chemical or enzymatic hydrolysis. PASP is as an anti-scalant additive in laundry detergents, dishwashing or as dispersants for variety of organic and inorganic solids (Frommeyer et al., 2014). Besides, cyanophycin is also a potential source for conversion to several nitrogen containing bulk chemicals such as, 1,4-butanediamine and acrylonitrile, 1,4-butanediol or urea (Mooibroek et al., 2007).

7.3.2. Cyanophycin based biopolymers

PASP is derived from cyanophycin after the hydrolytic removal of arginine. PASP

has properties that are very similar to polyacrylic acid. Moreover, the production cost of PASP is highly competitive for arginine. Commercial production up to 1,000 tons/year is also possible with PASP. Special applications in food and/or pharmaceutical industries offer higher market prices (Mooibroek et al., 2007).

Cyanophycin when produced in plants does not require any additional infrastructure or other special equipment. Hence, the product cost is highly compatible with other polyamino acids, which broadens the widespread applications mentioned for microbial cyanophycin (Neumann et al., 2005).

8. Outlook and perspective

The diversified applications of biopolymers in varied sectors have gained attention from various researchers all over the globe. However, out of many polyamides, only three have been identified in natural microorganisms. The widespread availability of microorganisms in various environmental conditions, offer a great possibility to identify more strains with high ability to produce novel polyamino acids and other biopolymers. These biomaterials fulfill the need-of-the-hour by offering environment friendly, highly water soluble and biodegradable polymers. Moreover, chemically modified polyamino acid could be used as a recyclable material and hence deserve more attention. Despite profuse applications and considerable progress in production and purification processes, the worldwide market for polymers is still dominated by non-renewable materials. The large scale production of polyamino acids has often been anticipated by high production costs, mainly because of complicated and inefficient downstream processing technologies. Therefore, extensive research is required to improve the production, separation and purification of polyamino acids, for efficient and cost-effective large-scale production.

Poly- ϵ -lysine is being used in food, pharmaceutical, biotechnology and other industries. In spite of huge research on poly- ϵ -lysine production, there are several hurdles that need to be tackled especially in its production and downstream processing. Commercially viable processes with new production strains are needed for economic large-scale production of this biomolecule. The biggest challenge in commercial production of poly- ϵ -lysine is the problem of biodegradation because of self-protecting mechanism of producing strains. Although poly- ϵ -lysine has long been used in various industries; technological innovation such as the use of immobilization supports and continuous flow

systems have been considered just recently.

Poly- γ -glutamic acid is yet another biodegradable and environment friendly biomaterial that has various applications. Recently, considerable research has been carried out for its efficient microbial production and applications. However, the complete understanding of poly- γ -glutamic acid biosynthesis is still not very clear. The regulation of poly- γ -glutamic acid production within the cell is still highly unclear. In addition, the large-scale poly- γ -glutamic acid production is also struggling to achieve its cost-effective manufacturing process. High production cost and low productivity is the biggest obstacle in its widespread application. Advanced production techniques with better understanding of biosynthetic pathway and application of genetic engineering will allow economic and stable poly- γ -glutamic acid production. Besides, the molecular weight control and D/L ratio of poly- γ -glutamic acid is also important during its production, as these properties directly associate with its commercial applications.

Cyanophycin has a homogeneous structure and composition, enabling its use in the production of nitrogen rich commodity products which are based on nitrogen-rich chemicals such as nylons. The derivatives and backbone polymers of cyanophycin offers various applications ranging from water softening or detergent applications to applications in the paper, building material, petroleum or leather industry, and in cosmetics. Several processes have been developed till date for the production of cyanophycin employing cyanobacterial cells as well as recombinant bacteria in larger quantities such as 30-500L. However, unlike poly- ϵ -lysine and poly- γ -glutamic acid, cyanophycin has not been commercialized yet because of higher production cost. Hence, intensive efforts are expected so that economical activities can be developed within fermentation industry, biopolymer production, processing, modification and product development (also for medical technology), packaging industry, food and feed supplementation industries, and state of the art technology. At present, attempts are underway to find culture conditions for *E. coli* to increase the yields, and to enable other industrially used bacterial hosts (*Corynebacterium glutamicum*, *Ralstonia eutropha*, *Pseudomonas* spp., to produce cyanophycin at lower cost. Besides, cyanophycin production in transgenic plants is also reported to be economic and efficient.

9. References

Aboulmagd, E., Oppermann-Sanio, F.B., Steinbuechel, A., 2000. Molecular characterization of the cyanophycin synthetase from *Synechocystis* sp. strain

- Ashiuchi, M., Nawa, C., Kamei, T., Song, J.J., Hong, S.P., Sung, M.H., Soda, K., Misono, H., 2001. Physiological and biochemical characteristics of poly- γ -glutamate synthetase complex of *Bacillus subtilis*. Eur. J. Biochem. 268, 5321–5328.
- Ashiuchi, M., Tani, K., Soda, K., Misono, H., 1998. Properties of glutamate racemase from *Bacillus subtilis* IFO 3336 producing poly- γ -glutamate synthesis. J. Biochem. 123, 1156–1163.
- Bajaj, I.B., Lele, S.S., Singhal, R.S., 2009. A statistical approach to optimization of fermentative production of poly (γ -glutamic acid) from *Bacillus licheniformis* NCIM 2324. Bioresour. Technol. 100, 826–832.
- Bajaj, I.B., Singhal, R.S., 2009a. Sequential optimization approach for enhanced production of poly (γ -glutamic acid) from *Bacillus subtilis* of marine origin. Food Technol. Biotechnol. 47, 313–322.
- Bajaj, I.B., Singhal, R.S., 2009b. Enhanced production of poly (γ -glutamic acid) from *Bacillus licheniformis* NCIM 2324 by using metabolic precursors. Appl. Biochem. Biotechnol. 159, 133–41.
- Bajaj, I.B., Singhal, R.S., 2011. Poly (glutamic acid) - an emerging biopolymer of commercial interest. Bioresour. Technol. 102, 5551–5561.
- Bankar, S.B., Chaudhary, S.A., Singhal, R.S., 2014b. Purification and characterization of poly- ϵ -lysine from *Streptomyces noursei* NRRL 5126. JSIR 73, 33–40.
- Bankar, S.B., Dhumal, V., Bhotmange, D., Bhagwat, S., Singhal, R., 2014a. Empirical predictive modelling of poly- ϵ -lysine biosynthesis in resting cells of *Streptomyces noursei*. Food Sci. Biotechnol. 23, 201–207.
- Bankar, S.B., Singhal, R.S., 2010. Optimization of poly- ϵ -lysine production by *Streptomyces noursei* NRRL 5126. Bioresour. Technol. 101, 8370–8375.
- Bankar, S.B., Singhal, R.S., 2011a. Metabolic precursors enhance the production of poly- ϵ -lysine by *Streptomyces noursei* NRRL 5126. Eng. Life Sci. 11, 253–258.
- Bankar, S.B., Singhal, R.S., 2011b. Improved poly- ϵ -lysine biosynthesis using *Streptomyces noursei* NRRL 5126 by controlling dissolved oxygen during fermentation. J. Microbiol. Biotechnol. 21, 652–658.
- Bankar, S.B., Singhal, R.S., 2013. Panorama of poly- ϵ -lysine. RSC Advances 3, 8586–8603.
- Berg, H., Ziegler, K., Piotukh, K., Baier, K., Lockau, W., Engert, R.V., 2000. Biosynthesis of the cyanobacterial reserve polymer multi-l-arginyl-poly-l-aspartic acid (cyanophycin). Mechanism of the cyanophycin synthetase reaction studied with synthetic primers. Eur. J. Biochem. 267, 5561–70.
- Bertini, F., Canetti, M., Patrucco, A., Zoccola, M., 2013. Wool keratin-polypropylene composites: Properties and thermal degradation. Polym. Degrad. Stab. 98, 980–987.

- Borbely, M., Nagasaki, Y., Borbely, J., Fan, K., Bhogle, A., Sevoian, M., 1994. Biosynthesis and chemical modification of poly(γ -glutamic acid). *Polym. Bulletin* 32, 127–132.
- Burnat, M., Herrero, A., Flores, E., 2014. Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst forming *Cyanobacterium*. *Proc. Nat. Acad. Sci. U S A* 111, 3823–3828.
- Cai, Z., Hou, C., Yang, G., 2012. Characteristics and bending performance of electroactive polymer blend made with cellulose and poly (3-hydroxybutyrate). *Carb. Polym.* 87, 650–657.
- Candela, T., Fouet, A., 2006. Poly- γ -glutamate in bacteria. *Mol. Microbiol.* 60, 1091–1098.
- Cao, M., Geng, W., Liu, L., Song, C., Xie, H., Guo, W., Jin, Y., Wang, S., 2011. Glutamic acid independent production of poly- γ -glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of pgs BCA genes. *Bioresour. Technol.* 102, 4251–4257.
- Carrea, G., Colonna, S., Kelly, D.R., Lazcano, A., Ottolina, G., Roberts, S.M., 2005. Polyamino acids as synthetic enzymes: mechanism, applications and relevance to prebiotic catalysis. *Trends Biotechnol.* 23, 507–513.
- Chen, X.S., Gao, Y., Zhen, B., Han, D., Zhang, J.H., Mao, Z.G., 2016. Separation and purification of ϵ -poly-L-lysine from fermentation broth. *Proc. Biochem.* 51, 134–141.
- Chheda, A.H., Vernekar, M.R., 2014. Improved production of natural food preservative ϵ -poly-L-lysine using a novel producer *Bacillus cereus*. *Food Biosci.* 7, 56–63.
- Cromwick, A.M., Gross, R.A., 1995. Effect of manganese(II) on *Bacillus licheniformis* ATCC 9945A physiology and γ -poly(glutamic acid) formation. *Int. J. Biol. Macromolec.* 16, 265–75.
- Dash, M., Chiellini, F., Ottenbrite, R.M., Chiellini, E., 2011. Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Prog. Polym. Sci.* 36, 981–1014.
- Do, J.H., Chang, H.N., Lee, S.Y., 2001. Efficient recovery of γ -poly (glutamic acid) from highly viscous culture broth. *Biotechnol. Bioeng.* 76, 219–223.
- 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.
- Feng, S., ZhiNan, X., PeiLin, C., 2007. Microbial production of natural poly amino acid. *Sci. China Ser B-Chem.* 50, 291–303.
- Finking, R., Marahiel, M.A., 2004. Biosynthesis of nonribosomal peptides. *Annu. Rev. Microbiol.* 58, 453–488.
- Fischbach, M.A., Walsh, C.T., 2006. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chem. Rev.* 106, 3468–96.

- Frommeyer, M., Wiefel, L., Steinbuechel, A., 2014. Features of the biotechnologically relevant polyamide family “cyanophycins” and their biosynthesis in prokaryotes and eukaryotes. *Crit. Rev. Biotechnol.* 36, 153-164.
- Gupta, P., Nayak, K.K., 2015. Characteristics of protein-based biopolymer and its application. *Polym. Eng. Sci.* 55, 485–498.
- Hai, T., Ahlers, H., Gorenflo, V., Steinbüchel, A., 2000. Axenic cultivation of anoxygenic phototrophic bacteria, *Cyanobacteria*, and microalgae in a new closed tubular glass photobioreactor. *Appl. Microbiol. Biotechnol.* 53, 383–389.
- 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. Letters* 181, 229-236.
- Han, S., Yoshida, D., Kanamoto, T., Nakashima, H., Uryu, T., Yoshida, T., 2010. Sulfated oligosaccharide cluster with polylysine core scaffold as a new anti-HIV dendrimer. *Carbohydr. Polym.* 80, 1111-1115.
- Hara, T., Fujio, Y., Ueda, S., 1982. Polyglutamate production by *Bacillus subtilis* (natto). *J. Appl. Biochem.* 4, 112-120.
- He, L.M., Neu, M.P., Vanderberg, L.A., 2000. *Bacillus licheniformis* γ -glutamyl polymer: physiochemical characterization and U(VI) interaction. *Environ. Sci. Technol.* 34, 1694–1701.
- Hezayen, F.F., Rehm, B.H., Tindall, B.J., Steinbüchel, A., 2001. Transfer of *Natrialbaasiatica* BIT to *Natrialbataiwanensis* sp. nov. and description of *Natrialbaegyptiaca* sp. nov., a novel extremely halophilic, aerobic, non-pigmented member of archaea from Egypt which produces extracellular poly (glutamic acid). *Int. J. Syst. Evol. Microbiol.* 51, 1133–1142.
- Hiraki, J., 2000. ϵ -Polylysine, its development and utilization. *Fine Chem.* 29, 18–25.
- Jeong, J.H., Kim, J.N., Wee, Y.J., Ryu, H.W., 2010. The statistically optimized production of poly (γ -glutamic acid) by batch fermentation of a newly isolated *Bacillus subtilis* RKY3. *Bioresour. Technol.* 101, 4533–4539.
- Jia, S., Fan, B., Wang, G., Peng, P., Jia, Y., 2010. Fractionation and characterization of ϵ -poly-L-lysine from *Streptomyces albulus* CGMCC 1986. *Food Sci. Biotechnol.* 19, 361-366.
- Jung, D.K., Jung, S., Sun, J.S., Kim, J.N., Wee, Y.J., Jang, H.G., Ryu, H.W., 2006. Influences of cultural medium components on the production of poly (γ -glutamic acid) by *Bacillus* sp. RKY3. *Biotechnol. Bioprocess Eng.* 10, 289–295.
- Kahar, P., Iwata, T., Hiraki, J., Park, Y.E., Okabe, M., 2001. Enhancement ϵ -polylysine production by *Streptomyces albulus* strain 410 using pH control. *J. Biosci. Bioeng.* 91, 190–194.
- Kahar, P., Kobayashi, K., Iwata, T., Hiraki, J., Kojima, M., Okabe, M., 2002. Production of ϵ -polylysine in an air lift bioreactor (ABR). *J. Biosci. Bioeng.* 93, 274–280.

- Kambourova, M., Tangney, M., Priest, F.G., 2001. Regulation of polyglutamic acid synthesis by glutamate in *Bacillus licheniformis* and *Bacillus subtilis*. Appl. Environ. Microbiol. 67, 1004-1007.
- Kavitha, V., Radhakrishnan, N., Madhavacharyulu, E., Sailakshmi, G., Sekaran, G., Reddy, B.S., Rajkumar, G.S., Gnanamani, A., 2010. Biopolymer from microbial assisted in situ hydrolysis of triglycerides and dimerization of fatty acids. Bioresour. Technol. 101, 337-343.
- Kito, M., Takimoto, R., Yoshida, T., Nagasawa, T., 2002. Purification and characterization of ϵ -poly-L-lysine-degrading enzyme from an ϵ -poly-L-lysine-producing strain *Streptomyces albulus*. Arch. Microbiol. 178, 325-330.
- Krehenbrink, M., Oppermann-Sanio, F.B., Steinbüchel, A., 2002. Evaluation of non-cyanobacterial genome sequences for occurrence of cyanophycin synthetase homologous genes and cloning of an active cyanophycin synthetase from *Acinetobacter* sp. DSM 587. Arch. Microbiol. 177, 371-380.
- Kreyenschulte, D., Krull, R., Margaritis, A., 2014. Recent advances in microbial biopolymer production and purification. Crit. Rev. Biotechnol. 34, 1-15.
- Kroll, J., Klintner, S., Steinbüchel, A., 2011. A novel plasmid addiction system for large-scale production of cyanophycin in *Escherichia coli* using mineral salts medium. Appl. Microbiol. Biotechnol. 89, 593-604.
- Kunioka, M., 1997. Biosynthesis and chemical reactions of poly (amino acid)s from microorganisms. Appl. Microbiol. Biotechnol. 47, 469-475.
- Kuraray, Co., 2001. Water swelling polymer gel and its production method. JP Patent 278984.
- Lin, B., Qiu, F., Lin, Q., Hong, Y., 2013. Optimization of breeding and cultivation conditions for mutant strain with high productivity of γ -poly glutamic acid. J. Pure Appl. Microbiol. 7, 2479-2488.
- Liu, H., Ray, W.K., Helm, R.F., Popham, D.L., Melville, S.B., 2016. Analysis of the spore membrane proteome in *Clostridium perfringens* implicates cyanophycin in spore assembly. J. Bacteriol. 198, 1773-1782.
- Liu, J., He, D., Li, X., Gao, S., Wu, H., Liu, W., Gao, X., Zhou, T., 2010. γ -Polyglutamic acid (γ -PGA) produced by *Bacillus amyloliquefaciens* C06 promoting its colonization on fruit surface. Int. J. Food Microbiol. 142, 190-197.
- Means, G.E., Feeney, R.E., 1990. Chemical modifications of proteins: History and applications. Bioconjug. Chem. 1, 2-12.
- Mooibroek, H., Oosterhuis, N., Giuseppin, M., Toonen, M., Franssen, H., Scott, E., Sanders, J., Steinbüchel, A., 2007. Assessment of technological options and economical feasibility for cyanophycin biopolymer and high-value amino acid production. Appl. Microbiol. Biotechnol. 77, 257-267.
- Neumann, K., Stephan, D.P., Ziegler, K., Hühns, M., Broer, I., Lockau, W., Pistorius, E.K., 2005. Production of cyanophycin, a suitable source for the biodegradable polymer polyaspartate, in transgenic plants. Plant Biotechnol. J. 3, 249-258.

- Nishikawa, M., Ogawa, K., 2002. Distribution of microbes producing antimicrobial ϵ -poly-L-lysine polymers in soil microflora determined by a novel method. *Appl. Environ. Microbiol.* 68, 3575-3581.
- Obst, M., Krug, A., Luftmann, H., Steinbüchel, A., 2005. Degradation of cyanophycin by *Sedimentibacter hongkongensis* strain KI and *Citrobacter amalonaticus* strain G isolated from an anaerobic bacterial consortium. *Appl. Environ. Microbiol.* 71, 3642–3652.
- Obst, M., Oppermann-Sanio, F.B., Luftmann, H., Steinbüchel, A., 2002. Isolation of cyanophycin-degrading bacteria, cloning and characterization of an extracellular cyanophycinase gene (cphE) from *Pseudomonas anguilliseptica* strain BI the cphE gene from *P. Anguilliseptica* Bi encodes a cyanophycin-hydrolyzing enzyme. *J. Biol. Chem.* 277, 25096-25105.
- Obst, M., Sallam, A., Luftmann, H., Steinbüchel, A., 2004. Isolation and characterization of gram-positive cyanophycin-degrading bacteria kinetic studies on cyanophycin depolymerase activity in aerobic bacteria. *Biomacromolecules* 5, 153-161.
- Obst, M., Steinbüchel, A., 2004. Microbial degradation of poly(amino acid)s. *Biomacromolecules* 5, 1166–1176.
- Ogunleye, A., Bhat, A., Irorere, V.U., Hill, D., Williams, C., Radecka, I., 2015. Poly- γ -glutamic acid: production, properties and applications. *Microbiol.* 161, 1–17.
- Oppermann-Sanio, F.B., Steinbüchel, A., 2002. Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. *Naturwissenschaften* 89, 11–22.
- Panchal, B., Bagdadi, A., Roy, I., 2013. Advances in Natural Polymers, in: Thomas, S., Visakh, P.M., Mathew, A.P. (Eds.), *Polyhydroxyalkanoates: The Natural Polymers Produced by Bacterial Fermentation*. Springer Berlin Heidelberg, 397-421.
- Pandey, A., 2003. Solid-state fermentation. *Biochem. Eng. J.* 13, 81–84.
- Pandey, A.K., Kumar, A., 2014. Improved microbial biosynthesis strategies and multifarious applications of the natural biopolymer epsilon-poly-L-lysine. *Proc. Biochem.* 49, 496–505.
- Ren, X.D., Chen, X.S., Zeng, X., Wang, L., Tang, L., Mao, Z.G., 2015. Acidic pH shock induced overproduction of ϵ -poly-L-lysine in fed-batch fermentation by *Streptomyces* sp. M-Z18 from agro-industrial by-products. *Bioprocess Biosyst. Eng.* 38, 1113–1125.
- Richard, A., Margaritis, A., 2001. Poly (glutamic acid) for biomedical applications. *Critical Reviews in Biotechnology* 21, 219–232.
- Richard, A., Margaritis, A., 2006. Kinetics of molecular weight reduction of poly(glutamic acid) by in situ depolymerization in cell-free broth of *Bacillus subtilis*. *Biochem. Eng.* 30, 303–307.
- Saimura, M., Takehara, M., Mizukami, S., Kataoka, K., Hirohara, H., 2008. Biosynthesis of nearly monodispersed poly (ϵ -L-lysine) in *Streptomyces* species. *Biotechnol. Lett.* 30, 377–385.

- Sallam, A., Steinbuechel, A., 2010. Dipeptides in nutrition and therapy: cyanophycin-derived dipeptides as natural alternatives and their biotechnological production. *Appl. Microbiol. Biotechnol.* 87, 815–28.
- Santos, S., Torcato, I., Castanho, M.A., 2012. Biomedical applications of dipeptides and tripeptides. *Biopolym.* 98, 288–93.
- Sherman, D.M., Tucker, D., Sherman, L.A., 2000. Heterocyst development and localization of cyanophycin in N_2 -fixing cultures of *Anabaena* sp. PCC 7120 (*Cyanobacteria*). *J. Phycol.* 36, 932–941.
- Shi, C., He, Y., Feng, X., Fu, D., 2015. ϵ -Polylysine and next-generation dendrigraft poly-L-lysine: chemistry, activity, and applications in biopharmaceuticals. *J. Biomater. Sci.* 26, 1343–1356.
- Shih, I.L., Van, Y.T., 2001. The production of poly(γ -glutamic acid) from microorganism and its various applications. *Bioresour. Technol.* 79, 207–225.
- Shih, I.L., Van, Y.T., Yeh, L.C., Lin, H.G., Chang, Y.N., 2001. Production of a biopolymer flocculant from *Bacillus licheniformis* and its flocculation properties. *Bioresour. Technol.* 78, 267–272.
- Shukla, S.C., Mishra, A., 2012. Optimization of process parameters for ϵ -polylysine production from mixed carbon source using “one factor at a time” Technique. *APCBEE Procedia.* 2, 120–124.
- Shukla, S.C., Mishra, A., 2013. ϵ -Polylysine production from sugar cane molasses by a new isolates of *Bacillus* sp. and optimization of the fermentation condition. *Ann. Microbiol.* 63, 1513–1523.
- Shukla, S.C., Singh, A., Pandey, A.K., Mishra, A., 2012. Review on production and medical applications of ϵ -polylysine. *Biochem. Eng. J.* 65, 70–81.
- Smith, A.M., Zeeman, S.C., 2006. Quantification of starch in plant tissues. *Nat. Protocols* 1, 1342–1345.
- Solaiman, D.K.Y., Ashby, R.D., Zerkowski, J. A., 2012. Substrate preference and oxygen requirement for cyanophycin synthesis by recombinant *Escherichia coli*. *Biocatal. Agric. Biotechnol.* 1, 9–14.
- Solaiman, D.K.Y., Garcia, R.A., Ashby, R.D., Piazza, G.J., Steinbuechel, A., 2011. Rendered-protein hydrolysates for microbial synthesis of cyanophycin biopolymer. *N. Biotechnol.* 28, 552–558.
- Steinle, A., Steinbüchel, A., 2010. Establishment of a simple and effective isolation method for cyanophycin from recombinant *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 85, 1393–1399.
- Stubbe, J., Tian, J., He, A., Sinskey, A.J., Lawrence, A.G., Liu, P., 2005. Nontemplate-dependent polymerization process: polyhydroxyalkanoate synthases as a paradigm. *Annu. Rev. Biochem.* 74, 433–80.
- Su, J.F., Huang, Z., Yuan, X.Y., Wang, X.Y., Li, M., 2010. Structure and properties of carboxymethyl cellulose/soy protein isolate blend edible films cross linked by Maillard reactions. *Carb. Polym.* 79, 145–153.

- Wang, J., Liu, Z., Wang, Y., Cheng, W., Mou, H., 2014. Production of a water-soluble fertilizer containing amino acids by solid-state fermentation of soybean meal and evaluation of its efficacy on the rapeseed growth. *J. Biotechnol.* 187, 34–42.
- Watzer, B., Engelbrecht, A., Hauf, W., Stahl, M., Maldener, I., Forchhammer, K., 2015. Metabolic pathway engineering using the central signal processor PII. *Microb. Cell Fact.* 14, 192.
- Wibowo, N., Yang, P.F., Lee, C.K., 2013. Epsilon-polylysine fermentation and its recovery using carboxymethyl cellulose (CMC)-conjugated magnetite. *Separ. Sci. Technol.* 48, 1086–1092.
- Wiefel, L., Steinbüchel, A., 2014. Solubility behavior of cyanophycin depending on lysine content. *Appl. Environ. Microbiol.* 80, 1091–1096.
- Wilming, A., Begemann, J., Kuhne, S., Regestein, L., Bongaerts, J., Evers, S., Maurer, K.H., Büchs, J., 2013. Metabolic studies of γ -poly glutamic acid production in *Bacillus licheniformis* by small-scale continuous cultivations. *Biochem. Eng. J.* 73, 29–37.
- Wu, Q., Xu, H., Shi, N., Yao, J., Li, S., Ouyang, P., 2008. Improvement of poly(γ -glutamic acid) biosynthesis and redistribution of metabolic flux with the presence of different additives in *Bacillus subtilis* CGMCC 0833. *Appl. Microbiol. Biotechnol.* 79, 527–535.
- Wu, Q., Xu, H., Xu, L., Ouyang, P., 2006. Biosynthesis of poly(γ -glutamic acid) in *Bacillus subtilis* NX-2: Regulation of stereochemical composition of poly(γ -glutamic acid). *Proc. Biochem.* 41, 1650–1655.
- Xia, J., Xu, Z., Xu, H., Liang, J., Li, S., Feng, X., 2014. Economical production of poly(ϵ -L-lysine) and poly(L-diaminopropionic acid) using cane molasses and hydrolysate of *Streptomyces* cells by *Streptomyces albulus* PD-1. *Bioresour. Technol.* 164, 241–247.
- Xu, G., Toh, G.W., Du, N., Liu, X.Y., 2012. Natural Polymers: Composites, in: John, M.J., Thomas, S. (Eds.), *Spider silk: the toughest natural polymer*. RSC, 275–304.
- Xu, H., Jiang, M., Li, H., Lu, D., Ouyang, P., 2005. Efficient production of poly(γ -glutamic acid) by newly isolated *Bacillus subtilis* NX-2. *Proc. Biochem.* 40, 519–523.
- Xu, Z., Bo, F., Xia, J., Sun, Z., Li, S., Feng, X., Xu, H., 2015. Effects of oxygen-vectors on the synthesis of epsilon-poly-lysine and the metabolic characterization of *Streptomyces albulus* PD-1. *Biochem. Eng. J.* 94, 58–64.
- Xu, Z., Feng, X., Zhang, D., Tang, B., Lei, P., Liang, J., Xu, H., 2014. Enhanced poly(γ -glutamic acid) fermentation by *Bacillus subtilis* NX-2 immobilized in an aerobic plant fibrous-bed bioreactor. *Bioresour. Technol.* 155, 8–14.
- Yao, D., Ji, Z., Wang, C., Qi, G., Zhang, L., Ma, X., Chen, S., 2012. Co-producing iturin A and poly- γ -glutamic acid from rapeseed meal under solid state fermentation by the newly isolated *Bacillus subtilis* strain 3-10. *World J. Microbiol. Biotechnol.* 28, 985–991.
- Yao, J., Jing, J., Xu, H., Liang, J., Wu, Q., Feng, X., Ouyang, P., 2009. Investigation on

enzymatic degradation of [gamma]-polyglutamic acid from *Bacillus subtilis* NX-2. J. Mol. Catal. B: Enzym., 56(2-3),158-164.

Yong, X., Cui, Y., Chen, L., Ran, W., Shen, Q., Yang, X., 2011. Dynamics of bacterial communities during solid-state fermentation using agro-industrial wastes to produce poly- γ -glutamic acid, revealed by real-time PCR and denaturing gradient gel electrophoresis (DGGE). Appl. Microbiol. Biotechnol. 92, 717–725.

Yoon, H.Y., Do, J.H., Lee, S.Y., Chang, H.N., 2000. Production of poly- γ -glutamic acid by fed-batch culture of *Bacillus licheniformis*. Biotechnol. Letters 22, 585–588.

Zeng, W., Chen, G., Wang, Q., Zheng, S., Shu, L., Liang, Z., 2014. Metabolic studies of temperature control strategy on poly (γ -glutamic acid) production in a thermophilic strain *Bacillus subtilis* GXA-28. Bioresour. Technol. 155, 104–110.

Zhang, D., Feng, X., Li, S., Chen, F., Xu, H., 2012. Effects of oxygen vectors on the synthesis and molecular weight of poly (γ -glutamic acid) and the metabolic characterization of *Bacillus subtilis* NX-2. Proc. Biochem. 47, 2103–2109.

Zhang, Y., Feng, X., Xu, H., Yao, Z., Ouyang, P., 2010. ϵ -poly-l-lysine production by immobilized cells of *Kitasatospora* sp. MY 5-36 in repeated fed-batch cultures. Bioresour. Technol. 101, 5523–5527.

Zhang, Y., Kumar, A., Vadlania, P.V., Narayanan, S., 2013. Production of nitrogen-based platform chemical: cyanophycin biosynthesis using recombinant *Escherichia coli* and renewable media substitutes. J. Chem. Technol. Biotechnol. 88, 1321–1327.

Zhao, C., Zhang, Y., Wei, X., Hu, Z., Zhu, F., Xu, L., Luo, M., Liu, H., 2013. Production of ultra-high molecular weight poly- γ -glutamic acid with *Bacillus licheniformis* P-104 and characterization of its flocculation properties. Appl. Biochem. Biotechnol. 170, 562-572.

Zhu, F., Cai, J., Wu, X., Huang, J., Huang, L., Zhu, J., Zheng, Q., Cen, P., Xu, Z., 2013. The main by products and metabolic flux profiling of γ -PGA-producing strain *B. subtilis* ZJU-7 under different pH values. J. Biotechnol. 164, 67–74.

Ziegler, K., Deutzmann, R., Lockau, W., 2002. Cyanophycin-like enzymes of non-cyanobacterial Eubacteria: characterization of the polymer produced by a recombinant synthetase of *Desulfito bacterium hafniense*. J. Biosci. 57, 522–529.

Zong, H., He, Y., Zhan, Y., Du, J., Feng, F., Li, D., 2010. Optimization of medium constituents for ϵ -poly-l-lysine fermentation with response surface methodology. J. Food Sci. 75, M552–M556.