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REVIEW ARTICLE

Poly-gamma-glutamic acid biopolymer: a sleeping giant with diverse applications and unique opportunities for commercialization

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Abstract

Poly-gamma-glutamic acid (γ-PGA) is a biodegradable, non-toxic, ecofriendly, and non-immunogenic biopolymer. Its phenomenal properties have gained immense attention in the field of regenerative medicine, the food industry, wastewater treatment, and even in 3D printing bio-ink. The γ-PGA has the potential to replace synthetic non-degradable counterparts, but the main obstacle is the high production cost and lower productivity. Extensive research has been carried out to reduce the production cost by using different waste; however, it is unable to match the commercialization needs. This review focuses on the biosynthetic mechanism of γ-PGA, its production using the synthetic medium as well as different wastes by L-glutamic acid-dependent and independent microbial strains. Furthermore, various metabolic engineering strategies and the recovery processes for γ-PGA and their possible applications are discussed. Finally, highlights on the challenges and unique approaches to reduce the production cost and to increase the productivity for commercialization of γ-PGA are also summarized.

Keywords Poly-gamma-glutamic acid, · Waste valorization, · Multi-nutritious, · Commercialization

1 Introduction

Microbial biopolymers are gaining worldwide attention due to their degradable, non-toxic, and eco-friendly nature compared to synthetic non-degradable materials. The market demand for biopolymer is higher due to its phenomenal properties; however, the high production cost and low yielding microbial strains is the major hurdle in the commercialization of biopolymer [1]. The main difference between biodegradable polymer (polycaprolactone) and bio-based polymer (poly-lactic acid) is that the former one undergoes degradation in the presence of microorganisms or other aerobic and anaerobic processes, whereas the latter one may be biodegradable (poly-lactic acid) or non-biodegradable (bio-polyethylene). Earlier biopolymers were derived from corn and other feedstocks, which raised a debate between food vs fuel, shifting the focus towards the use of lignocellulosic wastes such as rice straw and wheat bran [2]. With advancements in research and technologies, biopolymers are finding their way from being used as food thickeners and bioplastics into high-end regenerative medicines. Out of all the biopolymers known to date, poly gamma glutamic acid (γ-PGA) is one of the most expensive biopolymers with few milligrams at the cost of several dollars. Poly glutamic acid (PGA) is a poly(amino acid) in which glutamic acid monomers are coupled to each other *via* amide bonds between alpha or gamma carboxylic groups. The α-PGA are chemically synthesized by nucleophilic polymerization of L-glutamic acid in the presence of aprotic solvents like toluene and a metal catalyst. Naturally occurring microbes are not known to synthesize α-PGA unless genetically engineered [3]. Contrastingly, γ-PGA are synthesized by different microbes such as *Natrialba aegyptiaca*, *Natronococcus occultus*, and *Fusobacterium nucleatum.* Although there are different γ-PGA producer strains known, *Bacillus* species, especially *B.licheniformis* and *B.subtilis* are still considered as the most potent producers due to relatively higher production and productivity of γ-PGA [4, 5].

The biosynthesis of γ-PGA is solely based on the microenvironment and the type of organism producing it. Some may produce γ-PGA to evade antibodies and antimicrobial peptides, whereas others may use it as a source of nutrients during starvation [4, 5]. Depending on these factors, γ-PGA can either be cell-

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bound to elicit virulence or maybe secretory to withstand an extreme environment. As γ-PGA is not synthesized in a ribosomal-dependent manner, drugs such as erythromycin cannot cease γ-PGA production [6]. The γ-PGA producers can be broadly classified into three major classes: poly-γ-L-glutamic acid producer, poly-γ-D-glutamic acid producer, and poly-γ-DL-glutamic acid producer (Table 1). The Poly-γ-glutamic acid producer can be further sub-classified depending on the exogenous supply of L-glutamic acid as L-glutamic acid-dependent producers and L-glutamic acid-independent producers. The former one is a promising producer due to the comparatively high production of γ-PGA, but the production cost is high. The latter producer has an advantage of lower production cost; however, the γ-PGA yield is also less [3, 7]. Several research groups have explored various wastes in order to reduce the production cost of γ-PGA. The use of lignocellulosic biomass such as rice straw and corncobs can be an attractive alternative for carbon source [23, 24]. The carbon source can be further substituted using macroalgae, goose feathers, paper waste, swine, dairy, and chicken manure [8–12]. However, the major limitation with these wastes is the requirement of pretreatment for effective utilization of sugars as well as supplementing the production media with additional nutrients such as L-glutamic acid, citric acid, peptone and trace elements, further adding to the cost. Therefore, it is of utmost importance to have in-depth knowledge about different waste, their nutrient composition as well as their availability for cost-effective synthesis of γ-PGA. This review focuses on recent updates on the biosynthesis of γ-PGA by using various *Bacillus* strains for the economical production of γ-PGA. It also sheds light on the recovery processes, challenges that the researchers face in terms of high production cost and low-productivity, as well as strategies to overcome them.

2 Microbial production of γ-PGA

2.1 Screening of γ-PGA producers

For the microbial production of **γ-**PGA, screening of γ-PGA producer strain is the primary step. There are three screening strategies for γ-PGA producers: the basic approach is the UV based method to confirm γ-PGA at 216 nm absorbance**.** The second approach comprises the detection of a concentric zone around the colonies in the presence of neutral red. The final approach is the formation of fibrous precipitate in the production medium by the addition of methanol/ethanol, as depicted in Fig. 1. The final characterization of γ-PGA is done by thin-layer chromatography (TLC), Fourier transform infrared spectroscopy (FTIR), circular dichroism (CD-spectra), gel permeation chromatography (GPC), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and nuclear magnetic resonance (NMR) analysis [13, 14].

2.2 L-glutamic acid-dependent strains for γ-PGA production

2.2.1 *Bacillus subtilis*

Over the past decades, extensive efforts have been made for the production of γ-PGA from *Bacillus subtilis* after its discovery by Ivanovic and his co-workers in the form of a capsule in *B. anthracis* [15]. Wu *et al.* manipulated the cell membrane permeability with additives like tween80 or DMSO, which facilitated the utilization of abundant glucose and glutamate for enhanced production of γ-PGA. With the addition of glycerol, the 2-oxoglutarate dehydrogenase activity was suppressed, which helped to direct the carbon flux for glutamate synthesis, thereby stimulating γ-PGA production from 26.7 g/L to 31.7 g/L. Although Tween80 showed a negative impact on cell growth, it could trigger iso-citrate dehydrogenase activity for enhanced glutamate production, thus playing a pivotal role in γ-PGA synthesis with the yield of 34.4 g/L in *B. subtilis* CGMCC 0833 [16]. Lee *et al.* reported the ability of γ-PGA to inhibit angiotensin-converting enzyme at 1.25 ppm in *B.subtilis* D7, which could aid in reducing hypertension [17]. Bajaj and Singhal demonstrated that γ-PGA produced from *B. subtilis* R 23 had a flocculating ability of 30/optical density (O.D.) at 7.5 ppm in the presence of Ca^{2+}. The researcher explained that the purity of γ-PGA depends upon the carbon source utilized by *B. subtilis* IF03335 [18]. The

Table 1 Types of poly gamma glutamic acid producers

Poly-γ-L- glutamic acid producer	Poly-γ-DL- glutamic acid producer		Poly-γ-D-glutamic acid producer	Ref.
	L-glutamic acid-dependent producers	L-glutamic acid-independent producer		
• *B. halodurans*	*B. subtilis*IFO 3335	*B. subtilis* 5E	*B. anthracis*	[6, 15, 47, 66]
• *B. megateriums*	*B. licheniformis*ATCC 9945A	*B. subtilis* TAM-4		
• *Natrialbaaegyptiaca*	*B. subtilis*MR-141	*B. licheniformis* S173		
• *Natronococcusoccultus*	*B. subtilis*subsp. *chungkookjang*	*B. licheniformis* A35		

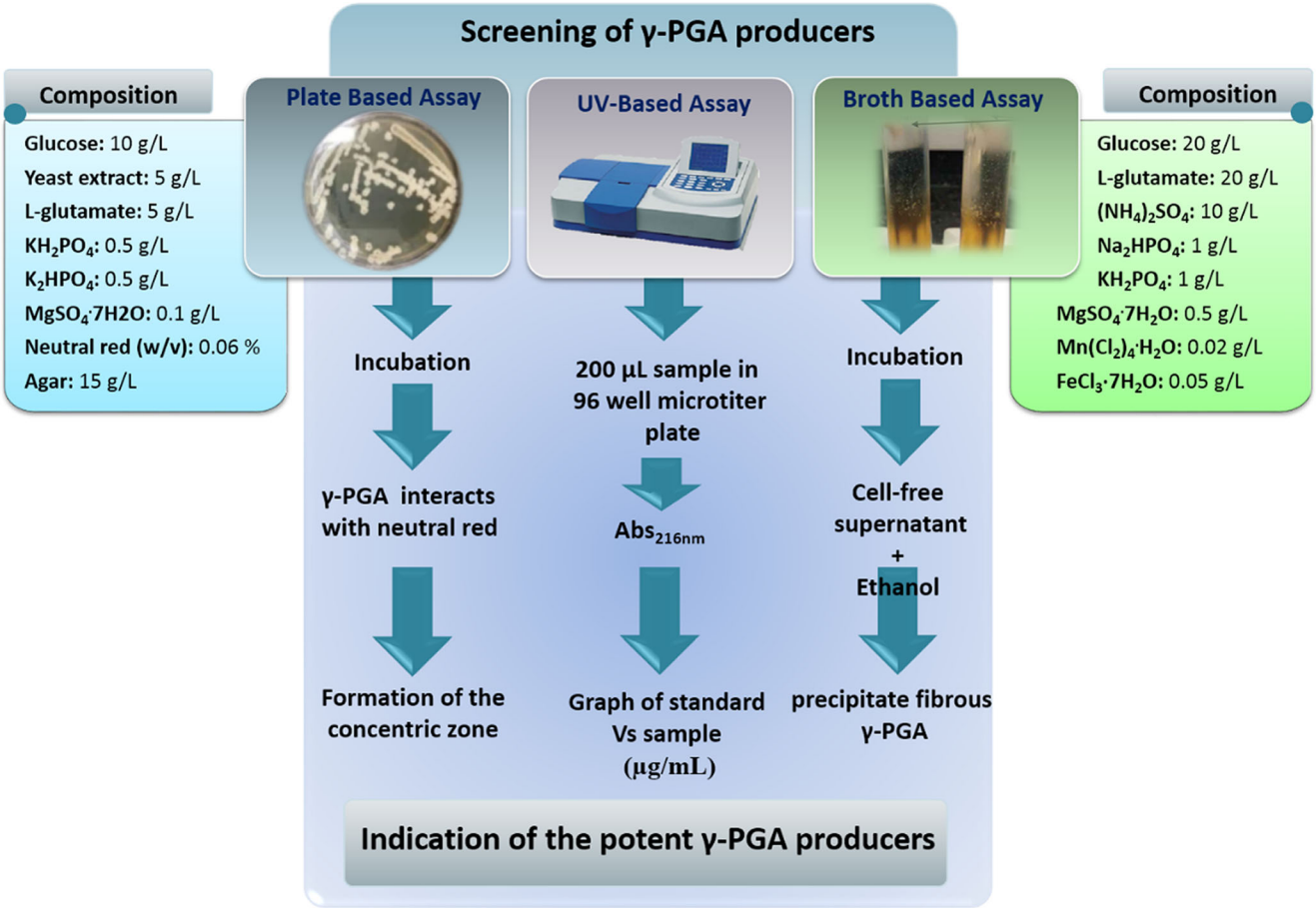


Fig. 1 Screening approach for isolating potential γ-PGA producing strain [13, 14]

addition of glucose produced polysaccharides as the byproduct, whereas the use of citric acid resulted in the synthesis of highly pure γ-PGA [19]. Zhang *et al.* statistically optimized solid-state fermentation for γ-PGA production in *B. subtilis* ZC-5 using Plackett-Burman and Box-Behnken design. The final optimized medium yielded 65 g/L of γ-PGA in a medium consisting of chicken manure, soybean cake, and glutamic acid extracts. This strategy has a multifarious advantage like waste valorization, lower substrate cost, decreased energy consumption, and the final fermented substrates can be used as bio-fertilizers [20]. Ju *et al.* demonstrated both the glutamate-dependent and independent property of *B. subtilis* MJ80 due to its ability to utilize L-glutamic acid and soybean from the production medium. This strain produced about 68.7 g/L of γ-PGA with a molecular size of 1500 kDa in a 300-L fermenter within 72 h of incubation. The γ-PGA production is solely dependent on the TCA cycle, and any approach that can improve the rate transfer of oxygen would enhance the γ-PGA yield [21]. Da Silva *et al.* checked the effect of metabolic precursors and an oxygen-carrying agent such as polydimethylsiloxane (PDMS) on γ-PGA production. Inclusion of L-glutamine and α-ketoglutaric acid as the metabolic precursors elevated the γ-PGA production up to 20%, and further addition of PDMS resulted in increment of oxygen mass transfer rate with the productivity of 0.97 g/L/h in *B. subtilis* BL53 [22].

Zhang *et al.* developed an eco-friendly and economical strategy to enhance the production of γ-PGA. The fishmeal wastewater can act as a cost-effective substitute for nitrogen sources, thereby decreasing its extrinsic supply into the production medium. The optimized production medium yielded 25 g/L of γ-PGA with glucose (3%), glutamic acid (2.5%), and fishmeal wastewater with chemical oxygen demand of 15 [23]. Huang *et al.* developed a fed-batch approach by maintaining the glucose concentration at 0.3 to 1% throughout the fermentation process, which yielded 200 times the γ-PGA production as compared to normal batch cultivation. With this strategy, the production of 101 g/L was achieved in a 10 L fermenter with a productivity of 2.19 g/L/h [24]. Kedia *et al.* investigated the impact of different media such as E, C, F, and GS medium on γ-PGA production in *B. subtilis* natto, one of the most widely used γ-PGA-producing strains. The E and F media mainly consisted of glycerol as the carbon source, whereas in GS and C media, sucrose and glucose act as carbon sources. It was observed that *this strain* could not utilize E medium; however, with GS medium, the yield reached around

28 g/L [25]. To ensure that the glutamic acid added to the medium is being polymerized to γ-PGA, Ogawa *et al.* radiolabeled L-glutamic acid monomers and supplemented it to the fermentation medium, which showed radioactivity in the γ-PGA secreted by *B. subtilis* natto MR-141 [26]. Zhang et al. also demonstrated another greener approach for the production of γ-PGA., wherein *B.subtilis* NX-2 could produce around 52 g/L of γ-PGA using cane molasses and waste liquor of monosodium glutamate, thereby reducing the cost associated with expensive media components [27]. Lignocellulosic biomass such as rice straw and wheat bran are the most widely generated agricultural biomass treated as waste in many countries. Due to a lack of knowledge about the proper disposal strategies, the biomass is burnt off, which raises many environmental concerns [28]. To address this issue, by using the same strain, Tang *et al.* demonstrated a two-stage hydrolysis strategy for efficient retrieval of sugars from rice straw. In first stage, approximately xylose (2.2%), glucose (0.2%), and arabinose (0.1%) were retrieved whereas in stage II, glucose (2.6%) and xylose (0.1%) were obtained. Using this approach, γ-PGA yield was 73 g/L by continuous fermentation with the productivity of 0.8 g/L/h [29]. Similarly, corncob hydrolysate was investigated for its efficient utilization by *B. subtilis* HB-1, which yielded 24 g/L of γ-PGA with the productivity of 0.6 g/L/h [30]. The metallic ions in the medium are also an important factor in the production of γ-PGA. Wu *et al.* reported the potential of metal ions such as Mn^{2+} to modulate the stereochemistry of γ-PGA secreted by *B. subtilis* NX-2. In the absence of Mn^{2+} ions, the γ-PGA yield was only 9.25 g/L; however, with the addition of Mn^{2+} ions, the yield reached up to 28.42 g/L. With a lower concentration of Mn^{2+} ions in the production medium, the ratio of D-glutamate in the γ-PGA elevated from 17 to 77% due to an increase in the activity of glutamate racemase [31].

2.2.2 *Bacillus licheniformis*

This Bacillus species is a mesophilic organism known for its high γ-PGA-producing ability. Shih *et al.* statistically optimized four variables, namely citric acid, L-glutamic acid, pH, and glycerol using factorial and central composite design for enhanced γ-PGA production by *B. licheniformis* CCRC 12826. With this approach, γ-PGA production increased from 5.2 to 19.8 g/L, which was about 372% higher than the traditionally used medium E [32]. By using the same strain, Yan *et al.* demonstrated the potential of the bio-flocculant activity of γ-PGA to replace polyacrylamide from the sugarcane industry for clarification. The presence of metal ions such as Mg^{2+} could enhance the flocculating ability and γ-PGA production, unlike *B. subtilis* NX-2, which requires Mn^{2+} ions [33]. Bajaj *et al.* reported the ability of metabolic precursors to improve the molecular size and yield of γ-PGA by *B. licheniformis* NCIM 2324. The addition of 0.5 mM L-Glutamine and 10 mM α-ketoglutarate raised the γ-PGA production to 35 g/l, indicating the enhanced activity of glutamine synthetase and aminotransferases. With this combination of metabolic precursors, the molecular size increased from 210 to 570 kDa [34]. Du *et al.* investigated the effect of glycerol in *B. licheniformis* WBL-3 on phospholipids and ester-linked fatty acids. With glycerol in the production medium, the level of phosphatidylglycerol and cardiolipin increased, whereas that of phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid decreased. Similarly, the addition of glycerol showed a substantial reduction in C18 fatty acid, such as oleic acid, thereby increasing the cell membrane permeability. A significant increase in C10 fatty acid was also observed, which might aid in enhancing the membrane fluidity implicating their function in increased γ-PGA secretion [35].

Wei *et al.* demonstrated a salt-induced production of γ-PGA by a halotolerant strain of *B. licheniformis* WX-02. The molecular weight and the yield of γ-PGA were reduced by increasing the salt concentration with a maximum yield of 13.86 g/L at 8% NaCl. Thus, the molecular weight of γ-PGA can be modulated accordingly depending on the application by increasing or decreasing the concentration of salts in the medium [36]. Cromwick *et al. i*nvestigated the effect of Mn^{2+} on the physiology and the production of γ-PGA in *B. licheniformis* ATCC 9945A. The presence of Mn^{2+} in the range of 6.15 to 615 μM can enhance the uptake of carbon sources as well as maintain high cell viability for a more extended period. However, for the production, maximum yield was achieved at $MnSO_4$ concentration between 6.5 and 61.5 μM, indicating the requirement of stress condition for improved γ-PGA production. Further, the presence or the absence of $MnSO_4$ in the fermentation medium have shown to modulate the stereochemistry of the γ-PGA (L and D glutamate isomeric ratio); however, the mechanism is still unknown[37]. Altun *et al.* reported an economically feasible strategy by using goose feathers and *B. licheniformis* 9945A strain. The goose feathers were hydrolyzed by keratinolytic enzymes and then added to the production medium, which yielded 5.4 g/L of γ-PGA [8]. Similarly, waste papers were also explored as a cost-effective alternative for carbon sources yielding around 6.46 g/L of γ-PGA by *B. licheniformis* WX-02 [10]. Other L-glutamic acid-dependent producers are *B. methylotrophicus* and *B.amyloliquefaciens; however*, they are least explored for their enhanced γ-PGA producing ability [12, 38].

2.3 L-glutamic acid independent strains for γ-PGA production

2.3.1 *B. subtilis*

In the previous section, we have mentioned this strain as L-glutamic acid-dependent strain, but several researchers reported this strain could produce γ-PGA without the addition of L-

Glutamic acid. Ito *et al.* reported a glutamic acid independent strain *B. subtilis* TAM-4, which showed *denovo* synthesis of γ-PGA in the production medium. Out of eight carbon and nitrogen sources investigated, glucose and ammonium chloride showed a maximum yield of 13.4 g/L within four days. The stereochemistry of γ-PGA remained constant throughout the cultivation period with the diastereomeric ratio of D/L isomers (78:22) *[39]*. Zhang *et al.* investigated the significant factors affecting the γ-PGA biosynthesis and the endogenous supply of glutamic acid in *B. subtilis* C10. Assuming that the γ-PGA production in glutamic acid independent strain can be triggered by providing TCA cycle metabolites and organic acids, the effect of succinic acid, citric acid, malic acid, acetic acid, and oxalic acid on the production was assessed. The γ-PGA was not detected in the presence of acetic acid, whereas it reached a peak value of 28.3 g/L with citric acid in the production medium. The addition of ammonium chloride further enhanced the γ-PGA production to 29 g/L. The activity of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complex was elevated by supplementation of oxalic acid, whereas citric acid improved the isocitrate dehydrogenase and glutamate dehydrogenase activity [40]. Furthermore, the ability of a novel thermotolerant strain *B. subtilis* GXG5 to produce γ-PGA was evaluated. In the absence of L-glutamic acid, this strain could produce around 19 g/L of γ-PGA at 50°C, indicating its glutamate independent and thermotolerant ability [41].

2.3.2 *Bacillus licheniformis*

Soliman et al. statistically optimized different variables using Plackett–Burman design for improved γ-PGA production in *B. licheniformis* SAB-26. Out of the four nitrogen sources investigated, ammonium sulfate showed a maximum yield of 11.2 g/L, whereas the presence of L-glutamic acid yielded just 1.7 g/L, suggesting the L-glutamic acid independent mode of production. About fifteen independent variables were analyzed for their significance in γ-PGA production from which K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, and casein hydrolysate showed a positive correlation for γ-PGA production by stimulating γ-PGA synthetase complex *[42]*. Similarly, Mabrouk *et al.* optimized the production with nine variables in *B. licheniformis* A13. The statistical analysis revealed that the yeast extracts positively impacted γ-PGA production, whereas the media volume had a negative correlation. The optimized medium composed of glucose (5%); yeast extract (0.2%); NH_4Cl (0.3%); $MgSO_4.7H_2O$ (0.08%); NaCl (0.08 %); K_2HPO_4 (0.64%) yielded around 28.8 g/L of γ-PGA which was five-folds higher compared to the conventional medium [43]. In another study, Kongklom *et al.* demonstrated a fed-batch approach using *B. licheniformis* TISTR 1010 by feeding citric acid, glucose, and NH_4Cl at a particular interval. This strategy yielded 39 g/L of γ-PGA with a productivity of 0.9 g/L/h, which was thrice compared to formerly reported strain by the same group [44, 45]. Other L-glutamic acid independent strains include *B. methylotrophicus* and *B. amyloliquefaciens* LL3 with a yield of 35 g/L and 4.84 g/L, respectively [5, 46].

3 The biosynthetic mechanism for γ-PGA production

As γ-PGA is quite expensive than traditional polymers it has conceptualized to replace, the only anticipated solution is to decrease the overall production cost. To address this issue, one should know the mechanism and the metabolic gene clusters impacting the γ-PGA biosynthesis, which may aid in improving the strains for enhanced production [6]. A metabolic pathway for γ-PGA production and its gene cluster has been shown in Fig. 2. The prerequisite component for γ-PGA production, L-glutamic acid, can be either exogenous or endogenous. When it is derived endogenously, the L-glutamic acid pathway is activated wherein carbon source supplemented through the medium is first converted to acetyl CoA *via* glycolysis followed by synthesis of α-ketoglutaric acid *via* Krebs cycle. The potential precursor, α-ketoglutaric acid, can be converted into L-glutamic acid in two distinct ways. When glutamine is absent, glutamate dehydrogenase converts α-ketoglutaric acid and ammonium chloride to L-glutamic acid. When L-glutamine is present, 2-oxoglutarate aminotransferase catalyzes the conversion of α-ketoglutaric acid and glutamine to L-glutamic acid. The metabolic pathway for γ-PGA biosynthesis includes the activity of enzymes such as racemase, synthase, and peptidase, *etc.* [6, 47, 48].

3.1 Racemase

The γ-PGA can either be a homopolymer such as poly-γ-L-glutamic acid, poly -γ-D- glutamic acid, or a heteropolymer like poly-γ-DL-glutamic acid. The incorporation of L-glutamic acid monomers in the growing peptide chain can either be derived from the medium or be synthesized by glutamate dehydrogenase or 2-oxoglutarate aminotransferase enzyme [49]. However, the synthesis of D-glutamic acid requires the activity of racemases, which catalysis the formation of D-glutamic acid from L-glutamic acid in the process known as racemization [5]. The *B. subtilis* consists of two glutamate racemase genes, which are homologous, namely *racE*, also known as *glr* and *yrpC* [6]. Although *racE* and *yrpC* are not involved in γ-PGA biosynthesis, they are essential for growth in minimal (*yrpC*) and nutrient-rich medium (*racE*) *[47]*. Contrastingly, *glr* is crucial in γ-PGA synthesis by the formation of D-glutamic acid from L-glutamic acid, and its overexpression can increase the enantiomeric ratio of D-glutamate in *B. licheniformis [50]*.

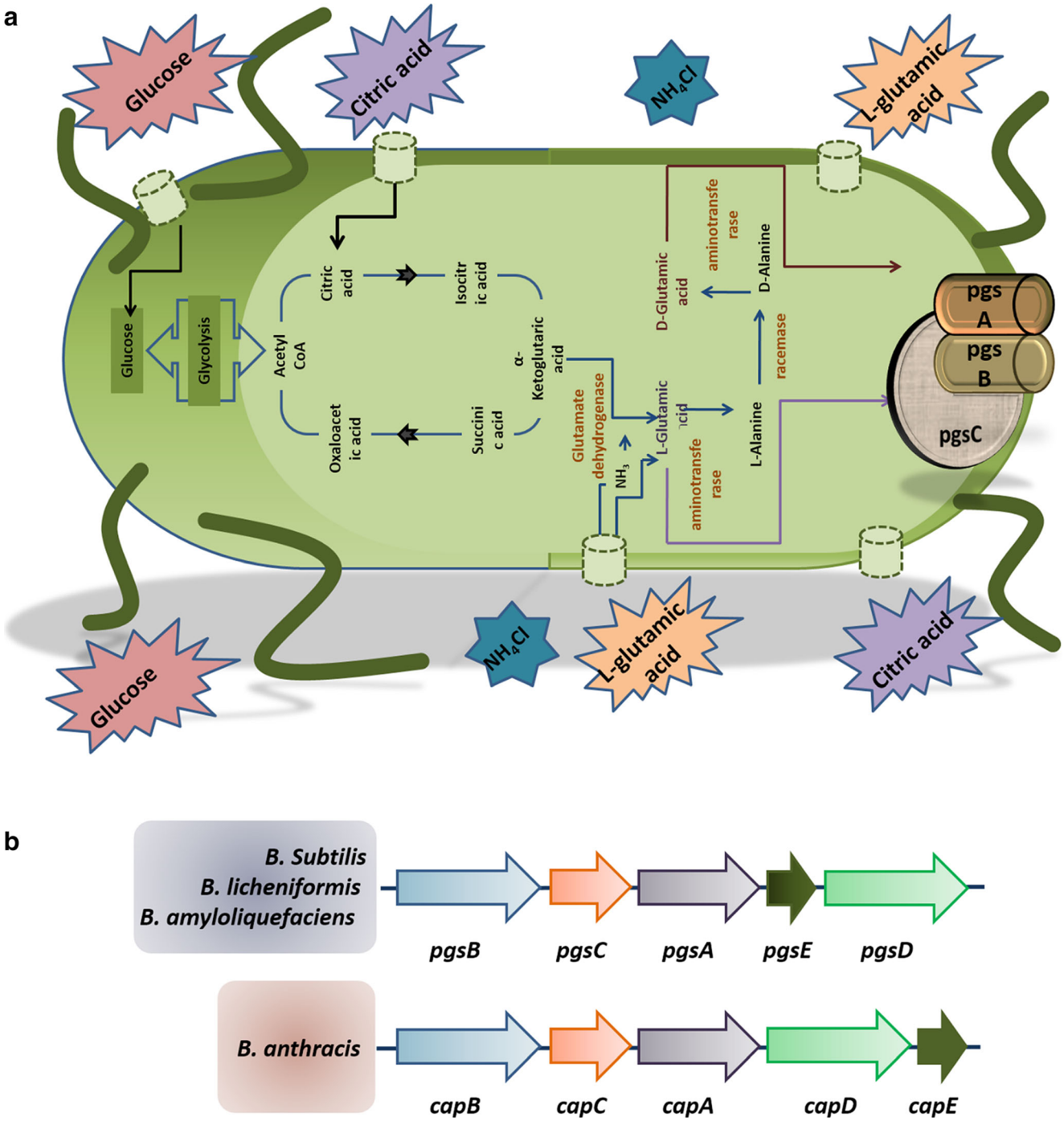


Fig. 2 A) Biosynthesis of poly gamma glutamic acid. B) The γ-PGA synthetase genes in *Bacillus* spp. [4, 15]

3.2 Synthase

In *B. anthracis*, the γ-PGA biosynthesis genes are encoded by the plasmid, whereas in some *Bacillus* species, these genes are inherited chromosomally [6]. The anchoring or release of synthesized γ-PGA is solely dependent on the genes involved. In the case of *B. anthracis*, the *cap* genes are essential for capsular formation in which γ-PGA is linked to the bacterial cell surface, whereas in *B. subtilis* or *B. licheniformis pgs* genes influence its release into the surrounding. The four sets of *cap* genes, namely *capB*, *C*, *A*, *E* in *B.anthracis* are homologous to *pgsB*, *C*, *A*, *E* in *B. subtilis* or *B. licheniformis.* The entire *cap* gene cluster is essential for γ-PGA production; however, the significance of all the four *pgs* genes is still controversial. Some researchers have demonstrated the importance of *pgs B* and *pgsC* genes, whereas others have reported *pgsE* gene as nonessential [5, 16]. In the γ-PGA synthase complex, *pgsB* and *pgs C* together constitute the catalytic unit, whereas *pgsA*

is involved in the inclusion of L-glutamic acid monomers and further transporting the biopolymer to the cell membrane [47, 51, 52].

3.3 Peptidase

During starvation or other adverse conditions, some *Bacillus* species secrete peptidase enzymes such as gamma-glutamyl-transpeptidases (GGT) that are capable of cleaving γ-PGA with their intrinsic exo-hydrolase activity, thereby serving as a source of nutrients. To the downstream of *pgsBCA* operon is the *pgdS*gene, that codes for gamma-glutamyl-hydrolase, with the ability to degrade γ-PGA between the two glutamate residues. Another member of the GGT family, *capD*, has the dual functionality of anchorage as well as depolymerization by cleaving γ-PGA and relocating it to an acceptor molecule or water, aiding in transpeptidation or hydrolysis [6, 16].

4 Bacterial fermentation and the downstream process for γ-PGA production

For the efficient production of microbial biopolymer γ-PGA, the media components and cultivation conditions play a crucial role as they influence the yield and the characteristic of γ-PGA. Researchers have utilized different production media containing L-glutamic acid for enhanced production of γ-PGA. However, some strains produce γ-PGA in the medium devoid of L-glutamic acid due to their intrinsic L-glutamic acid-producing ability. Apart from media, fermentation conditions are also crucial for the maximum yield of biopolymer. To date, extensive research has been carried out for the production of γ-PGA by using different fermentation media, both synthetic as well as diverse wastes, which are summarized in Tables 2 and 3, respectively.

4.1 Metabolic engineering for the production of γ-PGA

We have discussed microbial strain and its optimized fermentation processes for improved production of γ-PGA. The production via recombinant strain is necessary for the high production of γ-PGA in the fermentation medium. Overexpression of native γ-PGA gene cluster (*pgsBCA*) in *Bacillus* strain is the primary strategy of metabolic engineering for γ-PGA production. To date, various metabolic engineering strategies have been employed for enhancing the γ-PGA production in the native and other recombinant strains [53]. A xylose-induced plasmid pWH1520, which harbors *the pgsBCA* gene cluster, was inserted into a γ-PGA deficient strain *B. subtilis* MA41 resulting in γ-PGA production of 9 g/L [54]. Similarly, insertion of *pgs*BCA in a strong promoter into *B. subtilis* DB430 resulted in 28 g/L γ-PGA production [55]; however, this approach is not always reproducible. The excision of hydrolase genes such as *ggt* (encoding gamma-glutamyltransferase) did not increase the γ-PGA yield; however, it resulted in increased molecular weight. In *B. subtilis* NAFM5, deleting the *cwlO* gene resulted in the overproduction of γ-PGA compared to control with an increase in molecular weight from 240 kDa to 490 kDa [56]. Similar results were obtained in the case of *B. amyloliquefaciens* NK-c with about 28 % enhanced γ-PGA production by deleting the *cwlO* gene. Furthermore, the production was increased to 93% compared to control by excising both *cwlO* and *pgdS* genes [57]. In a recombinant strain of *B. subtilis* PB5522, γ-PGA production of 40 g/L was achieved after double deleting the genes such as *pgdS* and *ggt* [58, 59]. This is so far the highest production of γ-PGA using the recombinant strains. A slight increase in γ-PGA production was observed in *B. amyloliquefaciens* NK-E10 after the deletion of the *luxS* gene by making the cell more tolerant to toxic by-products [60]. In *B. amyloliquefaciens* LL3 , wherein genes responsible for intracellular glutamate supply such as *gudB* and *rocG* were deleted, γ-PGA production of 5 g/L was obtained, which was about 38 % higher than control [61]. In *B. licheniformis* WX-02, the genes responsible for NADPH regeneration, such as *zwf*, were overexpressed, resulting in 35% higher γ-PGA yield than control. The production of various polysaccharides can act as contaminants and cause difficulty in the γ-PGA purification [62]. In *B. amyloliquefaciens* NK-E5, the genes responsible for the production of levan and lipopolysaccharides were excised, resulting in increased PGA purity from 78% to 95%, although the production remained the same as control. Similarly, in *B. amyloliquefaciens* NK-E7, the deletion of the genes responsible for by-product formation, such as *pta,* enhanced γ-PGA production by 11% [60]. In another study, genes related to antibiotic production such as *itu* and *srf* were deleted, aiding in 36% of the γ-PGA yield. The carbon flux was manipulated in *B.licheniformis* WX-02 by replacing glpFK promoter with different promoters, causing an increasing utilization of glycerol (~31% more) followed by enhanced γ-PGA production. The increase in the γ-PGA production results in increased broth viscosity, followed by a decrease in the oxygen mass transfer rate. This problem can be tackled by the insertion of bacterial hemoglobin gene such as the *vgb* gene. Insertion of *vgb* gene in *B. amyloliquefaciens* LL3 via a plasmid or chromosomal route enhanced γ-PGA production to about 30 % [63]. Further deleting the gene such as *mreB* responsible for maintaining cell morphology and cell wall synthesis elevated γ-PGA production to 57% [64]. Apart from *Bacillus* strain, several heterologous expression of *pgsBCA* genes was done in *Corynebacteriumglutamicumand Escherichia coli which* resulted in γ-PGA production in *C. glutamicum* with a yield of 0.7 g/L in a medium devoid of glutamic acid [53, 65, 66]. Recently, Gao et al. used different metabolic engineering strategies wherein they partly blocked metabolic pathways by deleting *fadR*, *lysC*, *aspB*, *pckA*,

Table 2 Production of γ-PGA by *Bacillus* strains using different synthetic medium

Bacillus strain	Major nutrients	Time (h)	Temp. (°C)	Yield (g/L)	Productivity (g/L/h)	Ref.
B. licheniformis A35	Glucose, ammonium chloride	120	30	11.1	0.09	[88]
B. licheniformis ATCC 9945A	Glutamic acid, glycerol, citric acid, NH_4Cl	141	37	17	0.12	[37]
B. licheniformis ATCC 9945a	Glutamic acid, glycerol, citric acid, NH_4Cl	96	37	20.5	0.21	[89]
B. licheniformis ATCC 9945A	Glutamic acid, glycerol, citric acid, NH_4Cl	28	37	23.2	0.82	[90]
B. licheniformis CCRC 12826	L-glutamic acid, Citric acid, Glycerol, NH_4Cl	96	37	19.8	0.2	[32]
B. licheniformis WBL-3	Glutamic acid, glycerol, citric acid, NH_4Cl	88	37	29.4	0.33	[35]
B. licheniformis SAB-26	Glucose, glycerol, citric acid	48	37	33.5	0.69	[42]
B. licheniformis NCIM 2324	L-glutamic acid, citric acid, glycerol,L-Glutamine, α-Ketoglutaric Acid,	96	37	35.75	0.37	[34]
*B. licheniformis*WX-02	Glutamic acid, glucose, citric acid, NH_4Cl	96	37	13.86	0.14	[36]
B. licheniformis A13	Glucose, yeast extract, NH_4Cl	72	37	28.2	0.39	[43]
B. licheniformis CGMCC3336	Glucose, sodium glutamate, yeast extract	34	37	35	1.02	[91]
B. licheniformis P-104	Glucose, sodium glutamate, sodium citrate	39	37	41.6	1.06	[92]
B. licheniformis NCIM 2324	Sugarcane juice, glutamate, yeast extract	40	35	36.5	0.91	[93]
B. licheniformis TISTR 1010	Glucose, citric acid, NH_4Cl	96	37	27.5	0.28	[45]
B.licheniformis CGMCC 2876	Tri-sodium citrate, glucose, L-glutamic acid, glycerol, NH_4Cl	20	37	21.8	1.09	[33]
B. licheniformis TISTR 1010	Glucose, citric acid, tween-80, NH_4Cl	43	37	39.9	0.92	[44]
*Bacillus licheniformis*A14	Glucose, citric acid, monosodium glutamate	48	37	37.8	0.78	[94]
*Bacillus licheniformis*LMG 7559	Sodium glutamate, glucose, $(NH_4)_2SO_4$	72	37	0.5	0.0069	[95]
B. subtilis IF0333	L-glutamic acid, citric acid, ammonium sulfate	40	37	10.04	0.25	[19]
B. subtilis F-2-01	Veal infusion broth, glucose, L-glutamic acid	94	37	48	0.51	[96]
B. subtilis TAM-4	Fructose, ammonium chloride	96	30	22.1	0.23	[39]
B. subtilis MR-141	Sodium glutamate, maltose, soy sauce	96	40	35	0.36	[26]
B. subtilis var. chungkookjang	Sucrose, $(NH_4)_2SO_4$, L-glutamic acid	120	30	13.5	0.11	[49]
B. subtilis IFO 3335	Glycerol, citric acid, glutamic acid	30	37	23	0.76	[97]
B. subtilis C1	Glutamic acid, glycerol, citric acid, NH_4Cl	144	37	21.4	0.14	[98]
B. subtilis NX-2	Glucose, L-glutamic acid, yeast extract	24	37	30.2	1.25	[99]
B. subtilis ZJU-7	Sucrose, tryptone,L-glutamic acid	24	37	58.2	2.42	[100]
B. subtilis NX-2	Glucose,glutamic-acid,yeast extract	48	32.5	28.42	0.59	[31]
B. subtilis CGMCC 0833	Glucose, glutamate, $(NH_4)_2SO_4$, Tween 80	48	32.5	34.4	0.71	[16]
B. subtilis ZJU-7	Glutamic acid, glucose, tryptone	64	37	54	0.84	[101]
B. subtilis NX-2	L-glutamate, glucose, NH_4Cl	120	32.5	42	0.35	[102]
B. subtilis NX-2	Glucose, glutamate, glycerol	48	32.5	31.7	0.66	[103]
*B. subtilis*natto ATCC15245	Glutamic acid, sucrose, ammonium sulphate	96	37	28	0.29	[25]
B. subtilis CGMCC1250	Glucose, glutamate, yeast extract	46	37	101	2.19	[24]
B. subtilis NX-2	Glucose, glutamate, $(NH_4)_2SO_4$	72	32	40.5	0.56	[104]
B.subtilis R 23	Glucose,citric acid, L-glutamic acid, NH_4Cl	48	37	25.38	0.52	[18]
B. subtilis 2063	Glucose, citric acid, L-glutamic acid, NH_4Cl	96	37	21.42	0.22	[105]
B.subtilis D7	L-glutamate, glucose. Yeast extract	72	30	4.7	0.06	[17]
B. subtilis D7	Glutamic acid, mannitol, yeast extract	36	35	24.93	0.69	[106]
B. subtilis MJ80	L-glutamic acid, glycerol, citric acid, starch,urea	72	37	68.7	0.95	[21]
B. subtilis NX-2	Glucose, glutamate, $(NH_4)_2SO_4$	57	32	71.21	1.24	[107]
*B. subtilis*BL53	Glutamic acid, glycerol, citic acid, NH_4Cl	24	37	23.5	0.97	[22]
B. subtilis GXG-5	Glucose, ammonium nitrate	34	50	19.5	0.57	[41]
B. methylotrophicus SK19.001	Glycerol,sodium citrate, peptone	66	37	35.34	0.53	[108]
B. methylotrophicus	mannitol, monosodium glutamate, peptone	96	37	36	0.37	[38]
B. amyloliquefaciens LL3	Sucrose,$(NH_4)_2SO_4$	44	37	4.36	0.09	[60]
B. velezensis NRRL B – 23189	Molasses, citric acid, ammonium sulphate	72	27	4.82	0.06	[109]
Bacillus. sp. RKY3 KCTC 10412BP	Glutamic acid, glycerol, citric acid, NH_4Cl	48	38	28.4	0.59	[110]
Bacillus.sp. RKY3 KCTC 10412BP	Glutamic acid, glycerol, citric acid, NH_4Cl	24	38	48.7	2.02	[111]
Bacillus siamensis SB1001	Sucrose, L-glutamic acid, NH_4Cl	24	37	25.22	1.02	[112]
*B. sonorensis*44	Glycerol, yeast extract, α-ketoglutaric acid	72	30	11.84	0.16	[113]

proAB, *rocG*, and *gudB*. Furthermore, the srf and itu operons were deleted, and the NADPH level was improved by regulating the expression of *pgi* and *gndA*. The final engineered strain NK-A6 yielded 7.5 g/l γ-PGA, with a twofold increase

Table 3 Economical production of γ-PGA using different wastes

Bacillus strain	Wastes	Additional nutrient	Time (h)	Temperature (°C)	Yield (g/L)	Productivity (g/L/h)	Ref.
B. subtilis CCTCC202048	Swine manure, soy bean cake	Glutamic acid, citric acid	48	37	45	0.9	[11]
B. subtilis NX-2	Cane molasses	Glutamate, $(NH_4)_2SO_4$	96	32	51.1	0.53	[27]
*B. subtilis*HB-1	Corncob fibrehydrolysate	L-glutamate and yeast extract	40	37	24.92	0.62	[30]
B. subtilis NX-2	Rice straw hydrolysate	Glutamate, $(NH_4)_2SO_4$	90	32	73	0.81	[29]
*B. subtilis*ZC-5	chicken manure	Crude extract of glutamic acid, sodium glutamate waste liquor	48	37	65.7	1.3	[20]
B. subtilis A3	fish meal wastewater	glucose, L-glutamate	48	37	25	0.52	[23]
B. licheniformis 9945a.	Goose feather hydrolysate	L-glutamate, tri-sodium citrate dihydrate, glycerol	48	30	5.4	0.11	[8]
B. licheniformis WX-02	Paper waste hydrolysate	Glucose, sodium glutamate, sodium citrate	36	37	6.46	0.17	[67]
B. sp. SJ-10	Macroalgae (Ulva)	Sucrose, L-glutamate	48	37	6.29	0.13	[9]
B. amyloliquefaciens C1	Dairy manure compost, monosodium glutamate production residues	citric acid	48	37	60	1.25	[12]
B. *amyloliquefaciens* JX6	Corn stalk, Soybean meal	Industrial monosodium glutamate	72	37	112.82	1.56	[114]
*B. siamensis*IR10	molasses	L-Glutamic acid, NH_4Cl	20	37	45.42	2.02	[115]

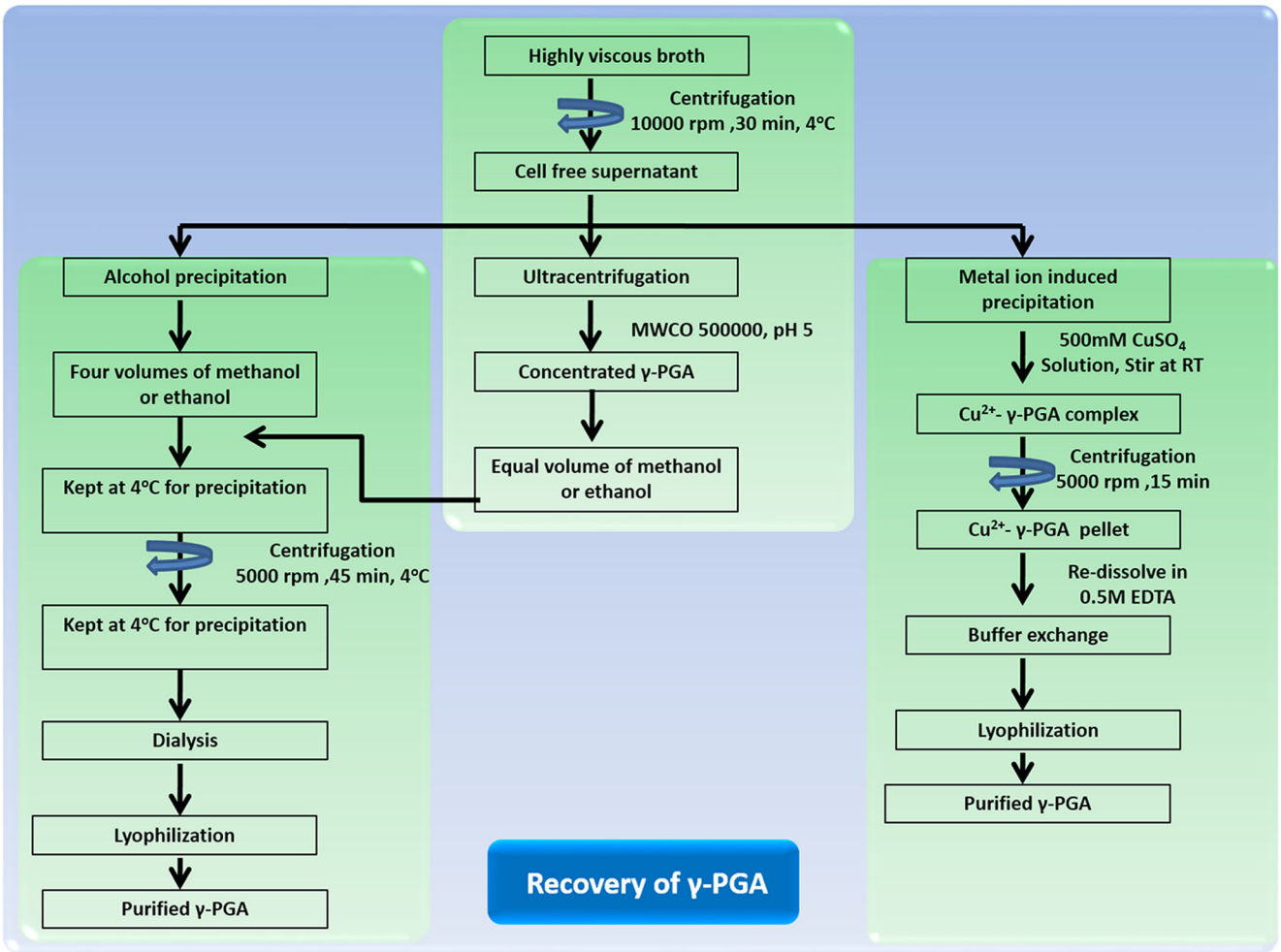


Fig. 3 Methods for efficient recovery of γ-PGA [68]

yield in comparison to *B. amyloliquefaciens* LL3 strain [46]. Despite all these metabolic engineering approaches, wild-type strains remain the potent and highest producers for γ-PGA.

4.2 Downstream process for recovery of γ-PGA

The downstream process is one of the main challenges for the scale-up of γ-PGA production with high purity. Various downstream processes are crucial for the recovery and characterization of γ-PGA synthesized in the fermentation medium. For efficient recovery of γ-PGA, three different strategies have been executed: precipitation (alcohol and metal ion-induced) and ultrafiltration (Fig. 3).

4.2.1 Precipitation

The precipitation process is the conversion of desired products (chemical substance) into a solid form or in crystals from a solution by converting the substance into an insoluble form. Alcohol precipitation is a standard method used for γ-PGA recovery. Alcohols such as methanol or ethanol are usually used for the precipitation of γ-PGA from the fermented broth. In this method, the fermentation broth is pellet down (centrifuged at 10,000 rpm for 30 min). Furthermore, four volumes of ice-cold methanol or ethanol are added to the cell-free supernatant and kept at 4°C for 12 h [34, 42]. In the final step, centrifugation (5000 rpm for 45 min) is carried out to obtain crude γ-PGA, followed by dissolving it in distilled water. The aqueous γ-PGA solution is desalted by dialysis (Mw 14 kDa cut-off) with multiple water exchanges for 12 h, and finally purified γ-PGA is achieved by lyophilization [34].

The other precipitation method is metal precipitation, where different metal ions such as $CuSO_4$, $FeCl_3$, $AlCl_3$, and $MnSO_4$ were used and optimized for γ-PGA precipitation in aqueous solution as well as from the fermented broth. In an aqueous solution, the addition of $CuSO_4$ formed a pale bluish-green precipitate with an optimum concentration of 50 mM. In the case of fermented broth supplemented with 50 mM $CuSO_4$ solution, no precipitation was observed; however, at 500 mM concentration, up to 95 % recovery was obtained. The tenfold difference in the concentration of $CuSO_4$ for precipitation may be because of other metal ions present in the fermentation broth that may bind to the anionic sites of γ-PGA. The Cu^{2+}-γ-PGA complex so formed is collected by centrifugation and re-dissolved in EDTA, followed by water exchange and lyophilization to achieve purified γ-

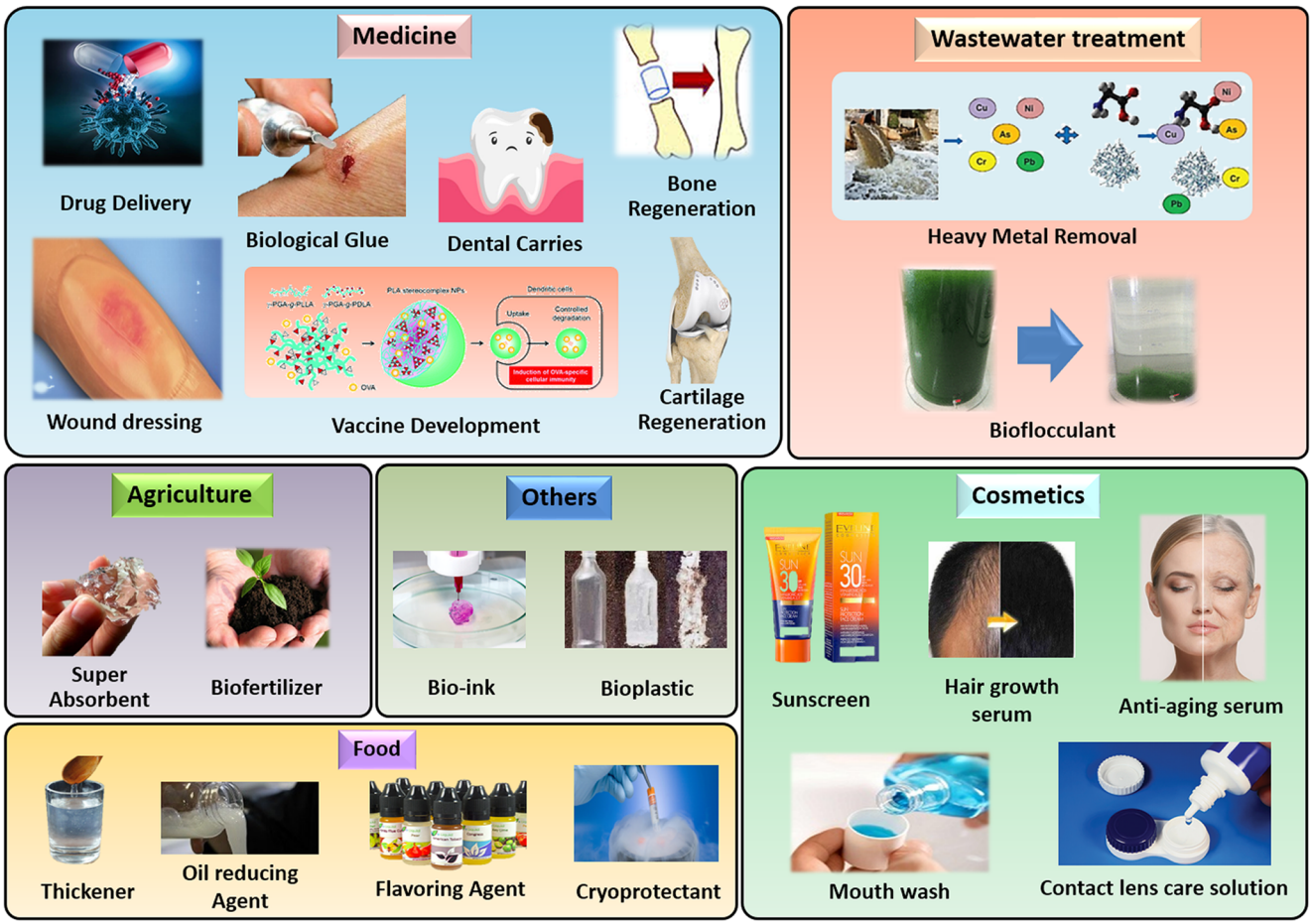


Fig. 4 Various γ-PGA products with potential industrial applications [6, 72]

Table 4 Application of γ-PGA in various fields

Field	Application	Description	Ref.
Agriculture	Super-absorbent	To enhance the water holding capacity, improve seed growth, the yield of cottonseed, increase the boll numbers per plant, length of cotton fibre, stem diameter, plant height.	[116]
		To retain water in the soil for a longer period, reduce the soil water infiltration.	[117]
	Biofertilizer	Boost fertility of the soil, increase utilization of essential minerals, enhance plant growth-promoting bacteria.	[118]
Wastewater treatment	Heavy metal removal	Ability to remove $Cr^{3+,}$ $Cu^{2+,}$ $Pb^{2+,}$ and Ni^{2+} at acidic pH using novel magnetic nanoparticles comprising of γ-PGA and Fe_3O_4.	[119]
		γ-PGA as a cation exchanger for removal of mercury.	[120]
		Nano-membrane technology consisting ofγ-PGA nanoparticles and ultrafiltration techniques for the effective removal of lead contaminants.	[121]
		Soil washing with γ-PGA can remove 56.5%,53.1%,50%,74.3% of Ni, Zn, Cr and Cu, respectively, from contaminated sites.	[122]
		Enhanced removal of cesium from radioactive wastewater using γ-PGA.	[123]
	Bio-flocculant	γ-PGA at 0.8ppm shows high flocculating ability,so it can replace polyacrylamide from sugarcane industry.	[33]
Medicine	Wound healing	Scaffold comprising of γ-PGA and PLA could efficiently accelerate re-epithelialization by infiltration of fibroblast and keratinocytes.	[124]
		Ag/ γ-PGA composite showed accelerated wound closure by preventing moisture evaporation and better water absorption.	[125]
		A versatile γ-PGA/gelatin hydrogel crosslinked with proanthocyanidins have shown to boost wound recovery by its swelling, antioxidant and fibroblast growth properties.	[126]
		γ-PGA/chitosan polyelectrolytic complex could reduce inflammation and enhance epithelial cell reestablishment, thereby effective healing.	[127]
		γ-PGA as an attractive alternative to hyaluronic acid in corneal wound closure.	[128]
	Dental caries	Promotes re-mineralization for protection of enamel.	[129]
	Bone regeneration	Electrospunfibres of γ-PGA/ β–tricalcium phosphate can be an interesting replacement material for bone regeneration as it exhibits high cell adhesion, osteogenesis, alkaline phosphatase activity aiding in bone deformity repair.	[130–132]
	Cartilage regeneration	Microsphere developed from γ-PGA/chitosan showed propagation and attachment of chondrocytes forming fibrous tissue.	[131]
		γ-PGA hydrogel encapsulated mesenchymal stem cell could re-establish cartilage in auricular defective rabbit.	[133]
	Rheumatoid arthritis (RA)	A unique nano-drug carrier was developed using γ-PGA, which could reduce the toxic nature of triptolide, thereby improving its efficacy for RA treatment.	[134]
		γ-PGA can quench osteoclastogenesis as well as decrease the destruction of bones in a mouse model	[135]
	Drug delivery	A nanoconjugate derived from γ-PGA and cisplatin exhibited improved antitumor activity against liver and breast cancer with reduced toxicity both in vivo and in vitro.	[136, 137]
		A self-assembling nanoparticle comprising of γ-PGA and chitosan could be used to deliver anticancer drug with high efficacy in ovarian cancer cells.	[138]
		A unique thermos-responsive microgel derived from γ-PGA/hydroxypropyl cellulose for oral delivery of insulin in a controlled manner.	[139]
		A light-inducible hydrogel based on γ-PGA/collagen could be used for controlled release of DOX, serving as an effective platform for drug delivery.	[140]
		A novel oral delivery platform for anti-diabetes was developed consisting of γ-PGA conjugated with an inhibitor of Na+/glucose cotransporter(phloridzin), which could decrease the hyperglycemic effect with high efficacy.	[141]
	Vaccine	Nanoparticle derived from γ-PGA has the potential to evoke cellular and humoral immunity serve as the basis of vaccination for the treatment of HIV, various cancers, Japanese encephalitis virus.	[72]
	Biological glue	γ-PGA has been used as an attractive substitute for fibrin glue for sealing air leakage after lung surgery.	[142]
Others	Food packing biomaterial/Bioplastic	Chemical modification of γ-PGA to form bioplastic with excellent properties such as resistant to acid and alkali, antimicrobial activity, hydrophobic and biodegradability.	[143, 144]
		Composite of γ-PGA /Poly lysine can be a suitable probiotic carrier with application in edible food packaging materials. Chemical modification of γ-PGA to form bioplastic with excellent properties such as resistant to acid and alkali, antimicrobial activity, hydrophobic and biodegradability.	[145]
	Bio-ink	γ-PGA/chitosan bio-ink has a remarkable cell survival rate inside3D bio-printed hydrogel.	[146]
Cosmetics	Sunscreen	-PGA formulation in next-generation sunscreens for protection against UVA and UVB.	[147]

Table 4 (continued)

Field	Application	Description	Ref.
	Mouthwash	γ-PGA can be used in mouthwash formulations as a replacement for alcohol since it is non-irritable, nontoxic and can inhibit *E.coli*, *S.aureus* with high efficacy.	[148]
	Hair growth promoter	γ-PGA with UHMW has shownto induce hair growth by acting on the anagenic stage of the hair growth cycle and suppressing the 5-alpha reductase activity.	[149]
		Nano-formulation based on γ-PGA hydrogel and herbal extract can enhance the size of the hair bulb and effectively deliver growth metabolites to the hair follicles.	[150]
	Contact lens care solution	γ-PGA as an effective cleaning agent of ionic contact lenses with lubricating property.	[151]
Food	Oil reducing agent	Oil uptake was reduced by five folds in doughnuts containing 0.2 g of γ-PGA/ g dough and also had a better appearance and taste than regular doughnuts.	[34]
	Thickener	Increases viscosity in fruit juices and sport drinks.	[5]
	Bitterness relieving agent	The addition of γ-PGA to compounds having a bitter taste such as caffeine relieved its bitterness.	[152]
	Cryoprotectant	Act as a better antifreeze agent than sucrose in protecting probiotic bacteria.	[153]

PGA. The recovery percent of γ-PGA using metal ion-induced precipitation is 85% compared to alcohol precipitation, 82%. Similarly, the co-precipitation of proteins is just 3% with this strategy compared to 50% using alcohol precipitation, indicating its higher selectivity [5, 67, 68].

4.2.2 Ultrafiltration

Another effective recovery approach for γ-PGA is ultrafiltration, which reduces the use of a solvent in the downstream process. It can retain macromolecules in the solution with the help of hollow fiber membranes aiding in concentrating high molecular weight γ-PGA [69, 70]. In this process, the γ-PGA was centrifuged (10000 rpm for 30 min), and the cell-free supernatant was then concentrated using ultracentrifugation. At the mol. wt. Cut-off (MWCO) of the membrane 30 to 100 kDa, the loss of γ-PGA was negligible, but the flow rate was low. However, with MWCO of 500 kDa, the flux was comparatively higher, with only 3% loss in γ-PGA. For ultrafiltration, the pH plays a crucial role in concentrating the cell-free supernatant with a considerable loss at lower pH due to a change in the conformation of γ-PGA. At lower pH, the hydrodynamic radius of γ-PGA decreases due to the change from random coil structure to helical form, enhancing the membrane permeability. Therefore, at pH 3, the concentrate had 110 g/L of γ-PGA, whereas, at pH 5, the value decreased to 60 g/L. With this strategy, the amount of alcohol utilized for γ-PGA precipitation can be significantly reduced [5, 71].

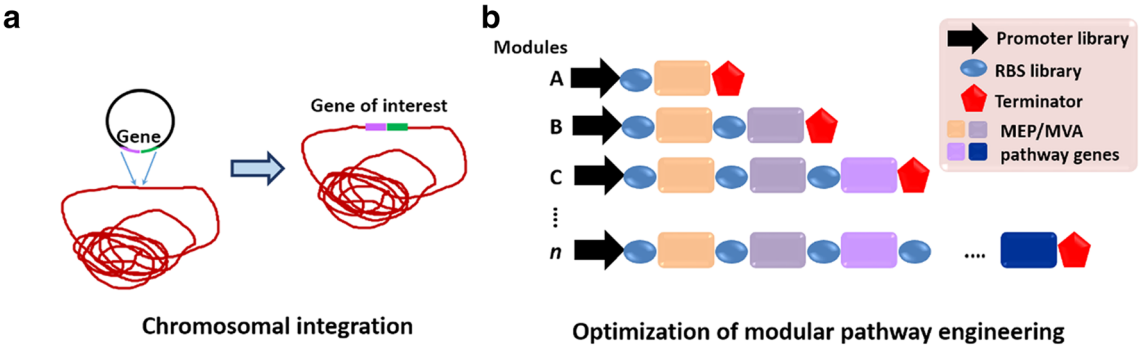
5 Application of γ-PGA

Due to the remarkable properties of γ-PGA, it has been chosen as a suitable candidate for applications in various fields as medicine (bone regeneration, dental carries, wound dressing, drug delivery, vaccine development, bio-glue), agriculture (biofertilizer, super-absorbent), wastewater treatment (bio-flocculants, metal removal), cosmetics (anti-ageing,mouth wash, sunscreen lotion), food industries (flavouring agent, thickener, packaging material), etc. as summarized in Fig. 4 and Table 4 [6, 72].

6 Challenges and strategies in the commercialization of γ-PGA

Recently, the interest in the wider commercialization of γ-PGA has been gaining immense attention due to its phenomenal properties and its use in diverse applications. Since *Bacillus* species are the most potent producers of γ-PGA, it is a boon for biotechnological industries as most of the *Bacillus* species are considered as GRAS (Generally Regarded As Safe) organisms [73]. The major bottleneck in γ-PGA production is the high cost associated with the media components. A probable solution is to reduce the overall production cost by using waste that can replace all the nutrients required for γ- PGA biosynthesis [29]. As there is enormous potential in India and European countries for the valorization of surplus waste, we can direct the waste for economic and

Fig. 5 Microbial genome editing tools for γ-PGA production: A) Chromosomal integration for inserting new genes in the microbial genome. B) Optimization of the metabolic pathway by inserting various combinations of modules. C) Cross-lapping-in vitro assembly (CLIVA) for inserting multiple modules in vector. D) CRISPR/Cas9-mediated genome editing. E) CRISPRi/CRISPRa-mediated transcriptional regulation of genes used for the downregulation(interference) or upregulation (activation) of specific genes. F) Eukaryotic MultiplexAutomated genome engineering (eMAGE) used for achieving specific chromosomal mutations with high efficiency. G) RNAi-mediated transcriptional regulation tool used to cleave specific mRNA sequences [82, 86, 87]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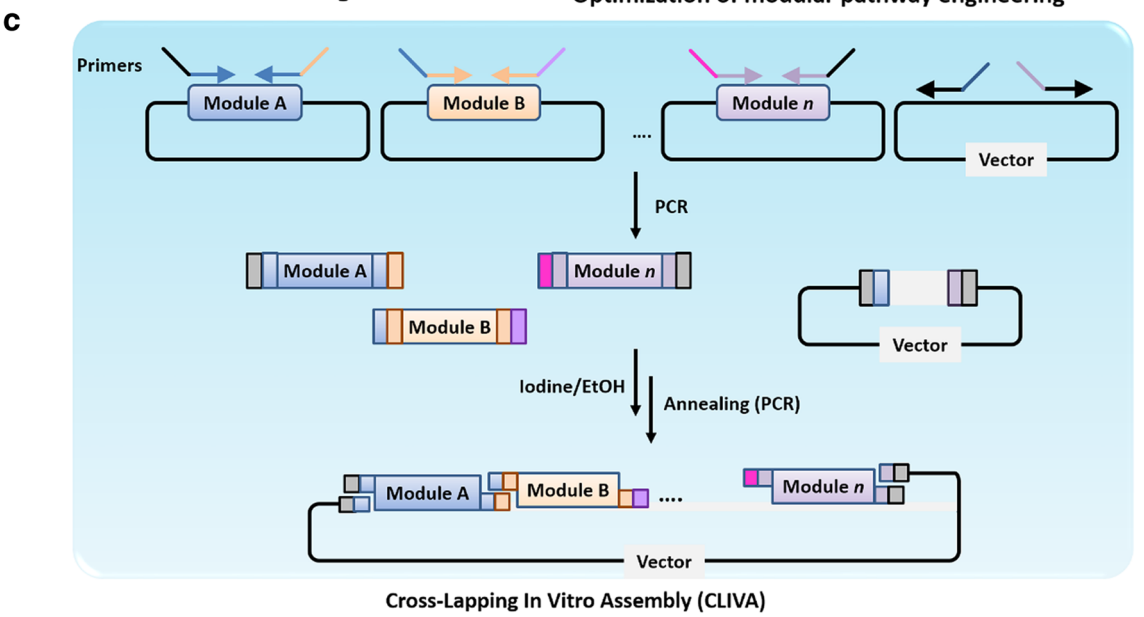
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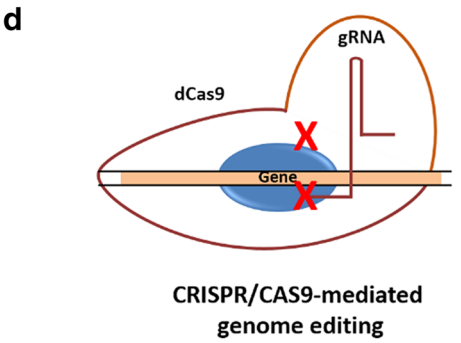
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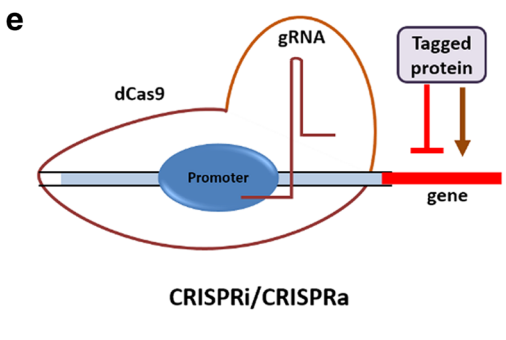
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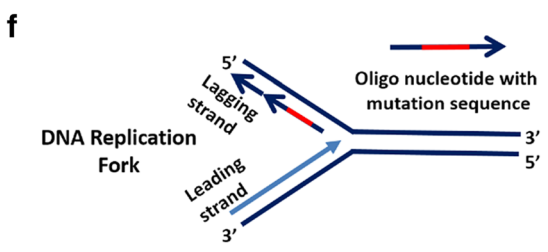
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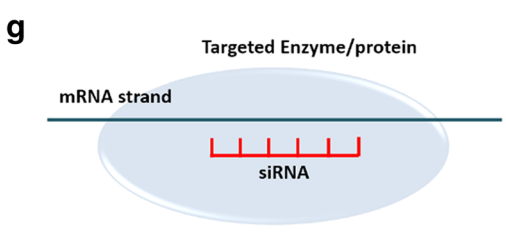
g
Targeted Enzyme/protein
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feasible production of γ- PGA. However, the waste selected for γ- PGA production must be accessible throughout the year to keep the process technology in place. Extensive research has been conducted to reduce the production cost of γ-PGA, such as the use of lignocellulosic biomass, waste manure, macroalgae, goose feathers, and fishmeal wastewater (Table 3) [5, 23]. Excessive accumulation of such waste will have a negative environmental impact, and thus it is essential to valorize this surplus waste into a value-added commodity, thereby strengthening our economic sustainability. However, these wastes require either acid/alkali pretreatment or supplementation with additional nutrients to be used in the production medium, further adding to the cost [74]. Thus, there is an utmost need for a waste that is multi-nutritious and does not require any pretreatment. Such a multi-nutritious waste medium that can serve as a source of carbon, glutamic acid, and nitrogen without the supplementation of any additional nutrients and pretreatment will help in reducing the overall cost of the process [75]. Another way to reduce the production cost is the implementation of a mixed culture strategy. The fermentation involving mixed culture has several advantages over pure culture, including higher yield as well as efficient and better utilization of diverse wastes. *B. subtilis* have been co-cultured with *C.glutamicum* using glucose and sucrose as a mixed carbon source, thereby shortening the fermentation time and reducing the production cost [76]. The production cost of γ-PGA using L-glutamic acid-dependent strains are higher as compared to L-glutamic acid independent strains due to external supply of L-glutamic acid in the fermentation medium. The strains of *C.glutamicum* are well-known for the production of glutamic acid from different wastes. Thus, in the case of L-glutamic acid-dependent strains, a stable association between the strains of *C.glutamicum* and *Bacillus* species could be used for enhanced γ-PGA production in the fermentation medium devoid of glutamic acid [15, 66, 77, 78]. The cost of γ-PGA is solely based on the cost of the production media as well as the productivity of the γ-PGA producing strains [79]. The lower the productivity of γ-PGA, the higher will be the cost of production, ultimately resulting in increased cost of γ-PGA. Thus, lower production, as well as productivity of γ-PGA, is another hurdle that can be addressed by goal-based screening for γ-PGA producers. For example, isolation and screening of osmophilic strains that could produce γ-PGA at higher sugar concentration will aid in higher production and productivity in a batch fermentation [5]. Similarly, extremophilic bacteria such as halophiles can be explored for their ability to produce γ-PGA. Since the presence of NaCl is known to reduce the molecular weight of γ-PGA [36], it would be worth exploring the characteristic of γ-PGA produced by different halophiles at a high salt concentration. Their ability to grow at higher salt concentrations will also reduce the risk of contamination in non-sterilized fermentation media. On the other hand, hyperthermophilic strains can also be screened for γ-PGA production to extract γ-PGA with higher thermo-stability [80]. The productivity of γ-PGA further can be increased by metabolic engineering strategies. The advancement in emerging tools, knowledge, and technologies in synthetic biology and protein engineering can be combined to develop combinatorial engineering strategies to boost carbon flux for the microbial production of γ-PGA. For achieving a higher titre of γ-PGA, new enzyme screening, novel pathway discovery, conjugation of multiple pathways from district systems could be used. The traditional homologous recombination and chromosomal integration techniques, the modular control systems [81–83], the pathway assembly methodsCross-Lapping-In Vitro assembly (CLIVA) [84], and the newly emerging techniques/tools for gene replacement/editing *e.g.*CRISPR/CAS9 [85] (Fig. 5) would boost the developments in the biosynthesis of γ-PGA in native and heterologous host systems. The cell growth pattern can be manipulated by excising genes involved in binary fission such as *micC* and *minD* resulting in multiple fission. This approach will increase the biomass concentration within a short period, thereby reducing the time required for the maximum production of γ- PGA, ultimately increasing the productivity [53]. Similarly, the genes involved in the byproduct formation, such as polysaccharides, can be knocked out by biotechnological tools such as CRISPR/CAS9 [85]. This will direct the carbon flux entirely towards γ- PGA biosynthesis, thereby increasing the γ- PGA production with high purity.

7 Conclusion and future prospects

In conclusion, γ-PGA is a naturally occurring biopolymer made up of repeating units ofL/D-glutamic acid, or both. Due to its biodegradable, edible, non-immunogenic, non-toxic properties, it has been applied in various industrial sectors. Since its discovery in 1937, to date, remarkable research has been carried out by different research groups around the globe. Recently, there is an increase in demand for the use of biopolymers worldwide, and they are on the verge of replacing conventional petro-based polymers. However, the main drawback that prevents the commercialization of biopolymers is the price when compared to conventional counterparts, which remains the main ultimatum for biopolymers. The γ-PGA is one of the most expensive biopolymers with diverse applications. Even though there are potent γ-PGA-producing strains available, the cost of production and recovery remains high. Therefore, the screening of potential γ-PGA producers using a low-cost medium is an upcoming need to reduce the overall production cost. This issue can be addressed by using a multi-nutritious waste that can replace all the essential nutrients required for γ-PGA biosynthesis. To achieve this, one should have in-depth knowledge about the various nutritional elements that influence the γ-PGA production. The future

research can be focused mainly on exploring various types of fruit and vegetable wastes that could be used directly as the fermentation substrate without supplementation of additional nutrients. These wastes are multi-nutritious, containing organic acids, amino acids, and even high sugar levels, and do not require any pretreatment step. Since they have a short shelf life, a large amount of waste will also be generated, which can be valorized to γ-PGA resulting in waste valorization and economical production of γ-PGA. This will ultimately lead to the circular economy, thereby awakening this sleeping giant for its broader commercialization.

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Declarations

Conflict of interest The authors declare no competing interests.

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