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Research review paper

## Building a circular economy around poly(D/L- $\gamma$ -glutamic acid)- a smart microbial biopolymer

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## ABSTRACT

Bio-derived materials have long been harnessed for their potential as backbones of biodegradable constructs. With increasing understanding of organismal biochemistry and molecular genetics, scientists are now able to obtain biomaterials with properties comparable to those achieved by the petroleum industry. Poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) is an anionic pseudopolypeptide produced and secreted by several microorganisms, especially *Bacillus* species.  $\gamma$ -PGA is polymerised via the pgs intermembrane enzymatic complex expressed by many bacteria (including GRAS member - *Bacillus subtilis*).  $\gamma$ -PGA can exist as a homopolymer of L- glutamic acid or D- glutamic acid units or it can be a co-polymer comprised of D and L enantiomers. This non-toxic polymer is highly viscous, soluble, biodegradable and biocompatible.  $\gamma$ -PGA is also an example of versatile chiral-polymer, a characteristic that draws great attention from the industry. Increased understanding in the correlation between microbial genetics, substrate compositions, fermentation conditions and polymeric chemical characteristics have led to bioprocess optimisation to provide cost competitive, non-petroleum-based, biodegradable solutions. This review presents detailed insights into microbial synthesis of  $\gamma$ -PGA and summaries current understanding of the correlation between genetic makeup of  $\gamma$ -PGA-producing bacteria, range of culture cultivation conditions, and physicochemical properties of this incredibly versatile biopolymer. Additionally, we hope that review provides an updated overview of findings relevant to sustainable and cost-effective biosynthesis of  $\gamma$ -PGA, with application in medicine, pharmacy, cosmetics, food, agriculture and for bioremediation.

### 1. Introduction

Poly(D/L- $\gamma$ -glutamic acid) ( $\gamma$ -PGA) is an extracellularly secreted protein-like polymeric material synthesised by an array of Prokaryotic and Eukaryotic organisms from glutamic acid monomers (Ashiuchi, 2010; Ogunleye et al., 2015; Luo et al., 2016). The first identification of  $\gamma$ -PGA occurred in 1921, when Kramar and co-workers associated the immunoinvisibility of *Bacillus anthracis* to the cell wall bound  $\gamma$ -D-PGA predominant capsule (Housewright and Thorne, 1950) (Shih and Van, 2001) (Ashiuchi, 2010). Although of similar physico-chemical properties to *B. anthracis*  $\gamma$ -D-PGA,  $\gamma$ -DL-PGA synthesised by other organisms (see Table 2) may serve very different biological functions (da Silva Filho et al., 2020).

Most Prokaryotic and Eukaryotic  $\alpha$ -polypeptidic material is of mRNA

origin and translated by ribosomes (see Fig. 2) (Lehninger et al., 2008). In contrast to other polypeptides, the biosynthesis of  $\gamma$ -PGA occurs through an enzymatic complex, responsible for the polymerisation of both D- and L- glutamic acid monomers (see Fig. 1 and Fig. 2) (Ashiuchi, 2010). By employing an enzymatic complex, producers can greatly alter the properties of the polymer in response towards changing environmental conditions.

Currently, numerous organisms have been identified as  $\gamma$ -PGA producers, however an array of differences have been evidenced amongst producers (Zeng et al., 2017). Glutamic acid dependant producers require exogenous glutamic acid to then polymerise into  $\gamma$ -PGA chains; conversely, glutamic acid independent producers are able to convert other sugars/amino acids into glutamic acid –intracellularly- and then polymerise it into  $\gamma$ -PGA chains (Mabrouk et al., 2012; Ito et al., 1996;

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Zeng et al., 2017). Enzymatic complexes and microorganismal biochemistry which allows these processes to operate have been further described by Ashiuchi (2010) (see Fig. 1). Although  $\gamma$ -PGA biosynthesis occurs within an intracellular membrane complex, producers further divide into  $\gamma$ -PGA secretors or cell wall-bound producers (see Fig. 3) (da Silva Filho et al., 2020). The former attach polymeric chains through a 'capD/capE' protein whereas the latter cleave polymeric chains through the pgdS enzyme (Candela and Fouet, 2006; Buescher and Margaritis, 2007; Ogunleye et al., 2015; da Silva Filho et al., 2020).

This difference in polymer chemical composition allows microbial  $\gamma$ -PGA producers to polymerise monomeric units into  $\gamma$ -bound chains with reduced susceptibility towards enzymatic biodegradation (Nabi and Das, 2015; Moradali and Rehm, 2020).

Given that the biosynthesis of  $\gamma$ -PGA is regulated by enzymatic activity (Leonard et al., 1958; Leonard and Housewright, 1963) (see Fig. 1 & Fig. 2), it is incredibly challenging to obtain intra and inter-batch reproducibility (Pereira et al., 2012). Most of the variability revolves around the molar mass of the polymeric chains, D/L ratio variability and variable chain association with salts. As (Pereira et al., 2012) states; difficulty in achieving homogeneous polymeric chains from bacterial cultures is a result of both the material's instability and its molecular complexity. Each one of these variation can significantly impact chain physical arrangement and ultimately polymeric behaviour. In nature, the variation in polymeric chemical structure, imparts improved survival during challenging conditions, including: nutrient shortage (Kimura, 2004; Moradali and Rehm, 2020), high salt (Hezayen et al., 2001) and heavy metal (McLean et al., 1990; Kaplan, 1998; Deol et al., 2022) concentrations.

Although challenging to control, the high variation in chemical properties that can be achieved makes this material very attractive for the industry. For instance, material chemical variability enables polymer use as antifreeze (Bhat et al., 2013), thickener (Shyu et al., 2008;

Shyu and Sung, 2010), food and feed additive (Tanimoto et al., 2001; Ho et al., 2009; Tanimoto, 2010), as a filtration membrane (Bhattacharyya et al., 1998; Hajdu et al., 2012), as a bioremediation agent (Chang et al., 2013; Inbaraj et al., 2006; Bai et al., 2022; Deol et al., 2022; Sakamoto and Kawase, 2016), humectant (Choi and Kunioka, 1995), as medical material (Ye et al., 2006), for cosmetics (Ben-Zur and Goldman, 2007), as thermoplastic (Borbely et al., 1994), and for pharmaceutical applications (Hsieh et al., 2005; Hsieh et al., 2006; Kurosaki et al., 2010; Uotani et al., 2011; Bhat, 2012; Hsueh et al., 2017; Khalil et al., 2018). At present, the majority of commercially available  $\gamma$ -PGA is synthesised by members of the *Bacillus* species (GRAS organisms).

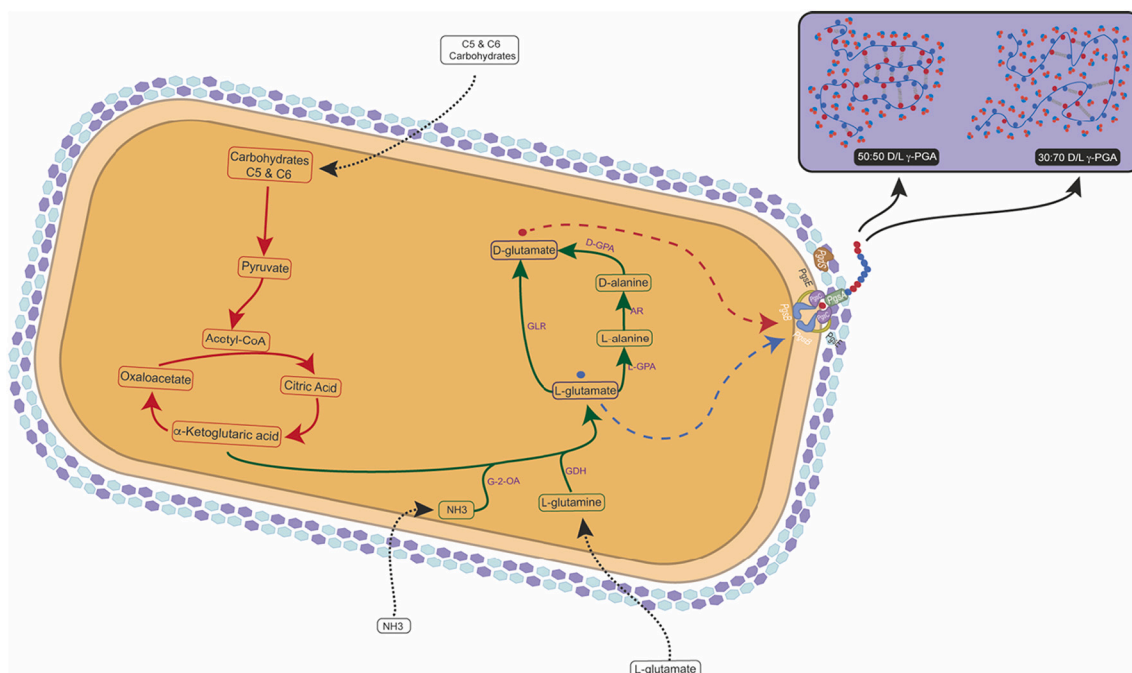
Provided the enormous potential of  $\gamma$ -PGA, this review paper aims to deliver a comprehensive summary of  $\gamma$ -PGA's biosynthesis, the association of fermentation conditions to chemical structures, the physical behaviour of  $\gamma$ -PGA with varying chemistry, potential cost optimisation strategies for  $\gamma$ -PGA biosynthesis, current and potential applications of  $\gamma$ -PGA as a cost-effective alternative to non-sustainable polymers.

Although up-to-date reviews have been published on the topic of poly- $\gamma$ -glutamic acid, this paper focuses on understanding the correlation between microorganismal genetics, substrate compositions, fermentation conditions and polymeric chemical characteristics with a commercial outlook. This is significantly different from current reviews which focus more on exploiting genetic engineering methods in order to obtain high-yield  $\gamma$ -PGA producers. With this review we hope to bring practical background for the biosynthesis of this sector a-specific, biomaterial.

## 2. Poly(glutamic acid) synthesis

### 2.1. Chemical synthesis of poly-glutamic acid

Poly-glutamic acid exists in two main isoforms: poly( $\alpha$ -glutamic



**Fig. 1.** Factors, actors and pathway involved in the biosynthesis of poly ( $\gamma$ -glutamic acid) ( $\gamma$ -PGA). Herein, the metabolic pathway (in red) of  $\gamma$ -PGA producers has been summarised. In red, the energy generation components have been shown, similarly,  $\gamma$ -PGA generation intermediates/components have been presented in green. The figure also illustrates polymerisation of D- and L- glutamic acid monomers (red and blue dots respectively) into a polymeric chain by the enzymatic complex. The physical arrangement of the polymer -with varying D/L ratios- in solution has also been summarised. The genetic fragments responsible for  $\gamma$ -PGA's enzymatic complex synthesis have been reported within Fig. 3. Light blue and purple hexagons represent cross-linked NAM & NAG moieties of Gram-positive peptidoglycan. G-2-OA- glutamate-2-oxaloglutarate aminotransferase; GDH- Glutamate dehydrogenase; L-GPA- L-glutamic acid:pyruvate aminotransferase; AR- alanine racemase; D-GPA- D-glutamic acid:pyruvate aminotransferase; GLR- glutamate racemase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid) and poly( $\gamma$ -glutamic acid). Interestingly, both chemical and biological methods can be employed to synthesise poly-glutamic acid. Poly ( $\alpha$ -amino acids) have numerous applications due to their biodegradable properties and biocompatibility (Kino et al., 2011; Sanda et al., 2001).

In this respect, Kino et al. (2011) found a novel catalytic activity of RimK, a ribosomal protein from *Escherichia coli* K-12, which resulted in the biosynthesis of poly- $\alpha$ -glutamic acid from L-glutamic acid. The robust RimK enzyme exhibited 86% activity after incubation at 55 °C for 15 min, and was able to synthesise poly- $\alpha$ -glutamic acid of various lengths (upwards of 6000 Da).

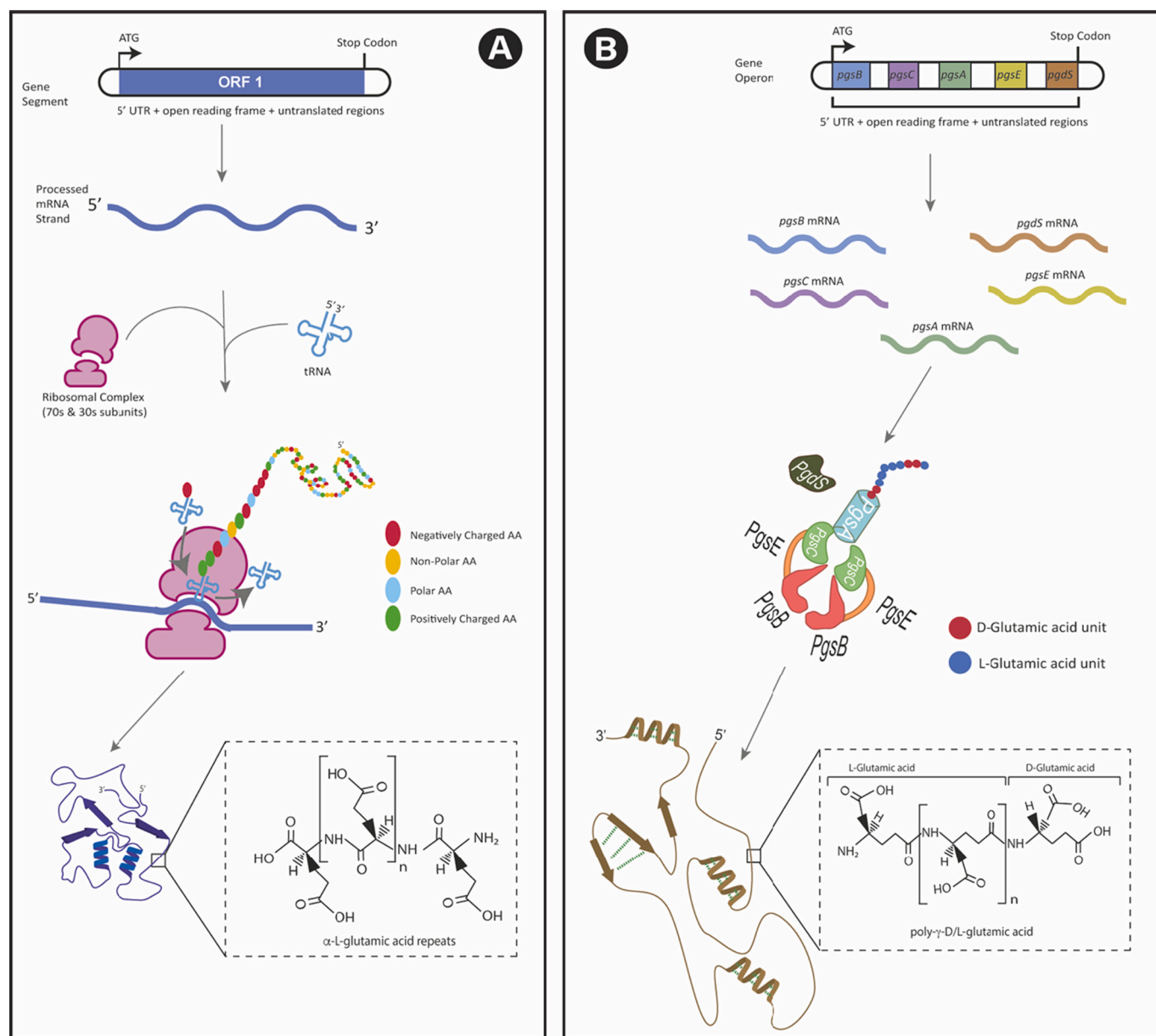
Poly( $\alpha$ -glutamic acid) can also be chemically synthesised (Fields, 2001). One method for synthesizing the poly( $\alpha$ -glutamic acid) is by reacting a glutamic ester monomer with a suitable initiator. An example of such reaction has been provided by Wang and Taylor (2011) wherein a benzyl ester glutamic acid N-carboxyanhydride can react with an amine initiator to produce a polyglutamic acid benzyl ester polymer through a ring opening polymerization mechanism (Wang and Taylor,

2011). With such type of chemical reactions, materials with different molar mass (30 to 185 kDa) can be obtained. The material molar mass can be precisely reduced by subjecting it to varying hydrolysing conditions. (Wang and Taylor, 2011) described the effect of time, temperature and acid upon the rate of poly( $\alpha$ -glutamic acid) hydrolysis.

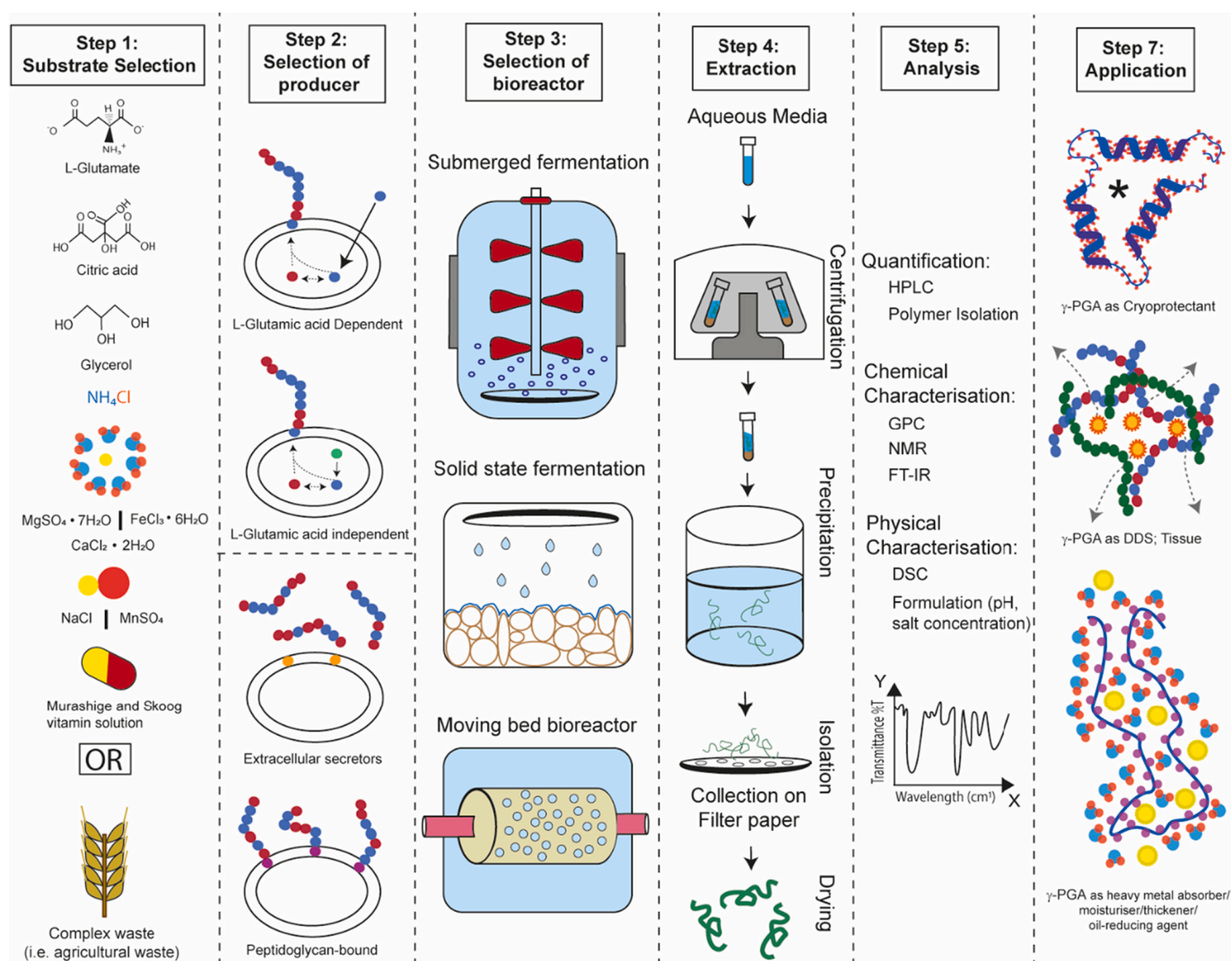
Poly-D/L- $\gamma$ -glutamic acid can be synthesised by biological or chemical means. Chemical synthesis of  $\gamma$ -PGA is achieved through nucleophile initiated polymerization of the  $\gamma$ -protected N-carboxyanhydride of L-glutamic acid. The material synthesised through this method does not present comparable characteristics (lower molar mass) nor properties to bacterially synthesised  $\gamma$ -PGA (Buescher and Margaritis, 2007; Ogunleye et al., 2015; Luo et al., 2016). For the aforementioned reasons discussions will be limited to  $\gamma$ -PGA of biological origin.

## 2.2. Biological synthesis of poly- $\gamma$ -glutamic acid

Production of  $\gamma$ -PGA depends greatly on the combination of substrate



**Fig. 2.** Biosynthesis of  $\alpha$ -polypeptidic material from mRNA (see A) against biosynthesis of poly( $\gamma$ -glutamic acid) through enzymatic complex polymerisation (see B). In the former, mRNA material is translated via ribosomes to synthesise a polypeptide; whereas, in the latter, an mRNA template is employed to construct an enzymatic complex responsible for the synthesis of poly( $\gamma$ -glutamic acid). AA- amino acid; UTR- Untranslated region.



**Fig. 3.** Stages of  $\gamma$ -PGA biosynthesis. Herein, the components of a defined media or complex waste substrate, producer type and biochemistry (L-glutamic acid dependent or independents & extracellular secretors or peptidoglycan bound producers), fermentation set-up (submerged fermentation, solid state fermentation or moving/rotating bed bioreactor), downstream processing (supernatant isolation, supernatant precipitation in ethanol, precipitate isolation and lyophilisation), analysis, and application have been schematically presented. HPLC- High Performance Liquid Chromatography; GPC- Gel Permeation Chromatography; NMR- Nuclear Magnetic Resonance ( $^1\text{H}$  &  $^{13}\text{C}$ ); FT-IR- Fourier Transform Infrared; DSC- Differential Scanning Calorimetry; DDS- Drug Delivery System. \*represents a probiotic strain (such as *Bifidobacteria* sp. or *Lactobacillus* sp.).

(Bajaj and Singhal, 2008), microorganism (Kedia et al., 2010; Ashiuchi et al., 2003a,b), fermentation vessel (Jian et al., 2005; Bajaj and Singhal, 2008), fermentation conditions (Jian et al., 2005; Ogawa et al., 1997) and extraction method (Manocha and Margaritis, 2010) (see Fig. 3).

The ability to modulate  $\gamma$ -PGA's chemical composition has been fundamentally achieved through understanding why the polymer is biosynthesised. The functions of this polymer in a biological system include: nutrient storage (Kimura, 2004; Moradali and Rehm, 2020), nutrient sequestration (Liu et al., 2010), resistance to variation in salt concentration (Kandler et al., 1983; Hezayen et al., 2001), heavy metal sequestration (McLean et al., 1990; Kaplan, 1998; Ogunleye et al., 2015), biochemical association of neuronal components (Edde et al., 1990) and Immunoprotection (Mesnage et al., 1998; Kaplan, 1998; Kocianova et al., 2005).

### 2.2.1. Fermentation conditions

The biosynthesis of  $\gamma$ -PGA is greatly dependent upon the fermentation environment. The fermentation system, mode and conditions play a pivotal role in determining polymeric yields and characteristics (see Fig. 3 and Table 1). Understanding of these, and how they are further

modulated by the substrate and the strain, is vital in obtaining the maximum amounts of specific polymeric composites in a consistent matter. Mimicking the native conditions for which  $\gamma$ -PGA production occurs, is fundamental in obtaining the maximal yields of the polymer. In the case of Gram-positive aerobic organisms, the presence of oxygen as an electron acceptor is crucial for synthesising the greatest amounts of polymer (Bajaj and Singhal, 2010). Further, provided the viscosity achieved following the biosynthesis of high molar mass  $\gamma$ -PGA (Park et al., 2005; Do et al., 2001; Luo et al., 2016), the operation mode of the fermenter is essential in providing adequate oxygen and nutrients to the cells; whilst also maintaining membrane integrity of the biological entities (Bajaj et al., 2008; Fang et al., 2020). This aspect is further assessed by Jiang et al. (2016), which correlates the fermentation strategy to  $\gamma$ -PGA productivity of different strains at different bioreactor volumes.

The biosynthesis of  $\gamma$ -PGA can occur in batch, fed-batch or continuous, with shake flasks (on a small scale) or with the use of bioreactors (medium to large scale fermentation) (Chen et al., 2008). Provided that  $\gamma$ -PGA is extracellularly produced, and secreted, one of the main issues encountered during fermentation involves the increase in viscosity of the media (Do et al., 2001). Such changes in physico-chemical properties

**Table 1**  
Summary of property-specific  $\gamma$ -PGA and correlation with bacterial strain, substrate composition and fermentation conditions.  $\gamma$ -PGA yields and characteristics have been summarised from literature and the main parameters have been presented.

Bacterial Strain	Substrate		Auxilliary components		Ferm. mode	Fermentation conditions						Polymeric yields			Polymeric composition			Reference	
	Component	g/L	Component	g/L		Temp (°C)	Inoc. Vol (mL)	pH	Aeration (L/min)	rpm	W.V. (L)	g/L	g/g sub.	g/g G.A	MW	PDI	D split (%)		
<i>B. licheniformis</i> (9945a)	L-glutamic acid, Citric acid, Glycerol, NH <sub>4</sub> Cl	20	MnSO <sub>4</sub>	0	Ferm. -Batch	37	5	6.5	1 to 5	250–800	3.50	4–5	0.014	0.25	N.S.	N.S.	N.S.	(Kedia et al., 2010)	
		12	NaCl	0	Shake fl. – Batch	37	0.4	N.S	N.A.	250	0.10	12–13	0.033	0.65	3.21 × 10 <sup>5</sup>	1.6	N.S.		
		80		13								0.108	0.65	1.00 × 10 <sup>6</sup>	N.S.	N.S.			
	7	L-G.A	0									5.7	0.047	0.29	2.10 × 10 <sup>6</sup>	N.S.	N.S.	(Bajaj et al., 2008)	
	Citric acid, Glycerol, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	15.2	L-G.A	0.00737	Shake Flask – Batch	37	20; with 3 × 10 <sup>7</sup> cells/mL	6.5	N.A.	200	0.05	26.00	0.298	353	5.30 × 10 <sup>5</sup>	N.S.	N.S.		
		62.4	α-K.A.	1.46															
		8		0.00737															
			Glycerol	0%								27.81 mg/gDS	0.028	0.11	N.S.	N.S.	N.S.		
		Soybean meal	5 g	Glycerol NH <sub>4</sub> SO <sub>4</sub> L-glutamine α-K.A.	0%	Solid State Ferm. – Batch	37	20; with 3 × 10 <sup>7</sup> cells/mL	6.5	N.A.	250	5.00	98.64 mg/gDS	0.099	0.37	N.S.	N.S.	N.S.	(Bajaj et al., 2008)
		L-glutamic acid, Citric acid, Moisture content	5% 2.5% 65%		2% 1.1% 0.05% 0.5%														
	Glycerol NH <sub>4</sub> Cl	80 7	L-G.A. Citric acid	8 4	Shake fl. – Batch	37	3 × 10 <sup>7</sup> cells/mL	7.4	N.A.	100	0.15	16.8 (r = 64)	0.166	2.10	N.S.	N.S.	44	(Thorne et al., 1954)	
	Sucrose, L-glutamic acid, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	50	NaCl	50	Shake Flask – Batch	30	0.5	7.0	N.A.	N.S.	0.05	11.75	0.077	0.59	1.00 × 10 <sup>6</sup>	N.S.	8	(Ashiuchi et al., 2006, 2007) (Ashiuchi and Shimizu, 2015)	
20		2.00 × 10 <sup>6</sup>		N.S.								N.S.							
10		6.2		0.031								0.31	2.00 × 10 <sup>6</sup>	N.S.	N.S.				
			Sugar	0								4.3	0.042	0.22	4.50 × 10 <sup>5</sup>	N.S.	25	(Ashiuchi, 2007), (Ashiuchi and Shimizu, 2015)	
			Sucrose	50								8.6	0.056	0.43	1.00 × 10 <sup>6</sup>	N.S.	8		
	L-glutamic acid, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20 10	Glucose	50	Shake Flask – Batch	30	0.4	7.0	N.A.	N.S.	0.05	6.6	0.043	0.33	6.00 × 10 <sup>5</sup>	N.S.	8		
			Arabinose	50								15.05	0.099	0.75	1.75 × 10 <sup>6</sup>	N.S.	5		
			Fructose	50								15.14	0.100	0.76	1.50 × 10 <sup>6</sup>	N.S.	8		
				20								5–6	0.032	0.06	N.S.	N.S.	N.S.	(Kedia et al., 2010)	
	Na-Glutamate	100	Glucose	40	Ferm. -Batch	37	5	6.5	1 to 5	250–800	3.50	9–11	0.053	0.11	N.S.	N.S.	N.S.		
	Peptone	15	60	24–26								0.115	0.26	N.S.	N.S.	N.S.			
	Yeast extract	10	Glycerol	60								12–14	0.062	0.14	N.S.	N.S.	N.S.		
	NaCl	30	Sucrose	60								12–14	0.062	0.14	N.S.	N.S.	N.S.		
			Citric acid	20								12–14	0.075	0.14	N.S.	N.S.	N.S.		
	Maltose	60		10	Ferm. -Batch	40	10	8.0	1	400	20.0	6	0.034	0.20	N.S.	N.S.	N.S.	(Ogawa et al., 1997)	
	Soy sauce	70	NaCl	30								19	0.096	0.63	N.S.	N.S.	N.S.		
	L-glutamate	30	50	12								0.055	0.40	N.S.	N.S.	N.S.			

(continued on next page)

Table 1 (continued)

Bacterial Strain	Substrate		Auxiliary components		Ferm. mode	Fermentation conditions				Polymeric yields				Polymeric composition			Reference
	Component	g/L	Component	g/L		Temp (°C)	Inoc. Vol (ml)	pH	Aeration (L/min)	rpm	W.V. (L)	g/L	g/g sub.	g/g G.A	MW	PDI	
	Maltose	60	N.A		Ferm. -Batch	40			300		15	0.066	0.25	N.S.	N.S	N.S.	(Ashiuchi et al., 2001a)
	Soy sauce	70	N.A								30	0.132	0.50	N.S.	N.S	N.S.	
	L-glutamate	60	N.A.								450	0.123	0.47	N.S.	N.S	N.S.	
<i>B. subtilis</i> (chung-kook-jiang)	Sucrose	50		5	Shake Flask - Batch	30	7.0	N.A.	N.S.	0.05	15.6	0.145	0.78	$1.00 \times 10^6$	N.S	N.S.	
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20	NaCl								11.0	0.072	0.55	$3.7 \times 10^5$	N.S	N.S.	
	L-glutamate	0.5									9.9	0.049	0.50	$1.10 \times 10^5$	N.S	N.S.	
				250							7.2	0.020	0.36	$1.10 \times 10^5$	N.S	N.S.	

L-G.A.- L-glutamic acid;  $\alpha$ -K.A.-  $\alpha$ -ketoglutaric acid, NH<sub>4</sub>Cl- ammonium chloride; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>- ammonium sulphate; inoc. Vol.- inoculum volume; rpm- revolution per minute; W.V.- working volume; g/g sub.- grams per grams of substrate; mg/gDS- milligrams per gram of dry substrate; g/g G.A.- grams per grams of L-glutamic acid; MM- molar mass; PDI- polydispersity index; N.A.- not applicable; N.S.- not specified.

of the system are directly correlated to a decrease in dissolved oxygen, required for cell growth and  $\gamma$ -PGA biosynthesis (Cromwick et al., 1996; Bajaj and Singhal, 2011). In this case, to cope with the increases in viscosity, both the stirring speed and the aeration rate can be increased. Bajaj and Singhal (2010) reported an optimal oxygen input of 1 vvm with optimal stirring at 750 rpm for *B. licheniformis* 9945a. To stimulate the production of some metabolites, culture stressing is often employed and, in some cases, staggered addition of carbon/nitrogen source might further stimulate the biosynthesis of the compound of interest (Poli et al., 2011; Tang et al., 2015c). Chen et al. (2008) employed a fed-batch strategy whereby fermentation media was supplemented with 2 L of 100 g/L glucose solution after 48 h from inoculation. The group achieved a + 14% increase in  $\gamma$ -PGA yield, compared to standard batch fermentation, with a significant increase in viscosity of the solution (Chen et al., 2008). The group were able to correlate increases in viscosity of the fermentation media to increases in molar mass of the polymer (Chen et al., 2008).

To decrease both the viscosity of the growth media and the cost of the substrate, Bajaj et al. (2008) cultivated *B. licheniformis* 9945a on soybean meal through a solid state fermentation (SSF) system (Bajaj et al., 2008; Wang et al., 2008). Although SSF pose significant issues with its up-scaling, such methods decrease: capital expenditures (CAPEX), wastewaters and fermentation media disposal costs; whilst usually increasing  $\gamma$ -PGA production rates (Zhang et al., 2019; Fang et al., 2020). The use of complex waste media for the biosynthesis of  $\gamma$ -PGA has been further discussed within section 5. Fang et al. (2020) have also shown the ability of *B. amyloxyquefaciens* JX-6 to synthesise  $\gamma$ -PGA in both sterile and non-sterile conditions. Such capabilities enable significant reductions in overall process costs and could thus provide a suitable solution towards commercialisation of  $\gamma$ -PGA (Yu et al., 2019). In an attempt to increase scalability of the fermentation systems, and to tackle issues face with increases in media viscosity, Jiang et al. (2016) assessed the use of propylene based (6.2 m<sup>2</sup>/Kg) beads as carriers onto which  $\gamma$ -PGA producers could form a biofilm, increasing the yields by 18.6%. This moving bed biofilm reactor (MBBR – see Fig. 3) type system provided a lower washout, during fed-batch fermentation, and increased oxygen transfer to the culture resulting in maximal yields of 67.4 g/L (16.5% increase over other feeding strategies assessed) (Jiang et al., 2016).

Fermentation substrate and media characteristics can significantly alter pendant group state. Optimal cell growth occurs at pH 7 for most  $\gamma$ -PGA producers, however, optimal pH for  $\gamma$ -PGA biosynthesis has been reported to be 6.5 (Bhat, 2012). At the latter pH, it has been show that optimal absorption of precursors (glutamic acid and citric acid) occurs (Cromwick et al., 1996; Richard and Margaritis, 2003). Lower pH points and a low salt medium incur in the production of an anionic  $\gamma$ -PGA (H- $\gamma$ -PGA). Differently, higher salt media usually incur in the production of salt- $\gamma$ -PGA (Bhat, 2012). Bhat (2012) report that *B. subtilis* natto produced Na- $\gamma$ -PGA at 85.38% (w/w) in GS media (pH 6.8). Differently, when subject to a lower salt Media (Medium E – pH 6.8), *B. subtilis* natto biosynthesised Na- $\gamma$ -PGA at 52.31 w/w% (w/w) and a 44.56% (w/w) fraction of H- $\gamma$ -PGA.

### 2.2.2. The microorganisms

Although  $\gamma$ -PGA biosynthesis is not limited to members of the *Bacillus* sp., these microorganisms are often employed for industrial  $\gamma$ -PGA biosynthesis. For this reason,  $\gamma$ -PGA producers are classified in two groups: Group I = *Bacillus* species; Group II = other bacteria (Luo et al., 2016). In addition to the genetics of the different producers, the activity of racemase enzymes, the membrane attachment or secretion of the polymeric chain can offer an additional layer of producer classification. With the focus on variable  $\gamma$ -PGA biosynthesis, the correlation between microorganismal strain, D/L split, molar mass and fermentation conditions has been summarised within Table 2.

### 2.2.3. Genetic aspects of $\gamma$ -PGA production and its optimisation

Depending upon the strain considered, the type of  $\gamma$ -PGA synthesised

**Table 2**  
Enantiomeric composition and molar mass of known  $\gamma$ -PGA producers.

Microorganism	Enantiomeric split		Mw (Da)	Ferm. Conditions/Notes	Reference		
	D-isomer (%)	L-isomer (%)					
<i>B. anthracis</i>	100	0	N.S.	N.A.	(Hanby and Rydon, 1946)		
	32	68	N.S.	Isolation at $t = 24$ h			
	41	59	N.S.	Isolation at $t = 184$ h			
	42	58	N.S.	Isolation at $t = 280$ h			
	44	56	N.S.	Isolation at $t = 64$ h			
	47	53	N.S.	Isolation at $t = 88$ h			
	49	51	N.S.	Isolation at $t = 42$ h			
	20–80	20–80	N.S.	Static flasks displayed higher percentages of D-fractions			
	86	14	N.S.	N.A.			
	50	50	550,000	No MnSO <sub>4</sub> , Isolation at $t = 140$ h			
<i>B. licheniformis</i> 9945a	48	52	565,000	0.615 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 140$ h	(Gross, 1998)		
	61	39	485,000	6.15 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 140$ h			
	59	41	225,000	33.8 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 140$ h			
	77	23	222,000	61.5 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 140$ h			
	91	9	275,000	615 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 140$ h			
	42	58	1,800,000	No MnSO <sub>4</sub> , Isolation at $t = 22$ h			
	66	34	1,925,000	0.615 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 22$ h			
	44	56	1,770,000	6.15 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 22$ h			
	59	41	1,000,000	33.8 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 22$ h			
	75	25	1,300,000	61.5 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 22$ h			
<i>B. subtilis natto</i> (IFO3335)	91	9	2,000,000	615 $\mu$ M MnSO <sub>4</sub> , Isolation at $t = 22$ h	(Ashiuchi, 2013)		
	10- ~ 100	0–90	10,000,000	N.A.			
	22	78	N.S.	N.A.			
	42	58	208,000	N.A.			
	50–80	20–50	10,000 - 10,000,000	N.A.			
	8	92	600,000	Glucose			
	9	91	750,000	Xylose			
	5	95	1,750,000	Arabinose			
	10	90	300,000	Lactose			
	25	75	450,000	0 g/L Sugar			
<i>B. megaterium</i> WH320	50	50	>200,000	N.A.	(Ashiuchi and Misono, 2002)		
	10–20	80–90	>1000,000	N.A.			
	N.S.	N.S.	2,000,000	10% NaCl			
	3	97 (92–98)	1,800,000-4,150,000	capB**CA under Ptac promoter(PGA009)			
	Engineered		1,450,000-				
	<i>C. glutamicum</i> F343	63	37 (25–35)	1,600,000		pZM1-capBCA-(1lacO) BsracE (PGA010)	(Xu et al., 2019)
				1000,000 -			
		40	60 (50–70)	8,000,000		pZM1-capBCA-(2lacO) BsracE (PGA011)	
	<i>N. aegyptiaca</i>	0	100	> 1000,000		N.A.	(Sung et al., 2005)

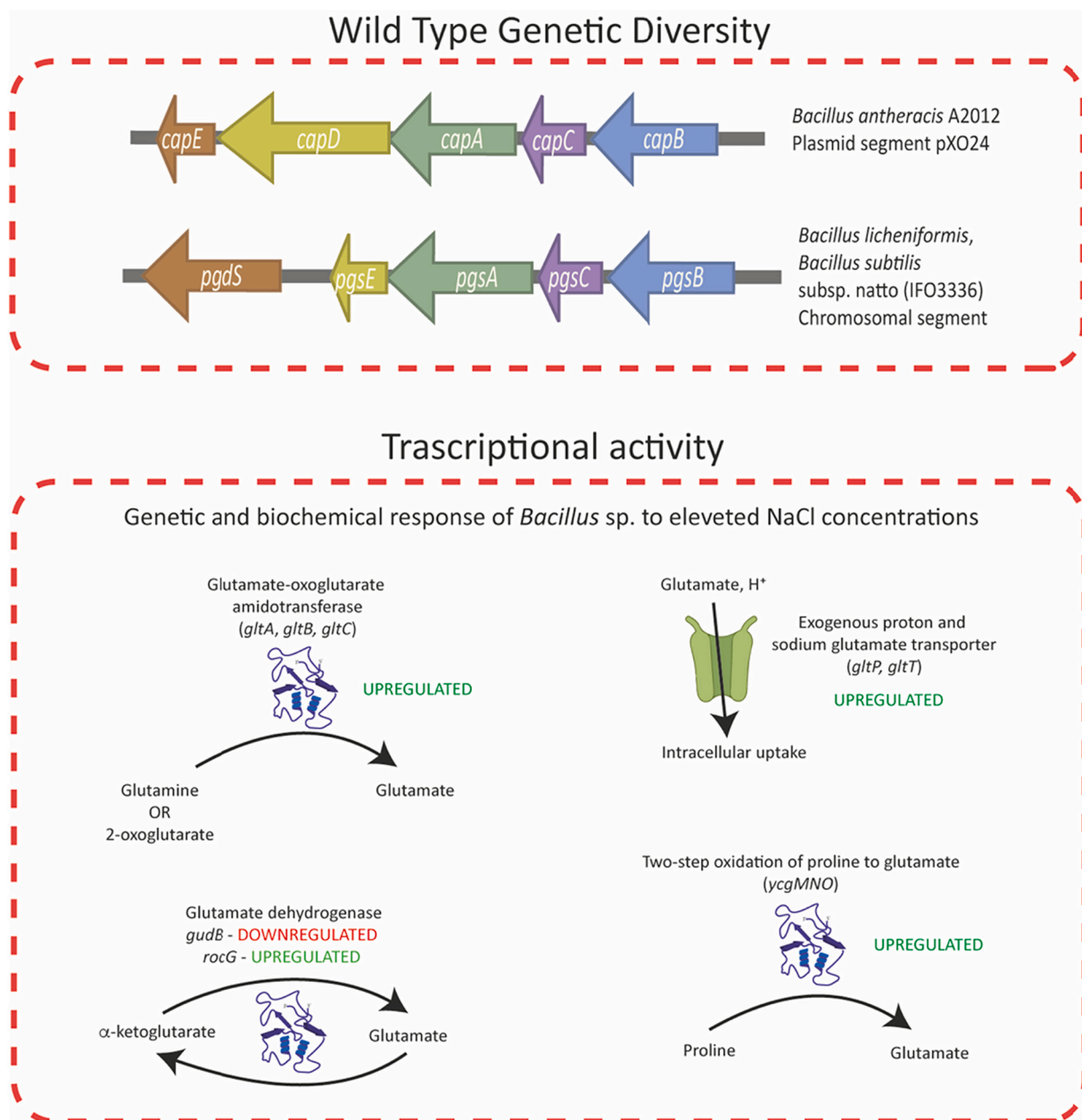
N.S. – Not Specified; N.A. – Not Applicable.

can differ in both composition and location (Gardner and Troy, 1979; Ogunleye et al., 2015). Some organisms synthesise the polymer extracellularly, but, present it bound to cell wall components (CapE protein derived from the *capE* gene (see Fig. 3) (Mesnage et al., 1998). This method of production is characteristic of Gram positive microorganisms with peptidoglycan-based cell walls. Such arrangement is suggested to confer virulence or act as a source of glutamate under cell starvation (Kimura et al., 2004; Kocianova et al., 2005; Candela and Fouet, 2006; Bhat, 2012; Ogunleye et al., 2015).

The differing components that regulate the attachment of the polymer to the peptidoglycan moiety or that enable extracellular secretion are the CapE and PgdS proteins, respectively (see location of these elements within Fig. 4). CapE has been correlated to  $\gamma$ -PGA attachment in *B. anthracis*, whereas the extracellular secretion of  $\gamma$ -PGA in non-peptidoglycan bound producers (i.e. *B. licheniformis*, *B. subtilis*) is suggested to be mediated by the PgdS enzyme, (Candela and Fouet, 2006; Buescher and Margaritis, 2007; Ogunleye et al., 2015). As far as we are aware, there are no known Gram negative producers presenting cell wall bound  $\gamma$ -PGA.

Different microorganismal strains are known to synthesise  $\gamma$ -PGA of varying D/L enantiomeric ratios whilst maintaining analogous fermentation conditions – as presented within Table 1, Table 2 & Fig. 2. Unless

supplemented within the fermentation media, the occurrence of D-glutamate moieties is derived by L-glutamate moieties. As such, the concentration of each molecule has to be controlled by the action of the racemase gene(s) (Ashiuchi, 2010). Kada et al. (2004) suggest that  $\gamma$ -PGA-producing *Bacillus subtilis* presents both *glr* and *yrcP*, two glutamate racemase genes. The same genetic structure has been reported for the  $\gamma$ -PGA non-producing *B. subtilis* strain 168. The group suggested that Glr has the ability to isomerize L-glutamate to the D-isomer when the concentration of the L-isomer is rather low, given its rather high affinity (Km value) towards L-glutamate. In contrast, YrcP presents a small Km value towards L-glutamate and a small Vmax value for the isomerisation. Thus, YrcP requires higher concentrations of L-glutamine for stimulating its isomerisation to D-glutamate. These differences in enzymatic properties suggest that YrcP is suitable for isomerisation of small amounts of L-glutamate when cell division progresses (Kada et al., 2004). Provided with such functional similarities of Glr and YrcP, Kada et al. (2004) over-expressed *yrcP* in *Escherichia coli* and, accumulation of the YrcP protein, caused growth inhibition in the bacteria. Analogous phenotypic variation is also observed with the MurI of *E. coli*, responsible for encoding the glutamate racemase essential in peptidoglycan biosynthesis. Differently, over-expression of the *glr* gene did not cause growth inhibition of *E. coli*. As such, Ashiuchi and Misono (2002)



**Fig. 4.** Genetic composition and arrangement of genes encoding the  $\gamma$ -PGA synthesising complex in various species and transcriptomic variability in response to increased NaCl concentrations. Comparison of genetic fragments within common  $\gamma$ -PGA producers have been summarised as well as common alterations in genetic expression as a response to increased NaCl.

assumed that *Glr* was involved in  $\gamma$ -PGA synthesis and *YrpC* was involved in peptidoglycan synthesis in  $\gamma$ -PGA-producing *B. subtilis*. However, contrary to this assumption, it was shown that the *glr* orthologue in *B. subtilis* 168 is essential for growth and supposedly for peptidoglycan synthesis, whereas *yrpC* is not. Kada et al. (2004) then determined which gene (*glr* or *yrpC* or both) was responsible for  $\gamma$ -PGA biosynthesis in *B. subtilis*. Following disruption of either or both genes, the group reports that *Glr* is essential for the conversion of L-glutamate into D-glutamate utilised for the synthesis of both  $\gamma$ -PGA and peptidoglycan in *B. subtilis*. This result was further supported by the fact that *glr* is transcribed actively during the exponential growth phase and also during the stationary phase when  $\gamma$ -PGA is produced (Kada et al., 2004).

Depending upon the bacterial strain, genes involved in  $\gamma$ -PGA biosynthesis can be embedded within the bacterial chromosome or can be located on a plasmid (Ashiuchi and Misono, 2002; Buescher and Margaritis, 2007). Such differences in genetic arrangement leads to

significant variation in the biosynthesis of  $\gamma$ -PGA as well as strain maintenance requirements (Ashiuchi and Misono, 2002; Sung et al., 2005). Some report that, after numerous subcultivations, the  $\gamma$ -PGA producing capacity of certain species can be altered. It has been shown that *B. subtilis* chungkookjang does not harbour a plasmid but owns high genetic competence (Ashiuchi et al., 2001a; Ashiuchi and Misono, 2002). Sung et al. (2005) have shown that the  $\gamma$ -PGA productivity of *B. subtilis* (chungkookjang) remains unchanged even after long-term storage and repeated subcultivations. Differently, strains such as *B. anthracis*, carry  $\gamma$ -PGA synthesis genes on a plasmid, which significantly increases the ability of producers to transfer competency to neighbouring microorganisms, at the cost of retention during subcultivation (Brézillon et al., 2015). These characteristics are particularly important when attempting to improve the yields of a particular colony through subcultivation; a common technique employed in biotechnology.

Microorganism type and its genetic makeup can also affect the final composition of the biomaterial in two ways (as summarised within Fig. 3, Table 1 and Table 2):

- a) Through the genes it possesses (and thus enzymes synthesised)
- b) Through the expression of such enzymes (transcribed and translated in response to external inputs)

**2.2.3.1. Synthetic optimisation of cell factories for  $\gamma$ -PGA biosynthesis.** As mentioned throughout,  $\gamma$ -PGA biosynthesis is greatly dependent upon the fermentation conditions (Leonard et al., 1958), such variation in growth environment has been shown to alter the expression of operon/genes involved in  $\gamma$ -PGA production (Leonard and Housewright, 1963; Ashiuchi et al., 2003a,b; Belitsky and Sonenshein, 1998; Cao et al., 2013; Cao et al., 2018). Although such mechanisms are particularly useful in nature with the producer able to limit energy expenditure when conditions are not harsh, in a commercial context, condition-dependent expression can significantly hinder high yields and increase the overall cost of the process (Gu et al., 2018). For these reasons, synthetic methods are often employed directly to overexpress genes involved in the production of the desired product or delete genes involved in  $\gamma$ -PGA catabolic processes or indirectly, by increasing the metabolic flux towards  $\gamma$ -PGA biosynthesis.

For example, by deleting *pgdS* and *ggt* (involved in the expression of degU32 protein), Scoffone et al. (2013) were able to increase the yields of  $\gamma$ -PGA from 25 to 48 g/L at  $t = 60$  h, however, the overall molar mass of the material was significantly lower compared to the wild type *Bacillus subtilis*. Differently, Ojima et al. (2019) showed that for *Bacillus licheniformis* RK14–46 only a single deletion of *ggt* was able to increase  $\gamma$ -PGA yields from 17 to 29 g/L at  $t = 72$  h, whereas a double deletion of *pgdS* and *ggt* did not yield any  $\gamma$ -PGA. As such, Ojima et al. (2019) suggest that *pgdS* gene is important for the growth of wild type RK14, as previously reported by (Tian et al., 2013). Using the principle presented by Tian et al. (2013), Wang et al. (2020) were able to precisely modulate the molar mass of  $\gamma$ -PGA using one chassis microorganism (*B. licheniformis* WX-02) through the optimisation of signal peptides. Halmschlag et al. (2019) further develop the fine-tuning of  $\gamma$ -PGA molar mass and D/L enantiomeric ratio by shuffling *pgs* and *racE* genes native of *B. subtilis*, *B. amyloliquefaciens*, *B. anthracis*. The group reports that the highest percentage of D-glutamic acid containing  $\gamma$ -PGA (50% w/w) was achieved from a *B. subtilis*  $\gamma$ -PGA synthetase-expressing strain with glutamate racemase from *B. amyloliquefaciens* (PG38). Compared to molar mass of  $\gamma$ -PGA produced by wild-type *Bacillus subtilis* (10 to 7000 kDa as reported by Ashiuchi and Misono (2002)), the group's *Bacillus subtilis* PG10 strain (native, PgsBCAE<sup>*B. subtilis*</sup>) yielded  $\gamma$ -PGA with molar masses up to 8500 kDa. Medium molar mass  $\gamma$ -PGA (170 to 660 kDa) was also successfully produced through *B. subtilis* PG25-E (with Pgsbcab<sup>*amyloliquefaciens*</sup>). The group also produced low molar mass  $\gamma$ -PGA (29 to 34 kDa) from Pgsbcae<sup>*B. anthracis*</sup>. The combination of genetic overexpression combined with external stimuli, also shows great promise in  $\gamma$ -PGA optimisation. In fact, Deol et al. (2022) demonstrated that overexpression of PgsE enhances Zn<sup>2+</sup> and Cu<sup>2+</sup> – dependent  $\gamma$ -PGA production. The addition of 250  $\mu$ M ZnSO<sub>4</sub> or 100  $\mu$ M CuSO<sub>4</sub> to PgsE overexpressed from the *hysp* promoter (with 1 mM IPTG, repressor site inhibitor) lead to  $\gamma$ -PGA biosynthesis of 10 mg and 17 mg of  $\gamma$ -PGA per gram of cell wet weight, a significant increase compared to 3 mg and 7 mg of  $\gamma$ -PGA per gram of cell wet weight produced in wild type *Bacillus subtilis* cells.

Differently from synthetic engineering of the *pgs* synthetic cassette, metabolic engineering efforts have also proven to be successful in increasing  $\gamma$ -PGA biosynthesis (Feng et al., 2017; Cao et al., 2013). In fact, Li et al. (2021) successfully enhanced carbon flux in *Bacillus licheniformis* WX-02 with significant increases in  $\gamma$ -PGA yields. By overexpressing pyruvate dehydrogenase (PdhABCD) and citrate

synthase (CitA) they achieved 34.9% and 11.1% increases in yields -respectively- compared to wild-type *B. licheniformis* WX-02. By rewiring the glyoxylate shunt, as a result of alteration of isocitrate lyase (AceA) expression (down-regulated strain WXP<sub>bacA</sub>aceBA), the group achieved a 23.2% increase in  $\gamma$ -PGA yield compared to wild-type *B. licheniformis* WX-02. Further, following the deletion of pyruvate formate-lyase gene *pflB*, the group demonstrated an increase in  $\gamma$ -PGA yield equal to 30.7% compared to wild-type *B. licheniformis* WX-02. As a result of such metabolic engineering, overall  $\gamma$ -PGA yields were improved by 69.3% (reaching a productivity of 12.02 g/L) by overexpressing *pdhABCD* and *citA*, repressing *aceA*, and deleting *pflB*.

Further, with an innovative approach, Yang et al. (2022) were able to increase the yields of  $\gamma$ -PGA by 40.5% through switching the cofactor specificity of glutamate dehydrogenase RocG from nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide (NADH) by creating the mutant RocG<sup>D276E</sup>.

Alongside  $\gamma$ -PGA, *Bacillus* sp. secrete a number of different proteins. These 'impurities' are usually removed during the isolation and purification steps of the process. However, due to the tendency of  $\gamma$ -PGA to interact with other biological material, an ultra-pure material is difficult to obtain. In this respect, He et al. (2022) were able to significantly decrease proteic impurity of the resulting material from 1.48% to 0.83% by knocking down the *fla-che* operon, *PBSX*, as well as the *yrpD*, *ywoF* and *yclQ* genes and other five genes encoding for extracellular proteins. The group was also able to decrease polysaccharide impurities (from 2.21 to 1.93%) by knocking out the *epsA-O* operon.

Although genetic manipulation of  $\gamma$ -PGA is very promising for the controlled synthesis of biological polymers, overall yields arising from these constructs are still significantly lower than wild-type counterparts. At present, low polymeric yields, in combination to genetically modified organism safety concerns, make fermentation dependant biopolymer tuning the most promising commercial method of biomaterial production.

#### 2.2.4. The effect of microbial cultivation on $\gamma$ -PGA yield and properties

Fundamentally, the synthesis of  $\gamma$ -PGA requires a source of carbon, a precursor, suitable micronutrients adequate temperature, oxygen and pH (Gross, 1998). However, modulation of these fundamental parameters, and other auxiliary factors (i.e. NaCl, Mg<sup>2+</sup>, Zn<sup>2+</sup> and Mn<sup>2+</sup> concentrations, aeration) greatly affects both the yields and chemistry of the resulting polymer. Provided with the genetic variation of  $\gamma$ -PGA producers, the impact of media alterations affect differently varying microorganisms. As such, a thorough investigation on the yields & polymer characteristics in relation to the substrate, microorganism and fermentation parameters has been summarised in Table 1.

**2.2.4.1. Use of defined media for  $\gamma$ -PGA production.** As presented within Table 1 and Table 3, an array of microorganisms/substrate combinations facilitate the achievement of high-yield, and mostly reproducible, synthesis of  $\gamma$ -PGA (Zhang et al., 2018; Ashiuchi, 2010; Ogunleye et al., 2015; Luo et al., 2016). However, as previously mentioned, this approach incurs in a greater process cost associated with the addition expensive media components (L-glutamic acid/citric acid/vitamin solutions). To tackle such issue, the substitution of a defined media with precursor presenting waste complex media can be employed. In this regard, clear understanding of the interaction between the media components and their effect upon microorganismal growth/biomaterial production is fundamental. Commonly, the commercial production of  $\gamma$ -PGA is achieved by employing GRAS *Bacillus* family members. These microorganisms require supplementation with a vitamin solution within their growth media for optimal duplication and synthesis of  $\gamma$ -PGA (Kedia et al., 2010). Further, the presence of manganese – Mn<sup>2+</sup> (Birrer et al., 1994; Kedia et al., 2010), zinc – Zn<sup>2+</sup> (Yamashiro et al., 2010), sodium – Na<sup>+</sup> (Birrer et al., 1994; Kedia et al., 2010) (Ogawa et al., 1997; Ashiuchi et al., 2006; Ashiuchi and Shimizu, 2015) calcium – Ca<sup>2+</sup>

**Table 3**

Summary of  $\gamma$ -PGA substrate components, substrate costs, polymeric yields and cost savings. Herein a summary of substrate, yields and costs related to  $\gamma$ -PGA biosynthesis has been reported.

Substrate Components	Material Cost	Standard Media <sup>a</sup>		Algal media <sup>b</sup>		Corn stalk, Soybean meal and industrial MSG <sup>c</sup>		Untreated cane molasses and MSG waste <sup>d</sup>		Rice straw <sup>e</sup>		Agroindustrial residues <sup>f &amp; f'</sup>			
	(\$/Kg)	(Kg/L)	(\$/L)	(Kg/L)	(\$/L)	(Kg/Kg)	(\$/Kg)	(kg/L)	(\$/L)	(kg/L)	(\$/L)	(kg/L)	(\$/L)	(Kg/Kg)	(\$/Kg)
Dried <i>Ulva</i> sp. powder	0.07			0.04	0.00										
Glucose	0.58							0.09	0.05					0.09	0.05
Sucrose	19.56	0.03	0.59												
L-glutamate	33.16	0.03	0.99	0.01	0.23			0.05	1.66	0.05	1.66	0.05	1.66		
NH <sub>4</sub> Cl	19.51	0.01	0.20												
NaCl	2.26	0.06	0.14	0.04	0.09										
KH <sub>2</sub> PO <sub>4</sub>	17.56	0.00	0.01					0.00	0.04	0.00	0.04	0.00	0.04		
MgSO <sub>4</sub> · 7H <sub>2</sub> O	17.56	0.00	0.01					0.00	0.00	0.00	0.00	0.00	0.00		
CaCl <sub>2</sub> · 2H <sub>2</sub> O	19.51	0.00	0.00												
MnSO <sub>4</sub> · 4H <sub>2</sub> O	31.21	0.00	0.00					0.00	0.00	0.00	0.00	0.00	0.00		
FeCl <sub>2</sub> · 7H <sub>2</sub> O	29.26	0.00	0.00												
NH <sub>4</sub> (SO <sub>4</sub> ) <sub>2</sub>	172.00							0.01	0.86	0.00	0.43	0.01	0.86	0.01	0.86
Corn Stover <sup>g</sup>	0.04					7.50	0.29								
Soybean Meal <sup>h</sup>	0.35					7.50	2.66								
MSG	1.53					1.50	2.29	0.06	0.09	0.02	0.14			0.06	0.09
MSG waste liquor	0.02									4.50	0.07				
Cane Molasses	0.15									0.15	0.02				
Rice straw hydrolysis	0.05											0.08	0.00		
Industrial waste glycerol	0.99													0.04	0.04
Dry mushroom residues	0.09													0.60	0.06
MSG production residues	0.27													0.40	0.11
Pre-treatment cost	0.09											1.00	0.09		
<b>Polymeric yields (kg/L)</b>		0.0262		0.0063		0.1025	0.0530			0.0521		0.0730		0.0336	0.1156
<b>Cost of substrate (\$/L)</b>		1.94		0.32		5.24	2.70			0.66		2.65		1.00	0.20
<b>Cost of 1 Kg polymer (\$/Kg)</b>		74.0		50.8		51.1	50.9			12.7		36.3		29.9	1.8
<b>Cost Saving (%)</b>		N.A.		31.4		30.9	31.2			82.9		50.9		59.6	97.6

a&b Batch fermentation, *Bacillus* sp. SJ-10, 5 L flask with 1 L working volume, 72 h fermentation (Kim et al., 2019b); <sup>c</sup> Solid state fermentation, *B. amyloliquefaciens* JX-6, 37.5 kg scale (150 L bioreactor), 60% hydration of the solid substrate, 72 hrh fermentation (Fang et al., 2020); <sup>d</sup> Fed batch fermentation, *B. subtilis* NX-2, 7.5 L bioreactor, 4.5 L fermentation volume, 72 h fermentation (Zhang et al., 2012); <sup>e</sup> Continuous fermentation, *B. subtilis* NX-2, 7.5 L bioreactor with 4.5 L of fermentation media, 90 h (Tang et al., 2015a); <sup>f</sup> Submerged fermentation, *B. subtilis* NX-2, 7.5 L fermenter volume, 4.5 L working volume, 48 h fermentation (Tang et al., 2015b); <sup>f'</sup> Solid state batch fermentation, *B. subtilis* NX-2, 5 L fermenter volume, 200 g substrate, 65% hydration, 48 h fermentation (Tang et al., 2015b); <sup>g</sup> Cost of corn stover: (Thompson and Tyner, 2014); <sup>h</sup> Cost of Soybean meal (Markets Business Insider, 2021). MSG: monosodium glutamate.

(Yamashiro et al., 2010), magnesium – Mg<sup>2+</sup> (Ashiuchi and Shimizu, 2015) and iron – Fe<sup>2+</sup> (Yamashiro et al., 2010) ions have all been shown to modulate the yields and/or chemical characteristics of the biomaterial (Ashiuchi et al., 2001a; Ashiuchi, 2007).

As such, if a specific polymeric chemistry needs to be obtained, the waste complex media has to be fully characterised and modified accordingly.

**2.2.4.2. Application of complex media for  $\gamma$ -PGA production.** Particular understanding in  $\gamma$ -PGA biosynthesis and properties, is recognised within the Asian continent has been catalysed by the traditional fermentation of natto beans. In these geographic regions, solid state fermentation (SSF) of corn stalk and soybean meal is common for  $\gamma$ -PGA production (Bajaj et al., 2008; Fang et al., 2020). (Fang et al., 2020) report that such practice lowers fermentation costs, leads to higher productivity, reduces initial freshwater input, lowers wastewaters and requires relatively simple facilities (Fang et al., 2020). This has also been supported by Bajaj et al. (2008) which obtained 98.64 g of  $\gamma$ -PGA per Kg of dry soybean meal through *B. licheniformis* 9945a fermentation. Similarly, Fang et al. (2020) assessed the solid state fermentation of corn

straw and soybean meal in 50 and 150 L bioreactors by *B. subtilis* JX-6, obtaining yields of 116.88 ± 5.05 g  $\gamma$ -PGA/kg and 102.48 ± 3.30 g  $\gamma$ -PGA/kg respectively. However, compared to submerged fermentation (SmF), SSF sees greater difficulty in scalability, due to the difficulty in aeration, agitation and heat transfer (Huang et al., 2011; Fang et al., 2020).

Macroalgae are another source of waste complex media capable of stimulating the biosynthesis of  $\gamma$ -PGA. Currently, macroalgae are protagonists of increasing numbers of blooms and have caused significant economic and environmental disruption across the world (Glibert et al., 2018; Kim et al., 2019b). Furthermore, there is a great increase in cultivation of micro and macro algae as a beneficial food ingredient/supplement for humans, animals and crops across Europe and the United Kingdom. In particular, macroalgae are being recognised as a precious source of antioxidants, thickeners and pigments (Jumaidin et al., 2018; Edwards, 2011; Leandro et al., 2019; Duarte et al., 2021). Similarly, microalgae have also been recognised as a valuable source of antioxidants and lipids for both the food and biofuel sector (Ahmad et al., 2011). However, for most of the aforementioned applications, only a portion of the cell is used. Currently, significant algal by-products are generated

(Bishop and Rahman, 2017). (Seratale et al., 2018) review the co-fermentation of algal waste and other complex waste to improve the anaerobic digestion process, for the synthesis of biomethane. Although quite successful, the group also discusses the issues derived with lack of process circularity. Differently, employing pre-extracted biomass for  $\gamma$ -PGA biosynthesis can both increase process circularity and stimulate production of a value-added compounds (Kim et al., 2019b; Parati et al., 2022). Further, the richness of algae in amino acids is particularly beneficial in stimulating the biosynthesis of  $\gamma$ -PGA. To the best of our knowledge, there is only one report of post fatty acid microalgae extracted biomass use as  $\gamma$ -PGA substrate (Parati et al., 2022). However, both Kim et al. (2019b) and Parati et al. (2022) were able to employ macroalgae as substrate for  $\gamma$ -PGA biosynthesis. Kim et al. (2019b) assessed the synthesis capabilities of *Bacillus* sp. SJ-10, a D- $\gamma$ -PGA producer, from *Ulva* sp., a common bloom-forming macroalgae. The study found that this bacteria presented immediate degradation of *Ulva*'s membrane but also employed these macronutrients as a substrate in non-sterile fermentation conditions. Employing macroalgae as a source of carbon and nitrogen provides a solution to an array of issues commonly encountered within SmF: the first being the cost of the substrate, as well as providing a source of value-added compounds and a reduction in operational cost as substrate/microorganism combination has been shown to be suitable also for  $\gamma$ -PGA biosynthesis through non-sterile conditions (Kim et al., 2019b; Parati et al., 2022). In this respect, substitution of defined media with algal waste has incurred in a reduction in process cost calculated at 32% compared to a defined media (Kim et al., 2019b). In addition, provided with the differences in cell wall composition, both macroalgae and microalgae are far easier for microorganisms to degrade and utilise compared to lignocellulosic waste. In fact, Guo et al. (2017) have demonstrated the ability of *Bacillus* sp. A0, *Bacillus* sp. A4, *Exiguobacterium* sp. AS2B, *Pseudomonas* sp. CDS3, *Bacillus* sp. CH2OS1, *Bacillus* sp. K1, *Raoultella* sp. X1, and *Bacillus subtilis* X4 towards the secretion of carboxymethyl cellulose, xylanase, laccase and filter paper activity enzymes; all of which have resulted in the lysis of the microalgae *Chlorella zofingiensis*. The group reported that, *Bacillus* sp. K1 allowed for high lipid extraction as well as 40% algal cell lysis after a 24 h incubation at room temperature (Guo et al., 2017). Such ability by members of the *Bacillus* sp. could benefit a biorefinery concept. To this end, algal biomass can be utilised as substrate for production of  $\gamma$ -PGA whilst also providing a 'green' route towards the extraction of intracellularly accumulated lipids and co-value added materials. Table 3 compares the cost of  $\gamma$ -PGA biosynthesis by employing an array of waste complex media.

Other adequate complex waste media sources, yet to be assessed/exploited, include beer, cider and wine by-products (Zacharof, 2017; Jun et al., 2017; Färçaş et al., 2017; Duarte et al., 2021). The UK is a significant producer of beer (39.5 million hectolitres in 2019 – by Wunsch, 2020) and of cider (2.93 million hectolitres in 2019 – reported by (H.Weston and Sons Ltd, 2019); of these 15 to 25% are carbohydrate and nitrogen rich, showing potential for  $\gamma$ -PGA synthesis. A similar argument is relevant in the southern regions of Europe wherein wine production is dominant (41.1 million hectolitres in France, 40.1 million hectolitres in Italy and 30.4 million hectolitres in Spain - (Zacharof, 2016)) and results in the generation of significant amounts of wastewaters, and grape pomace (10–20% of the grape). The pomace obtained from grape processing presents similar properties to cider pomace (Dávila et al., 2017) (Aivazidou and Tsolakis, 2020). Given the global widespread of wine production, between 10.5 and 13.1 million tons of grape pomace/annum are generated (Dávila et al., 2017), rendering its use as a substrate for  $\gamma$ -PGA biosynthesis relevant in the context of an integrated biorefinery concept. With all of these substrates, the analysis of components is limited to the solid/liquid fractions and content of polysaccharides, pectin and lignin; not accounting for the specific mineral content, amino acid content (i.e. percentage of L-glutamic acid) and/or other TCA intermediates (i.e. citric acid); all fundamental parameters modulating the synthesis of  $\gamma$ -PGA (see Fig. 1, Fig. 3 and

Table 1). Further, seasons greatly affect the chemical composition of the pomace and can thus significantly alter the yields and chemical composition of  $\gamma$ -PGA synthesised. However, as presented within Table 1, the chemical composition of each batch is conditional to the fermentation parameters, as such, the ultimate application of the  $\gamma$ -PGA can be varied, depending upon the polymeric characteristics of the batch. Unfortunately, for the majority of commercial commodities, such an approach would be challenging to implement given the need for reproducible material synthesis.

### 3. Controlling $\gamma$ -PGA chemical properties

#### 3.1. Effect of cultivation condition on enantiomeric composition of $\gamma$ -PGA

As discussed above, enzymatic polymerisation allows for the biosynthesis of  $\gamma$ -PGA as a D- or L- homo-polyamide or as a D/L-copolymer (Wu et al., 2006; Buescher and Margaritis, 2007; Ogunleye et al., 2015; Luo et al., 2016; Kim et al., 2019b). The occurrence of either homopolymer or the variation in the co-polymer is dictated by the genetic make-up of the microorganism, but in most cases, it can also be controlled through external inputs (i.e. media components, environmental conditions) (see Fig. 3, Fig. 4 and Table 2) (Buescher and Margaritis, 2007; Ashiuchi, 2010; Ashiuchi, 2011; Nabi and Das, 2015; Cao et al., 2018; Xu et al., 2019).

The synthesis and quality of  $\gamma$ -PGA is also modulated by the fermentation environment (Fig. 3). For example, the concentrations of  $Mn^{2+}$  and  $Mg^{2+}$  impact the D/L ratios of the resulting material (Ashiuchi and Shimizu, 2015). The addition of  $Mn^{2+}$  to the fermentation of *B. licheniformis* 9945a and *B. subtilis natto* modulated the D/L ratios within the biomaterial as well as the molar mass of the resulting material and the  $\gamma$ -PGA productivity of the two microorganisms (Kedia et al., 2010; Ashiuchi and Shimizu, 2015; Luo et al., 2016). Further, Ashiuchi and Shimizu (2015) report that, the addition of  $Mg^{2+}$  (at 0.5%  $MgSO_4 \cdot 7H_2O$ ) within the fermentation medium of *Bacillus megaterium* WH320, increases the concentration of L-glutamic acid units within  $\gamma$ -PGA. Conversely, the same group reports that, supplementation with  $Mn^{2+}$  (at 0.05%  $MnCl_2$ ) increases the concentration of D-glutamic units for  $\gamma$ -PGA synthesised by *B. megaterium* WH320. Further, the addition of both 0.5%  $MgSO_4 \cdot 7H_2O$  and 0.05%  $MnCl_2$  showed increased productivity and leads to a  $\gamma$ -PGA ratio of 55.6:44.4 D:L glutamic acid units.

Ashiuchi et al. (2004) also assessed the impact of other divalent cations during the fermentation of *Bacillus subtilis chungkookjang* and the productivity and D/L ratio of  $\gamma$ -PGA. The group suggests that  $Mg^{2+}$  was essential in catalysis and that  $Zn^{2+}$  further increased the production of  $\gamma$ -PGA (Yamashiro et al., 2010). Differently,  $Fe^{2+}$  and  $Ca^{2+}$  cations acted as inhibitors. Unlike *B. licheniformis* 9945a, *B. subtilis natto* and *Bacillus megaterium* WH320, Yamashiro et al. (2010) reported that, fermentation media supplementation with  $Mn^{2+}$ , did not significantly influence the formation of  $\gamma$ -PGA in *Bacillus subtilis chungkookjang*. In fact, Yamashiro et al. (2010) suggest that a further sub-classification of  $\gamma$ -PGA producers should arise, wherein strains are divided into  $Mn^{2+}$  or  $Zn^{2+}$  dependent groups.

Furthermore, Ashiuchi and Shimizu (2015) explored the impact of the nitrogen source upon the D/L ratio of  $\gamma$ -PGA. Herein they assessed the effect of L-glutamic acid and/or ammonium sulphate on the enantiomeric composition of the resulting biomaterial, synthesised by *B. megaterium* WH320. The group reported that fermentation with 2% L-glutamic acid and 1% ammonium sulphate increased productivity and lead to polymeric chains presenting a split 8.8:91.2 of D:L glutamic acid units.

The enantiomeric properties of the precursor are also important in affecting the final composition of the biomaterial. Ashiuchi et al. (2004) assessed the impact of feeding *B. subtilis chungkookjang* and *B. subtilis natto* various amounts of D-glutamic acid upon both the yields and the enantiomeric properties of the resulting  $\gamma$ -PGA. Their experiments suggest that supplementing the substrate with 60–80% D-glutamate

increases the yields of *B. subtilis* natto by three times and results in a polymer with ~95:10 D:L units (see Table 2). The group also reports that the same substrate does not alter the enantiomeric concentration of  $\gamma$ -PGA synthesised by *B. subtilis* chungkookjang (containing high glutamate racemase activity) wherein, supplementation with D-glutamic acid yielded comparatively constant (D unit contents ranging from 65 to 75%) (Ashiuchi et al., 2004).

Moreover, Ashiuchi et al. (2004) report that, given the inability of  $Mn^{2+}$  to interact with the glutamate racemase enzyme in some strains of *B. subtilis* (i.e. *B. subtilis* chungkookjang), the modulation in D/L ratios of these  $\gamma$ -PGA-producing strains should be obtained through the alteration in expression of the glutamate racemase gene (Ashiuchi et al., 2004).

As discussed above, correlation between metals and  $\gamma$ -PGA alteration of D/L enantiomeric ratios can be fine-tuned to a good degree (Ashiuchi and Shimizu, 2015; Halmeschlag et al., 2019). Such correlation is fundamentally explained by the mechanism by which D- or L-glutamic acid enzymes interact with divalent cations. In principle,  $\gamma$ -PGA biosynthesis is a ligase reaction for glutamate catalysed by ATP (as a cofactor), and further facilitated by divalent cations (Ashiuchi et al., 2001b; Ashiuchi et al., 2004). To establish the effect of different cations on the activity of enantiomeric enzymes, Ashiuchi et al. (2004) tested a number of different divalent metals. The group showed that  $Mg^{2+}$  and  $Zn^{2+}$  boosted the activity, whereas  $Ca^{2+}$  and  $Fe^{2+}$  inhibited enzymatic reactions involved in  $\gamma$ -PGA biosynthesis. To further demonstrate the fundamental role of divalent cations on the enzymatic operation, Ashiuchi et al. (2004) introduced EDTA within the media. This resulted in complete suppression of  $\gamma$ -PGA biosynthesis indicating that specific cations are required for the correct operation of glutamic acid enzymes (Ashiuchi et al., 2004).

### 3.2. Effect of process parameters on $\gamma$ -PGA molar mass

Similarly to the ratio of D/L glutamic acid units, the molar mass of the resulting polymer can be modulated by both the genetic make-up of the microorganism and by the fermentation environment.

In addition to the variation in producers' synthetases (either *pgsBCA* or *capBCA*),  $\gamma$ -PGA hydrolytic enzymes can modulate the molar mass of the polymer. In fact, deletion of genes encoding for  $\gamma$ -PGA hydrolases showed a higher molar mass polymer in *B. subtilis* (Kimura et al., 2004). There are three types of  $\gamma$ -PGA hydrolases, namely  $\gamma$ -PGA hydrolase (Pgds), D-/L-endopeptidase (i.e. Cwlo), and  $\gamma$ -glutamyl transferase (GGT); and all of these can be altered to prevent/modulate  $\gamma$ -PGA degradation (Feng et al., 2014; Liu et al., 2018; Sha et al., 2019). Feng et al. (2014) report that the highest  $\gamma$ -PGA production (7.12 g/L) was obtained from the *pgds* and *cwlo* double-deletion strain NK-pc (a mutant of the glutamate independent strain *B. amyloliquefaciens* LL3), which was 93% higher than that of wild-type LL3 strain (3.69 g/L). The triple-gene-deletion strain NK-pgc showed a 28% decrease in  $\gamma$ -PGA production, leading to a yield of 2.69 g/L. Nonetheless, all of the mutants showed significantly higher molar masses compared to the wild-type strain (see Table 2). The same group reported that  $\gamma$ -glutamyl transpeptidase (encoded by the *ggt* gene) could be involved in the inhibition of cell elongation (Feng et al., 2014). Further, Mitsui et al. (2011) reports that the disruption of the cell wall lytic enzyme Cwlo affects the amount and molar mass of  $\gamma$ -PGA produced by *B. subtilis* natto (Xu et al., 2019).

Although differences in microorganismal synthetases are evident, it appears that the molar mass of  $\gamma$ -PGA produced by differing microorganisms (*B. licheniformis* 9945a, *B. subtilis* natto, *B. megaterium* WH320 and *B. subtilis* chungkookjang) can be modulated by the concentration of sodium chloride (Birrer et al., 1994; Ogawa et al., 1997; Ashiuchi et al., 2006; Kedia et al., 2010; Ashiuchi and Shimizu, 2015), (see Table 2). Given that  $\gamma$ -PGA should protect the cell from stress, it is understandable that, with an increase in toxic concentrations of osmotically disruptive compounds, the cell triggers the production of higher molar mass  $\gamma$ -PGA (Shimizu et al., 2007; Hezayen et al., 2001; Quach et al., 2022).

It has been extensively reported that  $\gamma$ -PGA chains decrease in molar mass as the fermentation proceeds (Birrer et al., 1994; Ito et al., 1996; Ogunleye et al., 2015). Such behaviour has been attributed to the incremental synthesis of degradative enzymes, such as Pgds, Cwlo and GGT, as the fermentation progresses (Birrer et al., 1994; Ito et al., 1996; Ashiuchi et al., 2004; Mitsui et al., 2011; Feng et al., 2014; Cao et al., 2018; Xu et al., 2019). Such enzymatic action can be harnessed to decrease  $\gamma$ -PGA chain molar mass and polydispersity. The variation in molar mass, polydispersity, relative viscosity and D/L composition of  $\gamma$ -PGA synthesised by *B. subtilis* TAM-4 at various time intervals, has been further discussed by Ito et al. (1996).

Some have also investigated techniques for decreasing the molar mass of  $\gamma$ -PGAs following isolation of the polymer. Downstream techniques employed for such include: alkaline hydrolysis, ultrasonic degradation, and microbial or enzymatic degradation (Manocha and Margaritis, 2010; Luo et al., 2016).

Other fermentation parameters which affect the molar mass of  $\gamma$ -PGA include the concentration of dissolved oxygen, working volume and the stirring speed. Huang et al. (2011) suggest that within a lower working volume fermenter (40%) a relatively low agitation rate (<500 rpm) was sufficient to achieve dissolved oxygen (DO) levels above 10%. Lower agitations rates and higher DO levels were suggested to be beneficial towards the production of higher molar mass  $\gamma$ -PGA (Huang et al., 2011). Genetic and fermentative inputs which alter the enantiomeric composition and molar mass of  $\gamma$ -PGA chains have been summarised within Table 2.

### 3.3. $\gamma$ -PGA extraction from the fermentation media

The extraction process can enable selection of a material with a set physico-chemical properties (see Fig. 3). For example, L- or D-homopolymers of  $\gamma$ -PGA are soluble in ethanol, whereas an equimolar copolymer is not, and precipitates in ethanol (Candela and Fouet, 2006; Ogunleye et al., 2015; Luo et al., 2016; Kim et al., 2019b). Further, the complexity of the initial substrate might influence the purity and extraction process (Manocha and Margaritis, 2010; Luo et al., 2016).

Three main isolation techniques are employed for the extraction of the biomaterial from the aqueous portion of the media. In all cases, the first step is to remove the biomass from the fermentation broth through centrifugation or filtration through a 0.45  $\mu$ m filter (Manocha and Margaritis, 2010; Luo et al., 2016). If solid state fermentation is employed then the fermentation vessel is washed with sterile distilled water to dissolve  $\gamma$ -PGA before isolating it from the biomass with either centrifugation or filtration (see Fig. 3) (Bajaj and Singhal, 2011).

Commonly, solvent precipitation is one of the isolation techniques capable of providing the highest purity compounds, particularly important for medical applications (Candela and Fouet, 2006; Manocha and Margaritis, 2010; Ogunleye et al., 2015). However, the main disadvantages associated with these techniques include the cost of the solvent and the environmental burden associated with solvent disposal (Ogunleye et al., 2015; Luo et al., 2016). However, Manocha and Margaritis (2010) have shown that solvent isolation of  $\gamma$ -PGA leads to significant contamination with other proteinaceous material. To reduce both the cost of solvent extraction and the purity of the compound, tangential flow technologies have been assessed (Diorio and Stock, 1997; Kreyenschulte et al., 2012).

The second isolation technique is complex formation. Complex formation is one of the easiest and least expensive procedures involves the precipitation of  $\gamma$ -PGA through complex formation with  $Cu^{2+}$ ,  $Al^{3+}$ ,  $Cr^{3+}$ , and  $Fe^{3+}$  (Manocha and Margaritis, 2010; Ogunleye et al., 2015). Hisada and Kawase (2018) suggest that  $Cu^{2+}$  is the most efficient metal ion for selectively precipitating  $\gamma$ -PGA; this interaction has been shown to be the most effective even at a low concentration (Manocha and Margaritis, 2010; Luo et al., 2016). Manocha and Margaritis (2010) have demonstrated that addition of copper sulphate enables for 85%  $\gamma$ -PGA recovery with protein contamination limited to 3% when compared to

the 50% protein contamination observed with ethanol extraction. Unfortunately, this method cannot be employed for  $\gamma$ -PGA of any composition, as salts and oligomers of  $\gamma$ -PGA don't have enough available carboxyl groups capable of interacting with the metal ions and precipitate out of solution.

#### 4. $\gamma$ -PGA conformational properties

Similarly to other polymers, the behaviour of  $\gamma$ -PGA can be modulated by both polymer's chemical properties and its physical arrangement in solution (Fig. 5). In this section, variations in physical arrangements of  $\gamma$ -PGA will be discussed. Alteration in macromolecular arrangement of the polymeric chain, as a result of changing formulation environments, is of great importance towards understanding practical applications of the material (see Fig. 5 and Fig. 6). This section will focus on describing known and predicted behaviours of  $\gamma$ -PGA in varying conditions.

$\gamma$ -PGA's  $\gamma$ -peptidic bond structure lead to abundant availability of carboxylic groups. Chemically, such an abundance of available carboxyl groups allows for strong intramolecular association as well as strong affinity towards cations (if present in solution). As previously mentioned, such affinity is harnessed by producers to greatly increase organismal survival in otherwise toxic environments (high heavy metal concentrations or high salt concentration environments) (Ashiuchi, 2011). Further, differential stereochemistry can greatly affect intra and intermolecular interaction (Zanuy et al., 2000). For this reason, Understanding of such polymeric behaviours is pivotal in achieving desired formulation applications (Teraoka, 2002).

To understand and predict  $\gamma$ -PGA's 3D physical arrangement in solution, circular dichroism (CD) and infrared spectra investigations are two common analytical techniques employed (Wang et al., 2017).

In this section, current understanding of  $\gamma$ -PGA's behaviour at different concentrations, pH points and states (anionic state or salt state) has been presented.

As previously mentioned,  $\gamma$ -PGA's state differs based upon fermentation media properties, microorganism employed and pH. Generally, the combination of a low salt media and slightly acidic pHs result in the biosynthesis of  $\gamma$ -PGAs with a greater degree of free acids (H- $\gamma$ -PGA). In this state, four strong intra molecular hydrogen bonds existed between the -COOH group of the  $\gamma$ -peptide bond (pendant) and the -NH<sub>2</sub> group of the second  $\gamma$ -peptide monomer (see Fig. 5). Differently  $\alpha$ -helices of a poly- $\alpha$ -glutamic acid would present, on average, one hydrogen bond every 3.6 amino acid residues.

For  $\gamma$ -PGA with 100% of carboxyl groups acidified, the hydrogen bonds are designated with residue numbers 319, 317, 314 and 312 and formed within every 3 glutamic moieties (residues) instead of every 3.6 (See Fig. 5 - G). The tighter  $\alpha$ -helix conformation predicted by theoretical studies has been confirmed with Optical Rotatory Dispersion (ORD) studies, CD and infra-red studies. Such conformation results in an overall low interaction with solvent molecules and ultimately a strong hydrophobic character at low pH (Ho et al., 2006; Wang et al., 2017).

However, as pH rises, such motif is partially disrupted following the partial ionisation of the carboxyl group (from -COOH to -COO<sup>-</sup>). At pH 4.09, approximately 50% of the  $\alpha$ -helical motifs are still present, with the remaining portion of the chain loosing such secondary structure motif (See Fig. 5 - H). Once hydrogen-bonding is disrupted and the -COOH ionized,  $\gamma$ -PGA's conformation changes from the insoluble  $\alpha$ -helix (at pH = 2) into a soluble linear random-coil conformation. Such behaviour perpetuates, in an aqueous solution, until the pH reaches pH 6.0. In a pure water solution, at pH 6.5 or higher, poly- $\gamma$ -glutamic acid exists in linear random-coil conformation (no hydrogen bonding occurs) and all carboxyl groups change into free pendant anionic groups (NH<sub>2</sub> and COO<sup>-</sup> residues) (Ho et al., 2006). In general, the solubility increases as the pH of the media increases, resulting from the increase in the ionisation of -COOH groups. Such ionisation breaks the hydrogen bonding between the -COOH group of the  $\gamma$ -peptide bond (pendant) and

the -NH<sub>2</sub> group of a second  $\gamma$ -peptide monomer and increases molecular extension of  $\gamma$ -PGA chains. For  $\gamma$ -PGA, such increase in chain extension leads to increases in the intrinsic viscosity of the polymer as less intra-chain interaction occurs and greater chain interaction is established with water molecules. Such behaviour is maintained until the pH reaches 8.0 (Ho et al., 2006; Wang et al., 2017).

Differently, Wang et al. (2017), report that, in aqueous solutions presenting cations (i.e. sodium ion(s) – instead of hydrogen ions), the rod-like molecular structure morphs into a higher volume, sphere-like structure. This topic is further explored in the following sections.

When an aqueous solution presents cationic salts,  $\gamma$ -PGA does not form tight helical structures in acidic conditions, but behaves more like open random-coil chains, with greater solubility in solution (See Fig. 5 - D). Cations ionically interact with the -COO<sup>-</sup> pendant groups and prevent inter-chain hydrogen bond formation between -COOH group of the  $\gamma$ -peptide bond (pendant) and the -NH<sub>2</sub> group of a second  $\gamma$ -peptide monomer (See Fig. 5 - L). The overall reactive state of the  $\gamma$ -carboxyl group, in each of the glutamic acid moieties, is available for binding to the cationic entity of the other molecule or biopolymer, or for further modification as a free carboxylic acid (Ho et al., 2006).

The calcium salt of  $\gamma$ -PGA is very soluble in water, even at neutral pH. Such behaviour is largely attributed to the extended conformation (reduced hydrogen bonding between -COOH and NH<sub>2</sub> groups) as well as interaction of the ion with the negative dipole of the oxygen of the solvent molecule (See Fig. 5 - L). Ho et al. (2006) suggests that the characteristic coordinated complex ionic structure between calcium ion and poly( $\gamma$ -glutamate) ion largely contributes to the solubility and stabilizes the calcium ion in aqueous or body fluids at neutral pH conditions. The aforementioned complex can have important significance in health-care functionalities such as providing calcium bio-availability and facilitating the calcium absorption and bone formation in osteoblast cells or even reducing the osteoporosis conditions and the over-all growth and health conditions (Ho et al., 2006).

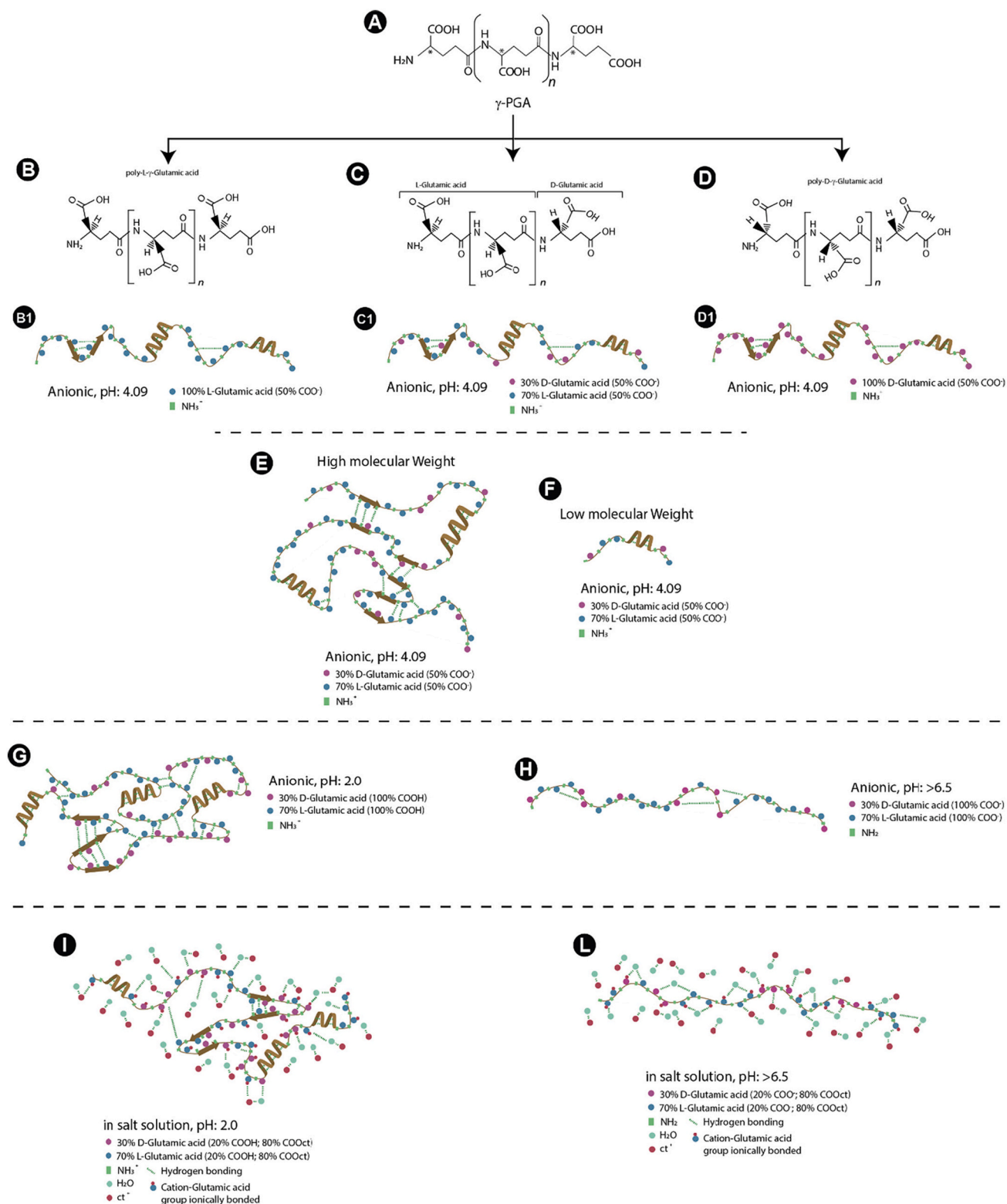
The linear random-coil conformation of poly- $\gamma$ -glutamic acid changes into chelated enveloped aggregate state upon binding with heavy metal ions, but changes into soluble coordinated ionic complex conformation upon binding with either calcium or magnesium ions. The strong hydrogen bindings and conformational changes of poly- $\gamma$ -glutamic acid make it a novel multiple-functional biopolymer with chemical characteristics suitable for application in diversified fields (Ho et al., 2006).

Nonetheless, any variation in  $\gamma$ -PGA's concentration in solution can significantly impact its ionised and unionised self-arrangement (Teraoka, 2002).

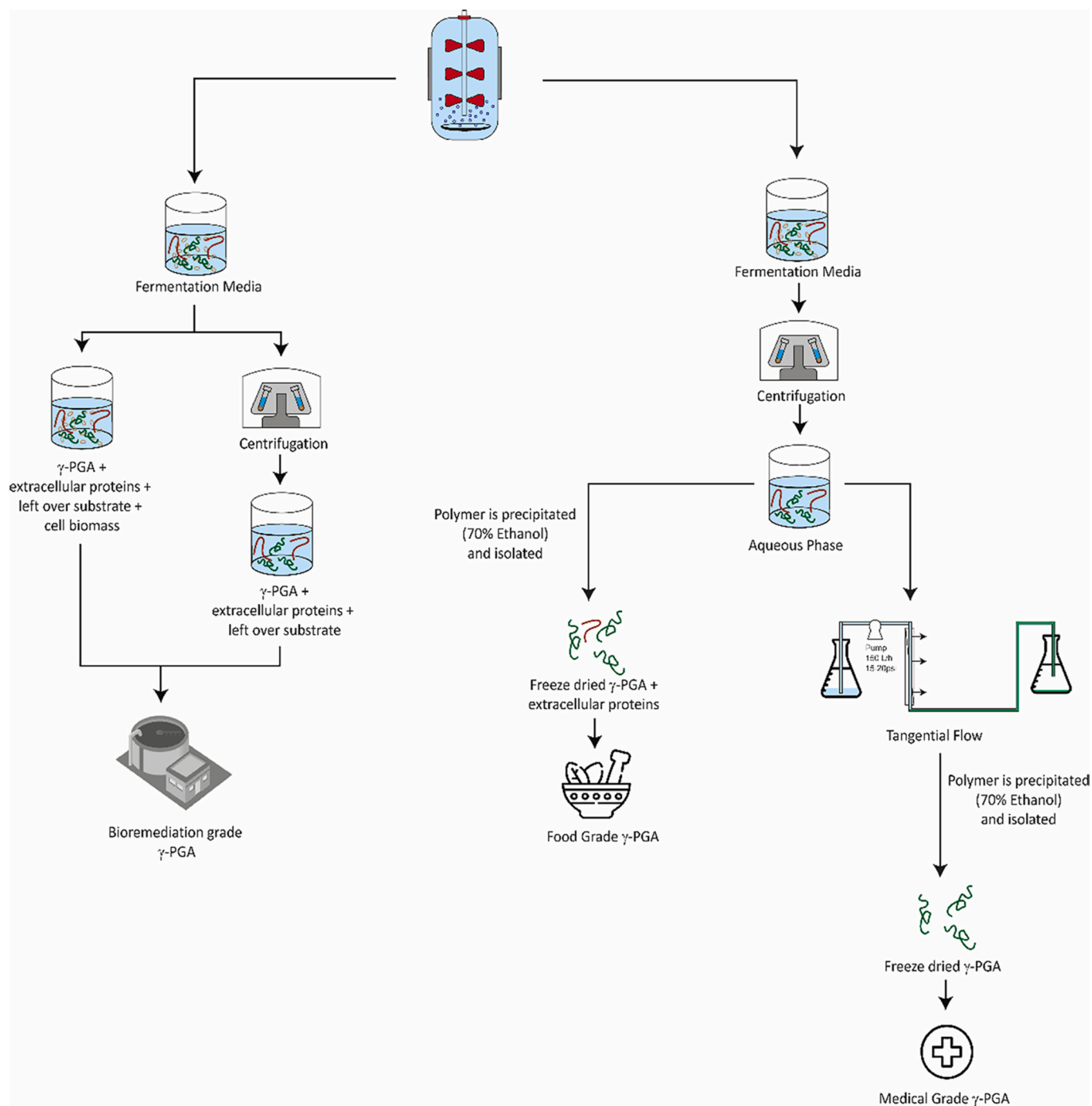
In dilute solutions,  $\gamma$ -PGA molecules behave independently and/or with other ions (as previously described). In these conditions, the polymer interacts maximally with solvent molecules (See Fig. 5). Differently, at higher polymer concentrations, the polymer chains overlap/interact and essentially become entangled. This physical interaction is initiated by inter-molecular interactions between -COOH and NH<sub>2</sub> groups of different polymeric chains (Wang et al., 2017).

#### 5. The cost of production

Currently, although  $\gamma$ -PGA has been found suitable for applications across an array of sectors, the main obstacle towards wide commercialisation of this polymer is the cost associated with the production of the polymer. As previously mentioned, substitution of the costly defined media with complex media/waste streams, attempts to reduce the cost of biosynthesis as well as increase the sustainability of its production. The art of employing complex waste media as substrate for polymeric biosynthesis revolves around the use of local and seasonal waste for the bioprocess. This makes the practice both sustainable and cheap. In Table 3 different complex waste media employed for  $\gamma$ -PGA biosynthesis have been presented and their relative yields and costs compared. The scientific literature presented within Table 3 suggests that the use of an



**Fig. 5.** Chemo-physical arrangement of  $\gamma$ -PGA in different aqueous solutions. [A] chemical structure of  $\gamma$ -PGA with \* indicating chiral centres [B] chemical structure of a L- $\gamma$ -PGA homopolymer [B1] physical arrangement of a L- $\gamma$ -PGA homopolymer in an aqueous solution (without cations) [C] chemical structure of a D,L- $\gamma$ -PGA co-polymer (30:70 D:L) [C1] physical arrangement of a D,L- $\gamma$ -PGA co-polymer (30:70 D:L) in an aqueous solution (no cations) [D] chemical structure of a D- $\gamma$ -PGA homopolymer [D1] physical arrangement of a D- $\gamma$ -PGA homopolymer in an aqueous solution (no cations) [E] physical arrangement of high molar mass D,L- $\gamma$ -PGA (30:70 D:L) in an aqueous solution (without cations) [F] physical arrangement of low molar mass D,L- $\gamma$ -PGA (30:70 D:L) in an aqueous solution (without cations) [G] physical arrangement of a D,L- $\gamma$ -PGA chain (30:70 D:L) in an aqueous solution (without cations) at pH 2 (100% COOH groups) [H] physical arrangement of a D,L- $\gamma$ -PGA chain (30:70 D:L) in an aqueous solution (without cations) at pH > 6.5 (100% COO<sup>-</sup> groups) [I] physical arrangement of a D,L- $\gamma$ -PGA chain (30:70 D:L) in an aqueous solution (with cations) at pH 2 (20% COOH groups, 80% COOct) [L] physical arrangement of a D,L- $\gamma$ -PGA chain (30:70 D:L) in an aqueous solution (with cations) at pH > 6.5 (20% COO<sup>-</sup> groups, 80% COOct).



**Fig. 6.** Application-specific downstream processing. Variation in  $\gamma$ -PGA purification strategy depending upon the grade required for bioremediation, food application or medical applications. For bioremediation use, a cheap biomass/polymer rich media or the polymer rich media can be employed; for food applications a cell free, low non- $\gamma$ -PGA contaminated powder is preferable; whereas an ultra-pure  $\gamma$ -PGA powder is required for medical grade applications. Precipitation and isolation of the polymer would involve the use of ethanol and centrifugation respectively.

untreated cane molasses + Mono Sodium Glutamate waste combination yielded a 82.9% cost reduction compared to standard GS media (Zhang et al., 2012; Kim et al., 2019b). The cost per Kg to produce  $\gamma$ -PGA reported by Tang et al. (2015b), by employing a combination of Industrial waste glycerol, Dry mushroom residues and Mono Sodium Glutamate production residues was reduced 97.6% cost compared to standard GS media (as presented by Kim et al. (2019b). In general, the various waste sources summarised within Table 3 suggest great cost reduction compared to the standard GS media. Nonetheless, the actual composition of the material synthesised has not always been reported, and thus

its suitability might be limited to a specific set of applications. At present, a number of known  $\gamma$ -PGA manufacturers have been identified, these have been summarised within Table 4.

## 6. Applications of $\gamma$ -PGA

The ever increasing industrial interest in  $\gamma$ -PGA has been largely attributed to the versatility of the polymer, for which numerous applications have been studied. In literature,  $\gamma$ -PGA has been used as anti-freeze (Bhat et al., 2013), thickener (Shyu et al., 2008; Shyu and Sung,

**Table 4**  
Main  $\gamma$ -PGA producers, their capacity and sector of interest.

Company	Product	Main characteristics	Production capacity (tons/year)	Sector of interest
Polyglu Ltd.	PG $\alpha$ 21Ca	Molar mass not specified	Not specified	Multi Functional High Polymer Flocculant
Crescent innovations Inc.	PG-M PGA-OA	Molar mass not specified	Not specified	magnetic body polymer flocculant Medical – Osteoarthritis
YR ChemSpec Technology Co., Ltd.	Polyglutamic acid	Molar mass > 700KDa 70-100KDa <10KDa	Not specified	Cosmetics
Bonding chemical Co.,Ltd.	Polyglutamic acid, product code: 439522	Mw = 1,100,000 Mw = 200,000 Mw = 100,000 Mw = 10,000 Mw = 2,000,000	Not specified	Food / cosmetic
Sancai Industry Co., Ltd.	Polyglutamic acid Product code: L-103,107,110	Available from 10,000 to 2,000,000 Da	10 Million tons/month	Cosmetics
	3.5% , 7% , 10% $\gamma$ -PGA liquid formulation Product code: L-203,207,210			Liquid, quick absorption, plant biostimulant
Shandong Freda Biotechnology Co., Ltd.	3.5% , 7% , 10% $\gamma$ -PGA liquid formulation Product code: S-230	Molar mass not specified.	Not specified	Liquid, Long-action slow-release, plant biostimulant
	30% concentrated powder Product code: C-110 10%, liquid formulation			Concentrated plant biostimulants
	OEM-1	Customisable concentration of $\gamma$ -PGA		Improving pesticide utilization rate, Micro element chelator, plant biostimulant
Creative biolabs Inc.	Not applicable	Not specified	Not specified	Cosmetics

2010), food and feed additive (Tanimoto et al., 2001; Ho et al., 2009; Tanimoto, 2010), as a filtration membrane (Bhattacharyya et al., 1998; Hajdu et al., 2012), as a bioremediation agent (Inbaraj et al., 2006; Bai et al., 2022; Deol et al., 2022), humectant (Choi and Kunioka, 1995), as medical material (Ye et al., 2006), for cosmetics (Ben-Zur and Goldman, 2007), as thermoplastic (Borbely et al., 1994), and for pharmaceutical applications (Hsieh et al., 2006; Kurosaki et al., 2010; Uotani et al., 2011; Bhat, 2012; Hsueh et al., 2017). Application of  $\gamma$ -PGA specific to its physico-chemical properties have been discussed in the following sections.

### 6.1. Applications of low molar mass $\gamma$ -PGA

As previously mentioned, chemical variations in  $\gamma$ -PGA's structure significantly impact the physical properties of the resulting material. Lower molar mass  $\gamma$ -PGA oligomers (45 to 60 kDa) can be functionalised in order to increase effectiveness of active compound delivery. Delivery of active pharmaceutical ingredient (API) loaded onto  $\gamma$ -PGA hydrogels or through  $\gamma$ -PGA conjugation presents four main advantages (Singer, 2005): firstly, compounds of above 50 kDa can accumulate (passively) within tumour tissue as a result of the tissue's increased permeability and retention. Molecules above 50 kDa in size cannot be processed by the kidney, and thus, the overall concentration of the conjugate is increased within tumour tissue. Secondly, the pharmacokinetic profile of the conjugate is increased as a result of pulsatile API release. Thirdly, if a polymeric matrix is employed as a delivery system, the staggered API diffusion is limited to tumour tissue, decreasing toxicity of the agent and increasing its availability. Fourthly, formulation of hydrophobic API with toxic solubilising agents is not required, thereby increasing the potential dose of administration. Ye et al. (2006), have reported that, the conjugation of cisplatin (cis-dichlorodiammineplatinum (II)) to  $\gamma$ -PGA, increased its solubility, reduced the toxicity of the cisplatin derivative and decreased the tumour size of xenografted human breast in nude mice. Human cell uptake of  $\gamma$ -PGA (30 kDa) has been shown through interaction with  $\gamma$ -glutamyl transpeptidase; a membrane protein (Liao

et al., 2012). Association of  $\gamma$ -PGA with membrane properties allows for far superior delivery of molecules intracellularly, in fact, Dai et al. (2018) has demonstrated the ability of stereo-complexed  $\gamma$ -PGA-graft-PLA micelles to provide far greater intracellular anti-cancer drug delivery. In addition to drug delivery,  $\gamma$ -PGA oligomers (>20 kDa) have also demonstrated adequate cryoprotective properties, without compromising the organoleptic characteristics of the food investigated (Mitsuiki et al., 1998).

### 6.2. Applications of high molar mass $\gamma$ -PGA

On the other hand, high molar mass  $\gamma$ -PGA (>1000 kDa) behaves much differently in solution, and is capable of arrangement in numerous different ways. Such self-arrangement capability was shown to improve cryoprotective properties towards probiotic organisms both during lyophilisation and simulated gastrointestinal tract passage (Adebayo, 2018; Bhat et al., 2013). Similar protective properties of  $\gamma$ -PGA were observed following coating/fixation of enzymes (Yamasaki et al., 2010; Yasuzawa et al., 2011). High molar mass  $\gamma$ -PGA has also been shown to have remarkable potential towards wastewater treatment. In fact, Shih and Van (2001) presented flocculation capabilities of  $\gamma$ -PGA towards an array of organic and inorganic compounds. Bhattacharyya et al. (1998) developed a microfiltration membrane with  $\gamma$ -PGA bound to membrane's pore surface. The latter model presented significant heavy metal absorption capabilities. Further, Inbaraj et al. (2006) discussed the potential of  $\gamma$ -PGA towards sequestration of basic dyes as well as recovery of these, following solution acidification to pH 1. High molar mass  $\gamma$ -PGA presents remarkable oil recovery properties however, the costs associated with biosynthesis of the material might be prohibitive for such application (Azarhava et al., 2020). In this respect, for harnessing  $\gamma$ -PGA as a bioremediation agent, cheaper downstream processing could be employed instead of expensive organic extraction procedures.

### 6.3. Applications of $\gamma$ -PGA with varying D/L monomeric ratios

Contrary to  $\gamma$ -PGA's role as flocculating agent and cryoprotectant, the D/L monomeric ratio significantly affects its suitability for both cosmetic and dental application (Ogunleye et al., 2015). A high L-glutamic acid polymer is preferred for the former, whereas high D-glutamic acid concentrations are required for the latter. In fact, the high water retention observed with predominantly L-glutamic acid rich biocomposites provides suitable humectant properties for cosmetic application (Hasebe and Inagaki, 1998; Ho et al., 2005; Ben-Zur and Goldman, 2007) whereas it has been shown to be inadequate as a dental filling (Ogunleye et al., 2015). The D/L enantiomeric ratio of  $\gamma$ -PGA has not shown to modulate interaction with ions as these present similar physical properties. In this respect, the material can be employed to increase the solubility of calcium – beneficial for bone density, or to increase absorption of minerals for improved plant growth (Zhang et al., 2017) and eggshell strength, reduced livestock body fat and reduction in phosphorus excretion (Saito et al., 1974; Tanimoto et al., 1996; Tanimoto et al., 2001; Ho et al., 2009).

### 6.4. Bioremediation

Depending on the use of the material, the characteristics and purity of the polymer might vary. For polymers destined towards bioremediation purposes, a high degree of purity might not be necessary. Although Hajdu et al. (2012), Bajaj and Singhal (2009), Bhattacharyya et al. (1998), Inbaraj et al. (2006) and other attained great bioremediation results by employing pure  $\gamma$ -PGA chains for the removal of dyes, heavy metals and other pollutants; the polymer might fulfil its function with partial contamination from its producers (cells) and other soluble protein/fermentation media matter. For such applications, the semi-purified polymer could still be capable of effectively sequestering heavy metal cations. In fact, the great advantages in this case would include: the significantly lower cost of the polymer (per Kg), the green biosynthesis (no organic solvents employed for polymer isolation) and therefore the greater competitiveness of this solution against other non-biological alternatives. To achieve a polymer with such properties, the supernatant would be subjected to a centrifugation cycle (10,000 rpm for 30 or more minutes depending on the viscosity of the solution) and subsequently the supernatant would be employed for bioremediation purposes. This has been schematically illustrated within Fig. 6.

### 6.5. Food grade

For food related application, the polymer is purified by adding 3 to 4 parts of ethanol (or other primary alcohols) to the supernatant. In most cases, the resulting  $\gamma$ -PGA precipitate will present some organic residue used for fermentation media, however, such residues are completely safe for human consumption (Pradhananga, 2018). The isolation procedure proposed has been schematically illustrated in Fig. 6.

The applications for which food grade  $\gamma$ -PGA would be employed include: nutritional additive to promote calcium absorption for prevention of osteoporosis (Tanimoto et al., 2001; Tanimoto et al., 2007), additive to increase the viscosity of liquid foods and as a stabilizer to improve the texture and taste of baked, fried, or frozen foods and beverages (Tanimoto, 2010; Hsueh et al., 2017) Bitterness relieving agent (Sonoda et al., 2000), thickener (Yamanaka, 1991; Shih and Van, 2001) as oil reducing agent (Lim et al., 2012) and as an antioxidant (Quach et al., 2022).

### 6.6. Medical grade

Although most of  $\gamma$ -PGA producers do not threaten human health, contamination of  $\gamma$ -PGA with water soluble proteins and fermentation media residues (He et al., 2022) could limit its use in medical-grade application. The relatively lower degree of purity observed with food

grade  $\gamma$ -PGA, cannot satisfy the tight, high-purity criteria required by the medical sector. As such, to remove unwanted proteinaceous aqueous fractions, a membrane based technique can be employed. Tangential flow is a membrane purification technique which selectively allows permeation of medium-or-high molar mass  $\gamma$ -PGA fractions, as displayed within Figure (Diiorio and Stock, 1997; Ogunleye et al., 2015). Medical applications of  $\gamma$ -PGA vary significantly from direct to indirect; some of these have been described below.

Differently to other synthetic/semi-synthetic glues,  $\gamma$ -PGA based suturing glues could provide adhesive properties (lacked by fibrin glue) without the drawbacks commonly observed by synthetic/semi-synthetic glues (cyanoacrylates, urethane pre-polymers, gelatin-resorcinol-formaldehyde) which include cytotoxicity, low degradation rates and chronic inflammation (Otani et al., 1999; Shih and Van, 2001). In addition, Xue et al. (2021) showed that a  $\gamma$ -PGA (442,000 Da) -co- Chitooligosaccharide -co- papain hydrogel inhibited excessive collagen deposition as well as the generation of hyperplastic scars effectively during wound healing, holding promise for solving numerous skin disorders.  $\gamma$ -PGA displays a great degree of versatility thanks to its ability to associate with other organic compounds and form complex structures. Recently, Wei et al. (2021) described the development of novel  $\gamma$ -PGA based, highly tunable, injectable, hydrogel systems with furfurylamine and tyramine-modified  $\gamma$ -PGA and a dimaleimide poly(ethylene glycol) (MAL-PEG-MAL) crosslinker.

$\gamma$ -PGA has also shown remarkable properties as a modulator of insulin sensitivity in adipocytes and neuronal cells (Lee et al., 2010; Jeong et al., 2018) and towards the stimulation of hair growth following complexation with promoting factors (Kim et al., 2019a). In addition to the known delivery properties (Akagi et al., 2010; Kurosaki et al., 2010; Dai et al., 2018), Association of  $\gamma$ -PGA with chitosan also presents bioink capabilities (Pisani et al., 2020).

Recently it was shown that  $\gamma$ -PGA can effectively inhibit enamel demineralisation (Qamar et al., 2016; Qamar et al., 2019; Parati et al., 2022). Effectively, Parati et al. (2022) suggest that  $\gamma$ -PGA's -COOH groups can bind to teeth enamel and prevent calcium dissociation in acidic environments. Additionally, Su et al. (2021) showed that a  $\gamma$ -PGA/tricalcium phosphate composite is able to promote inter- and intratubular dentin remineralization, holding great promise in childhood caries treatment.

Other benefits of  $\gamma$ -PGA include antifungal activity (Dinh et al., 2017), antibacterial activity (Stephen Inbaraj et al., 2011; Su et al., 2019), inhibition of influenza viruses (Ogata et al., 2009), lubricating agent for contact lenses (Su et al., 2020), metal chelation (Inbaraj and Chen, 2012), tissue engineering (Tsao et al., 2011; Gonçalves et al., 2012) and vaccine adjuvant (Ahn et al., 2016).

## 7. Future outlook

Novel materials hold an immense potential to fulfil the ambitions of a green dreaming industry, nonetheless, bioderived biodegradable and non-toxic materials are yet to compete with conventional materials both in terms of cost and properties. To that end, the topics discussed throughout this review have focused on the potential of  $\gamma$ -PGA and the methods involved in rendering  $\gamma$ -PGA competitive for both properties and cost. Unfortunately, when it comes to bioderived materials, the standard-demanding, chemistry-driven industry has to adapt to some chemical variability faced by employing biosynthesised materials.

For  $\gamma$ -PGA, and other biopolymers, to displace non-renewable counterparts; specific property/purity requirements have to be evidenced for each application. To do so, appropriate fermentation conditions can be employed to obtain application-specific properties. Competitiveness of  $\gamma$ -PGA against synthetically derived polymers can also be achieved through a purity specific application approach.

Apart from the applications already identified,  $\gamma$ -PGA derivatives could also provide sustainable solutions towards other sectors. Yu et al. (2021), for example, are expanding the application of bioderived,

biodegradable materials by co-formulating these with other organic and/or inorganic materials. In one of the groups' most recent published works, they present the co-formulation of  $\gamma$ -PGA with bacterial nacre, to create novel materials with much higher strengths.

### Declaration of Competing Interest

None.

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