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Microbial production of poly (γ-) glutamic acid and its use as an anti-thawing agent and as prebiotic

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Abstract

Poly-γ-glutamic acid (γ-PGA) is a natural, edible, non-toxic, non-immunogenic, and an anionic homo-polyamide, produced by various microorganisms. At an industrial level, for preparing probiotic foods, probiotic cultures are added in powdered forms. Here, cultures are formerly freeze-dried. Freeze-drying process is itself very detrimental to the viability of such organisms as it utilizes very low temperatures of -80 °C. A bacterial strain was isolated from fertilized soil and was statistically optimized in this study. Upon optimization using Plackett-Burman model, a significant increase in the yield from 4.51 ± 0.32g/L to 19.0 ± 0.5g/L was observed. To investigate the cryoprotectant activity during freeze-drying process, *Lactococcus lactis* was freeze-dried and was subjected to a series of thawing temperatures. Cultures where then treated with a polyelectrolytic combination of γ-PGA (anionic polymer) and Gelatin (cationic polymer). This combination showed a synergistic effect in protecting *Lactococcus lactis* during freeze-drying wherein a log reduction of only 0.23 was noted, compared to unprotected cells, 2.85. In addition to this, the prebiotic activity of γ-PGA was also assessed in vitro. It was found that there was a significant increase in the growth of *Lactococcus lactis* (32.14%) considering the effect of γ-PGA alone. Thus, the combinatorial effect of this biopolymer i.e., as a cryoprotectant and as a prebiotic can definitely bring out a beneficial probiotic product into the market with improved health effects.

Keywords: Poly-γ-glutamic acid; γ-PGA; Freeze-drying; *Bacillus thuringiensis*; *Lactococcus lactis*; Probiotic; Prebiotic

1. Introduction

Poly-γ-glutamic acid (γ-PGA) is a natural, biodegradable, non-toxic, non-immunogenic and, water-soluble anionic homopolyamide with repeating units of D- and L-glutamic acid units [29]. γ-PGA being a natural biopolymer has received much attention in the last decade, since it is substantially produced by many microorganisms chiefly by *Bacillus* species by biofermentation using different media that use simple constituents.

γ -PGA was first discovered by Ivonovics and Bruckner (1937) from a capsule of *Bacillus anthracis* [29]. It was also found to be one of the major constituents of natto, which is a traditional Japanese dish made from fermented soybeans. γ-PGA gives it a slimy, sticky, and stringy texture. Owing to the fact that γ-PGA is a natural biopolymer, it has been extensively used in many areas such as medicine, agriculture, eco-friendly products, wastewater treatment, and in the food industry [26]. The popularity of this biopolymer has motivated extended research in strain improvement, bioprocess engineering for producing γ-PGA, and advancements in certain analytical methods and applications. There are certain features of γ-PGA that will still remain a challenge. On the other hand, it may provide researchers with a lot of scope to seek deeper insights into the behavior of these biomolecules and thus pave the way towards optimizing new products in the market industry.

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Probiotic products like yogurt, fruit juices, milk, and fermented-based products are becoming more and more popular. The introduction of bacteria into the foodstuff as dry cultures and its storage is one of the most significant production procedures for making a probiotic food product. Working with dry cultures is preferable to working with wet cultures since they are easier to handle and have a longer shelf life [3, 12, 14]. However, health benefits solely rely on the viability of these probiotic cultures and on the maintenance of stock cultures during storage and transportation. Even though there are various techniques like freeze drying (the most popular method for creating dry bacterial powders) is used for probiotics, but it has been documented that there is always a loss of probiotic culture viability during the freeze-thaw process [15]. Furthermore, the methods used to prepare freeze-dried probiotic bacteria were often found to be detrimental to cell structure and viability [7, 8]. It has been previously shown that the viability of lactic acid bacteria reduces by 3 log CFU/g upon freeze drying [15]. Thus, in order to prevent the loss of culture viability cryoprotectants are now commonly used [27]. In recent years, there has been extensive work carried out with cryoprotectants applied to various types of food and cultures.

The freeze-thaw process is a major hurdle in many food industries, especially food products like probiotics. Cryoprotectants include all compounds that help to prevent deleterious effects in food and beverages caused by such repeated freeze-thaw processes. Taking into consideration the commercial importance of probiotics, γ-PGA will be used as a cryoprotectant. γ-PGA is known to protect prebiotic cultures significantly better than 10% sucrose (a widely used cryoprotectant) [8].

There are various sources to isolate γ-PGA producing microorganisms like food, especially natto. γ-PGA is one of the biopolymers produced by several *Bacillus* species, which help bacteria survive under harsh conditions by adhering to each other [25]. Thus, such soil would act as a good source to isolate γ-PGA producers. Also, Wang *et al.* (2008) found that *γ*-PGA can significantly increase the dry weight of both roots and shoots and the roots to shoots ratio (R/S).

Topsoil (TS), is the upper layer of soil, usually between 2 to 8 inches in depth, that contains most of the ground's nutrients and fertility. There are various synergistic *γ*-PGA producers that help contribute to soil fertility [5]. On the other hand, rhizospheric soil (RS) is subjected to the influence of diverse microbial communities like symbiotic microorganisms that contribute to plant growth [22]. *Trigonella foenum-graecum* is one of them that shows the presence of nodules that are sites of nitrogen fixation by these symbiotic microorganisms.

Very recently, polyelectrolyte complexes (PEs) that include a polyanion (γ-PGA) and a polycation have been widely used. The complexation occurs by a self-assembly process through electrostatic bonds present between these two polymers. Complex formation is thus a simple and versatile process, which uses mild conditions. It allows the incorporation of any molecules (drug, vaccine, protein, or genes) within their structure. Here probiotic cultures can be incorporated [2]. Thus, an attempt was made to understand a major application of γ-PGA-Gelatin PEs as a cryoprotectant by incorporating the probiotic cultures within the γ-PGA-Gelatin complex. This will efficiently protect them from the freeze-thaw process or other detrimental conditions.

Moreover, since γ-PGA is edible and generally regarded as safe, a study was carried out in vivo wherein mice were injected with a specific dosage of γ-PGA. It was found that the decrease in the number of *Clostridium* species corresponds to the increase in the level of *Lactobacillus* [17]. In laboratories, we demonstrated this activity in vitro while critically observing a type of LAB (Lactic Acid Bacteria) cultures growing in presence of this γ-PGA.

Thus, we hope that the combinatorial effect of γ-PGA as a cryoprotectant and having a prebiotic activity will definitely bring out a beneficial product into the market with improved health effects.

2. Material and methods

2.1. Sample collection

A total of 4 types of soil samples were collected. Topsoil from a depth of 5 cm was collected, this included, 3 plants (2 non-fertilized and 1 fertilized soil- Organic phosphate based was added 3 weeks before). Such a source was taken into consideration since many γ-PGA producers were found to be phosphate solubilizers. Rhizospheric soil was collected from *Trigonella foenum-graecum* (Methi) roots.

2.1.1. Primary screening

Each soil sample was weighed (1 g) and diluted by 10-fold and isolated on Nutrient agar medium supplemented with 0.5% L-Glutamic acid. pH of the medium was adjusted to 7.2 ± 0.2 by using 2N NaOH and/or 2N HCl. The medium was further autoclaved for 20 min at 121 °C. Plates were incubated at 37 °C for 24 h. *Bacillus cereus* (ATCC® 11778™), a well-known organism for producing γ-PGA, was taken as a positive control (PC) for all the experiments.

2.1.2. Secondary screening

Herein, a property of γ-PGA as a dye remover/ dye absorber was being made in use.

Part I: Distinct colonies with mucoidal texture were selected for a second-level screening. Colonies were inoculated into a Nutrient Broth medium supplemented with 0.5% L-Glutamic acid. After an incubation for 24 h at 37 °C, tubes were centrifuged for 12 min at 3000 rpm to obtain a cell-free supernatant [32].

Part II Agar diffusion: a drop of 0.02% of Methylene Blue was added into 1.3% agar. 50 µl of the supernatant obtained from Part I, was added into the wells. Plates were incubated at room temperature for 48 h [32].

2.2. Production and isolation of γ-PGA biopolymer using fermentation method

2.2.1. γ-PGA production in Fermentation medium

After the successful screening of γ-PGA producing microorganisms, production of γ-PGA in high yield was carried out by a simple fermentation method. γ-PGA was produced as described previously by Xavier *et al.,* 2020 with some modifications. The medium comprised of (in % w/v) 1.5 L-Glutamate, 2 Glucose, 1 Casein hydrolysate, 2 Glycerol, 0.05 $MgSO_4$, 0.5 $(NH_4)_2SO_4$, 0.015 $CaCl_2$, 1 Yeast Extract and 0.05 KH_2PO_4. Briefly, 1% of adjusted density culture suspension (approximately $3*10^8$ CFU/ml density adjusted by McFarland standards) was cultured in a γ-PGA production medium (250ml capacity Erlenmeyer flasks) for 2 days. Tubes were centrifuged for 15 min at 3000 rpm to obtain a cell-free supernatant. Further, chilled ethanol was added to the supernatant (Ratio of supernatant to ethanol, 1:3 ml) and all the tubes were maintained at 4 °C overnight for precipitation to occur.

2.2.2. Precipitate purification

Solvent was decanted, and 5 ml distilled water was added to the precipitate. This mixture was boiled for 25 min at 100 °C to denature unwanted proteins. This was resuspended in 5 ml distilled water and autoclaved for 20 min at 121 °C and re-precipitated using an equal volume of ethanol [9]. The precipitate thus obtained was processed for γ-PGA detection and chemical analysis.

2.3. Detection and quantification of produced γ-PGA biopolymer

2.3.1. Ninhydrin chemical assay (For standards)

Standard concentrations of L-Glutamate ranging from 20-100 µg/ml were taken into consideration. Distilled water was used to prepare different concentrations of this amino acid. Once prepared, 1 ml of 1% Ninhydrin reagent was added to each tube, and the tubes were kept in a boiling water bath for 15 min. The tubes were cooled and O.D. (Optical Density) was determined at 570 nm. *(SIGMA ALDRICH Product information, product number: N 7285).*

2.3.2. Sample preparation for chemical assay

Acid hydrolysis using an equal amount of 6N HCl was performed. In order to initiate hydrolysis, tubes were incubated at 100 °C for 6 h in a hot air oven. The next day, the hydrolysate was neutralized with 6 M NaOH. The quantity of glutamic acid in the solution was determined using ninhydrin with L- glutamic acid as a standard. The amount of PGA is given as glutamic acid equivalent to the difference between the hydrolyzed and unhydrolyzed samples [18].

2.4. Optimization of γ-PGA production

2.4.1. Optimization using "One-variable-a-time" (OVAT) method

Only one factor i.e., carbon source was kept varying. Rest all other variables were kept constant. The two important carbon sources for the formation of γ-PGA, glucose (2%) and rice bran (2%) were taken into consideration. Other ingredients as stated in section 2.2.1 were added along with the carbon sources. The best source was evaluated in order to utilize that particular carbon source for the Plackett-Burman design method of optimization.

2.4.2. Optimization using "Plackett-Burman Design" method

An experimental design with 7 factors and 8 runs was set up in order to screen for the most important factor component that is influencing γ-PGA production using Plackett-Burman Design. It was based on the first-order model.

Two-factor levels i.e., a low level (-1) and a high level (1) was taken into consideration for each independent nutritional variable (Table 1). Herein, rows represent the trials and columns represent the variable-independent factor. Along with this, two dummy variables were also included, raising the number of trials under study from n to n+1 (Table 2).

Table 1 Variables considered for Plackett-Burman design method

Sr. No.	Factor name	Code	Levels (g%)	
			-1 (Low value)	1 (High value)
1	L-Glutamate	X1	0.50	2.00
2	Ammonium Chloride	X2	0.50	1.00
3	Ferric Chloride	X3	0.02	0.08
4	Magnesium Sulphate	X4	0.01	0.05
5	Dummy variable 1	X5 (D1)	-	-
6	Glycerol	X6	1.00	4.00
7	Dummy variable 2	X7 (D2)	-	-

Table 2. Base design for 7 factor 8 run Placket-Burman first order experimental design for γ-PGA production

Trials/Runs	Variables						
	X1	*X2*	*X3*	*X4*	*X5 (D1)*	*X6*	*X7 (D2)*
1	1	1	1	-1	1	-1	-1
2	-1	1	1	1	-1	1	-1
3	-1	-1	1	1	1	-1	1
4	1	-1	-1	1	1	1	-1
5	-1	1	-1	-1	1	1	1
6	1	-1	1	-1	-1	1	1
7	1	1	-1	1	-1	-1	1
8	-1	-1	-1	-1	-1	-1	-1

2.5. Applications of γ-PGA biopolymer

2.5.1. Probiotic strain and medium used

A bacterial strain of *Lactococcus lactis* NCDC 309 from National Dairy Research Institute, Karnal was used in the present study. The medium used for the growth and maintenance is M -17 medium *(HiMedia Lab Pvt Ltd).* Bacterial culture in agar slants was incubated at 37 °C for 24 h and stored at 4 °C.

2.5.2. Use of γ-PGA as an anti-thawing during the freeze-drying process of probiotic cultures.

To prepare cells for freeze drying, *Lactococcus lactis* were cultured anaerobically at 37 °C. After incubation, viable counts were performed on M – 17 agar media *(HiMedia Lab Pvt Ltd)* to determine the number of viable cells prior to freeze drying. The cultures were centrifuged (10,000 rpm for 6 min) and were dried at 30 °C for 18 h. Further, obtained cell pellets was resuspended in 1 ml solutions of γ-PGA (0.5% w/v), Gelatin (0.5%), and γ-PGA (0.25%) with Gelatin (0.25%). For cells without a cryoprotectant, 1 ml of sterile PBS (Phosphate Buffered Saline) was added. Treated and untreated cells were stored using Eppendorf® vials. The suspensions were incubated at room temperature for 1 h and then frozen at −80 °C for 24 h. The frozen cultures were then incubated at 0 °C for 72 h. Further, cells were enumerated by the Miles and Misra technique [24]. Plates were incubated at 37 °C for 24 h [8].

2.5.3. Analyses of γ-PGA as a prebiotic

In order to check whether γ-PGA has a prebiotic activity or not, briefly, density *of Lactococcus lactis* was adjusted to approximately ~3*10^8 cfu/ml using McFarland's Standards. Later on, enumeration was carried out to find the exact count. This initial count was evaluated using the Miles and Misra technique on the M-17 medium *(HiMedia Lab Pvt Ltd)*. Plates were incubated anaerobically at 37 °C for 24 h. 1% of the culture suspension was inoculated into two flasks. First flask with 10 g/L γ-PGA, 3g/L Yeast extract, and 5g/L Casein Peptone, and the second flask is the control flask with the above ingredients except for γ-PGA. The volume of each medium was adjusted to 50 ml. Flasks were incubated at 37 °C for 24 h. Enumeration was carried out using the Miles and Misra technique on the M-17 medium. Incubation at 37°C for 24 h.

2.5.4. Bacterial species identification

The statistically optimized γ-PGA strain was grown on Nutrient Agar plates and sent for analysis using the VITEK® -2 compact system (bioMérieux, vitek, Inc., Lyon, France).

2.6. Statistical analysis

The data generated was subjected to descriptive statistics using Minitab 20.3.0 software (computer statistical software) and Microsoft Excel Data analysis tool. Mean and Standard deviation were estimated for each experiment that was carried out in either duplicates or triplicates. For the Plackett-Burman model, the F-test value and Degrees of freedom were taken into consideration to calculate P-value. The p-value <0.05 was considered to be significant.

3. Results

3.1. Isolation of γ-PGA producing microorganisms from soil

3.1.1. Results of Primary Screening

Upon isolating on the primary screening agar medium (Nutrient agar supplemented with L-Glutamate) variations in colony characteristics were observed. These are summarized in Table 3 Colonies that appeared distinct with mucoidal texture were selected for the second-level screening test. From each

Table 3 Colony characteristics of isolates on Nutrient agar supplemented with L-Glutamate

Particulars	RS-1	RS-2	TS-1	TS-2	TS-3	TS-4	PC
Size	Pinpoint	Pinpoint	1 mm	3 mm	Pinpoint	2 mm	5 mm
Shape	Round	Round	Round	Round	Round	Round	Round
Color	Yellowish	Off-white	Off-white	Off-white	Yellowish	Off-white	White
Margin	Entire	Entire	Entire	Undulate	Entire	Undulated	Undulate
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Texture	Mucoidy	Slightly mucoidy	Slightly mucoidy	Highly mucoidy	Slightly mucoidy	Slightly mucoidy	Slightly mucoidy
Elevation	Elevated	Elevated	Slightly elevated	Slightly elevated	Elevated	Slightly elevated	Slightly elevated

3.1.2. Results of Secondary Screening

The isolates that showed positive results in case of primary screening method were taken forward for a final level screening that confirms the production of γ-PGA biopolymer. TS, RS, and *Bacillus cereus* showed a visible zone of clearance. The highest was observed for TS-2 isolate (29 ± 1mm) and the lowest for TS-3 (6 ± 1mm) and no zones were observed in the case of TS-1, TS-4, RS-2, and negative control. The table below (Table 4) highlights the isolates showing zones of clearances. These isolates were further selected as an inoculum for the γ-PGA production medium.

Table 4 Zone diameter of clearance around wells

Isolates	Mean ± SD* (mm)
TS-1	0
TS-2	29 ± 1
TS-3	6 ± 1
TS-4	0
RS-1	12 ± 1
RS-2	0
Positive Control (*Bacillus cereus*)	17.5 ± 0.5
Negative control (uninoculated media)	0

*Standard deviations for 2 determinations.

3.1.3. Production and isolation of γ-PGA biopolymer using fermentation method

Using an appropriate medium, the biopolymer was produced for all isolates under shaker conditions for 3 days. Upon adding ice cold ethanol to the culture-free supernatant of fermentation broth, the precipitate readily formed at 4ºC for 24 h. The topsoil isolate showed the highest amount of precipitate, followed by *Bacillus cereus* and rhizosphere isolate. And the clear solution was observed for the uninoculated fermentation broth (negative control) indicating no precipitate formed. This was performed in triplicates and tubes were taken for quantitative analysis.

3.1.4. Purification of precipitate

Purified precipitate obtained appeared mucoid and fibrous in nature. This was dried and re-suspended in ethanol to preserve the γ-PGA biopolymer.

3.2. Detection and quantification of produced γ-PGA biopolymer

3.2.1. Ninhydrin chemical assay

A graph of O.D. (570 nm) v/s standard concentration of L-Glutamate (20-100 µg/ml) was plotted (Figure 1). These values gave a straight line passing through the origin obeying Beer and Lambert's law. The graph was further utilized for calculating the amount of free L-Glutamic acid units present in test samples.

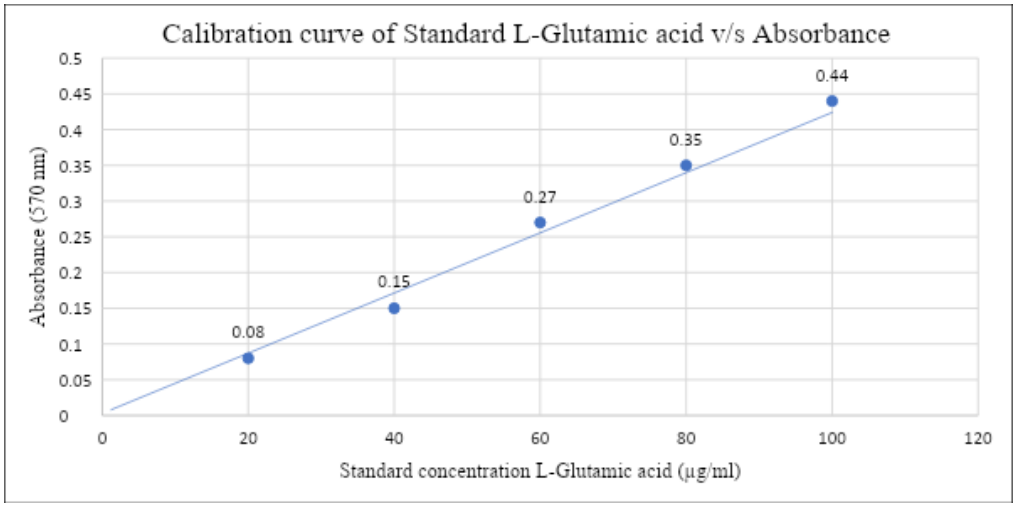

Figure 1 Standard plot for L-Glutamic acid by Ninhydrin chemical assay

Table 5 Yield of γ-PGA (g/L) from isolates using fermentation method (**Mean and Standard deviations for 3 determinations)*

Sample Type	Concentration (g/L) of treated samples	Concentration (g/L) of untreated samples	Concentration (g/L) (Mean ± SD) *
Rhizosphere isolate (RS)	0.89	0.00	0.89 ± 0.18
Top Soil isolate (TS)	4.73	0.22	4.51 ± 0.32
Bacillus cereus (PC)	2.26	0.00	2.26 ± 0.21
NC (Uninoculated)	0.00	0.00	0.00 ± 0.00

3.2.2. Sample preparation for chemical assay (Quantification of produced γ-PGA by isolates)

Sample preparation of all isolates is of paramount importance. This is because, in the utilized chemical assay, ninhydrin detects only free L-Glutamate residues and not polymer of L-Glutamate. Since all of our samples are nothing but a polymer of L-Glutamic acid, acid hydrolysis was performed as described in the methods. It was observed for RS, TS, and PC that the O.D. values differed greatly between treated and untreated samples (Table 5). For each of them, treated samples gave a higher O.D. value (the highest being for topsoil isolate 0.21 O.D. units). As far as untreated samples are concerned (containing L-Glutamate in polymer form), each sample gave 0.00 O.D. units except for topsoil isolate. Upon extrapolating the values, the highest yield was found to be for the top soil isolate 4.51 ± 0.32 g/L and the lowest for rhizosphere isolate 0.89 ± 0.18 g/L.

3.3. Optimization of γ-PGA production

3.3.1. Optimization using "One-variable-a-time" (OVAT) method

Since the topsoil isolate gave the highest, it was taken for further optimization to obtain higher yields of γ-PGA. Herein, the best source was evaluated in order to utilize that particular carbon source for our next-level optimization technique. The experiment was performed in triplicates taking standard deviations into consideration. Upon using glucose as a carbon source, γ-PGA was obtained in higher yields (4.8 ± 0.3 g/L) as compared to medium containing rice bran (2.5 ± 0.1 g/L). Thus, a significant increase of 92 % was observed upon using glucose as carbon source.

3.3.2. Optimization using the "Plackett-Burman Design" method

Table 6. Yield obtained for each trial using "Plackett-Burman Design" method

Trials/ Runs	Yield (g/L) Mean ± S.D.*	Error
1	3.4 ± 0.1	0.303
2	19.0 ± 0.5	0.303
3	3.7 ± 0.3	0.303
4	2.5 ± 0.1	0.303
5	18.1 ± 0.7	0.303
6	2.8 ± 0.4	0.303
7	3.3 ± 0.1	0.303
8	4.3 ± 0.1	0.303

*Standard deviations for 3 determinations.

Most important variables in the system were determined using this biostatistical method. The number of trials run were 8. Table 6 shows the yield obtained (yield was calculated using the method as described under 3.2.2) after taking all possibilities of variables into consideration. Experimental Error was found to be nearly zero (0.303). The highest yield was found for Trial no. 2 (19.0 ± 0.5) that needed variables Ammonium chloride (X2), Ferric chloride (X3), Magnesium sulphate (X4), and Glycerol (X6) at a high-level concentration whereas L-Glutamic acid (X1) required a low-level concentration. Further certain statistical parameters were calculated to determine which specific variable was having the most significant effect on the system (Table 7). F-values highlight that the variables X1, X2, and X6 were found to be having a large effect, X4 had a little effect and X3 had no effect on the system. Upon considering the degrees of freedom, the p-value was calculated

for each variable at a 95% confidence level, it was found that all the high F-test values were statistically significant. The correlation test gave value -0.880 which signifies the extent to which the two variables F-test value and P-value are moving in opposite directions.

Table 7 Statistical analysis of variables

Variable	Name of Constituent	Σ(H)	Σ(L)	Difference	Effect	Mean square	F test value	DoF	P-value
X1	L-Glutamic acid	12.0	45.1	-33.1	-8.20	136.951	451.353	1	0.0219
X2	Ammonium chloride	43.8	13.3	30.5	7.60	116.281	383.760	1	0.0325
X3	Ferric chloride	28.9	28.2	0.7	0.18	0.061	0.202	1	0.7311
X4	Magnesium sulphate	25.2	28.6	-3.4	-0.85	1.445	4.760	1	0.2736
X5 (D1)	Dummy variable 1	27.7	29.4	-1.7	-0.46	0.361	0.361	1	0.6556
X6	Glycerol	42.4	14.7	27.7	6.96	95.911	361.530	1	0.0357
X7 (D2)	Dummy variable 2	27.8	29.2	-1.3	-0.33	0.245	0.245	1	0.7074

3.4. Bacterial species identification

VITEK-2 Compact automated system identified the isolate (TS-2) as *Bacillus thuringiensis.* Table 8 gives detailed biochemical results of the isolate.

Table 8 Biochemical details of the isolate from VITEK-2

Test Name	Results	Test Name	Results	Test Name	Results	Test Name	Results	Test Name	Results	Test Name	Results
BXYL	-	LysA	-	AspA	-	LeuA	+	PheA	+	ProA	-
BGAL	-	PyrA	+	AGAL	-	AlaA	-	TyrA	+	BNAG	-
APPA	+	CDEX	-	dGAL	-	GLYG	+	INO	-	MdG	-
ELLM	-	MdX	-	AMAN	-	MTE	+	GlyA	-	dMAN	-
dMNE	-	dMLZ	-	NAG	+	PLE	-	IRHA	-	BGLU	-
BMAN	-	PHC	-	PVATE	+	AGLU	+	dTAG	-	dTRE	+
INU	-	dGLU	+	dRIB	+	PSCNa	-	NaCL 6.5%	+	KAN	-
OLD	-	ESC	+	TTZ	-	POLYB I	+				

3.5. Applications of γ-PGA biopolymer

3.5.1. Use of γ-PGA as an anti-thawing agent for probiotic cultures during freeze-drying process

In this particular experiment we checked the deleterious effect of the freeze-drying process on *Lactococcus lactis*. Further, investigated if γ-PGA and Gelatin polyelectrolyte is showing an anti-thawing activity or not. (Table 9) For this initial count was determined using Miles and Misra's technique performed in duplicates ($3.26*10^9$ cfu/ml). The final count was taken after a series of thawing steps that were performed for the freeze-drying process. When PBS was suspended into the prebiotic culture, a drastic 99.86% reduction (log reduction approaching 3) was observed. γ-PGA and Gelatin alone showed a moderate effect, but when added together as a polyelectrolyte in equal amounts, it showed a very significant effect as an anti-thawing agent. Percentage reduction was found to be only 40.79%. In comparison to the unprotected cells, this polyelectrolyte showed to protect the probiotic cells by 59.17%.

3.5.2. Use of γ-PGA as a prebiotic supplement

Prebiotic potency of γ-PGA in-vitro was assessed. A simple basal medium containing yeast extract and casein peptone was used. This broth was supplemented with γ-PGA. The former medium constituted our control. This was to verify that the growth stimulation of *Lactococcus lactis* is only because of γ-PGA biopolymer and not due to other constituents. (Table 10) In order to nullify the effect of other constituents, the difference between the cfu/ml was taken into consideration. It was found that there was a significant increase in growth (32.14%) considering the effect of γ-PGA alone. Enumeration was performed in duplicates using Miles and Misra's technique.

Table 9 Enumeration of *Lactococcus lactis* before and after freeze-drying process (Miles and Misra technique on M-17 medium, 24h 37ºC incubation)

Sr. No.	Test aliquots taken	Initial count (cfu/ml)	Final count (cfu/ml)	Log reduction	Percentage reduction (Mean ± SD*)	Anti-thawing effect (%) *
1	γ-PGA (0.5%)	3.26*109	4.11*108	0.90	87.38 ± 0.54%	12.48%
2	Gelatin (0.5%)	3.26*109	6.92*108	0.67	78.77 ± 1.23%	21.09%
3	γ-PGA (0.25%) + Gelatin (0.25%)	3.26*109	1.93*109	0.23	40.79 ± 0.48%	59.07%
4	Control	3.26*109	4.58*106	2.85	99.86 ± 1.36%	-

*Anti-thawing effect i.e., prevention of cell viability (compared with Control value), *Standard deviations for 2 determinations.

Table 10 Assessment of γ-PGA's prebiotic potency (**Standard deviations for 2 determinations)*

Count CFU/ml	Control (No added γ-PGA)	Test (Added γ-PGA)
Initial count	2.8*108 CFU/ml	2.8*108 CFU/ml
Final count (After 24 h incubation)	5.4*108 CFU/ml	9.1*108 CFU/ml
Percentage increase (Mean ± SD*)	48.14 ± 0.62%	69.23 ± 1.33%

4. Discussion

In more than 70 years of research related to γ-PGA, in-depth knowledge of its production, metabolic regulation, and application has been gained. Due to its high biodegradability and stability, γ-PGA has a wider range of applications from the food industry to the pharmaceutical industry. Efficient and cost-effective methods to screen for γ-PGA producing microorganisms from diverse sources are the need of the hour. Thus, in this study, we used a simple nutrient agar medium and supplemented it with L-Glutamate which acts as a precursor for the formation of our biopolymer. Once γ-PGA is produced by the organism externally, the entire colony appears to be having mucoid texture. In various studies also it was demonstrated that the mucoid phenotype is due to the production of the secreted polymer, γ-PGA [11, 30, 34]. These colonies were further selected for a second-level screening that utilizes the dye remover property of γ-PGA.

Topsoil isolate gave the highest zone of clearance for methylene blue (29 ± 1 mm) which is in comparison to the highest zone of clearance of 31 mm obtained for *Bacillus licheniformis* DFR63 strain, wherein samples were collected from sugarcane soils [33]. The reason as to why soil gives rise to high γ-PGA producers is described below.

Upon quantifying the amount of γ-PGA produced by the isolates, it was found that the top soil isolate gave the highest amount of biopolymer (59%). This was followed by rhizosphere isolate (12%) and *Bacillus cereus* having a moderate yield. The reason for this is that the topsoil is always subjected to harsh conditions [28] and drastic changes in pH and temperature, and γ-PGA is such a biopolymer which is produced by bacteria to survive under such dynamism of environment [25].

This isolate was further optimized using the Plackett-Burman design method. By optimizing our isolate, we saw a significant increase in the yield from 4.51 ± 0.32 g/L to 19.0 ± 0.5 g/L (78.9%). Glucose was found to be one of the important carbon sources. This is because, while considering the biosynthetic pathway for γ-PGA synthesis, it involves the Tricarboxylic Acid cycle. This suggests that carbon source like glucose is very useful for normal cell growth and for the synthesis of γ-PGA [6, 33]. This is also in line with the previous findings that reported glucose as the major carbon source for γ-PGA production [23].

The variables that were found to be very significant were glycerol, ammonium sulphate, and Glutamic acid. Ammonium sulphate acts as a nitrogen source thus it is the main ingredient of the production medium. Cromwick & Gross *et al. and* Zhang *et al.* [10, 36] reported that L-Glutamic acid and citric acid are the main precursor substrates for the production of γ-PGA biopolymer. Thus, in this present study both substrates were utilized.

Glycerol variable in the system gave a high F-test value. To date, the mechanism by which glycerol enhances the production of γ-PGA biopolymer is not clear. According to Troy *et al.* [31], glycerol may stimulate polyglutamyl synthetase which catalyzes the polymerization of L-Glutamic acid. Du *et al.* [13] investigated the quantity of intracellular and extracellular PGA in *Bacillus licheniformis* with and without the addition of the glycerol variable. In the absence of glycerol, intracellular PGA levels were found to be extremely high, inhibiting further PGA synthesis. Glycerol enhanced the permeability of the cell membrane for PGA, facilitating PGA secretion from *Bacillus licheniformis* and thereby increasing PGA output. Thus, it was confirmed that glycerol metabolism is very important for enhanced production of γ-PGA [35].

Further Bajaj *et al.* [6] also used the Plackett-Burman design method for optimizing their isolate *Bacillus licheniformis* NCIM 2324. γ-PGA production increased from 5.27 to 26.12 g/L (79.8%). This was because, Glycerol, Ammonium sulphate (N-source) supported maximum production of γ-PGA in this study, thus similar to what we obtained in this study.

In a study, different growth media was employed for obtaining higher yields. The yield was 12 g/l for the medium containing 2.46 mM $MnSO_4$ whereas it was only 4-5 g/l for the medium without $MnSO_4$. The yield obtained from a medium named 'GS' was the highest (26g/l). Thus, from this, we can say that the essential salts added to our medium (i.e., $MgSO_4$), had an impact on the system [19].

Vitek-2 Compact automated system identified the isolate (TS-2) as *Bacillus thuringiensis.* To date, there are no studies carried out on γ-PGA obtained from this species. Here, an attempt was made to explore the applications of this culture.

The introduction of bacteria into the foodstuff as dry cultures and its storage is one of the most significant production procedures for making a probiotic food product. Working with dry cultures is preferable to working with wet cultures. However, health benefits solely rely on the viability of these probiotic cultures and on the maintenance of stock cultures during storage and transportation. Even though there are various techniques like freeze drying used for probiotics, it has been documented that there is always a loss of probiotic culture viability during hypothermia, single and repeated freeze-thaw processes [15, 20]. Also, such procedures used to prepare freeze-dried probiotic bacteria are frequently harmful to the cell structure and viability [8].

It has been previously shown that the viability of lactic acid bacteria reduces by 3 log CFU/g (99.9%) when freeze-dried [15]. In our study, *Lactococcus lactis* is the first probiotic streptococci culture to be analyzed for the effect of γ-PGA biopolymer as an anti-thawing agent. It was observed that unprotected cultures gave a log reduction of 2.85 log CFU/g (99.86%) which is comparable to the later study. This is due to temperature changes during a series of thawing temperatures after freezing (-80°C). This may have a significant impact on bacterial viability due to changes in electrolyte concentrations or water recrystallization [1].

Also, the Polyelectrolytic combination of γ-PGA (anionic polymer) and Gelatin (cationic polymer) was found to be giving a synergistic effect of protecting the probiotic culture during freeze-drying (a log reduction of only 0.23 i.e., 40.79%). Gelatin being a polycation and γ-PGA being polyanion, forms a stable complex by a self-assembly process. Thus, probiotic cultures can be easily encapsulated with PEs for cryoprotection [21].

Most of the studies carried out so far were on *Lactobacillus* species Without γ-PGA, *Lactobacillus plantarum* that was freeze-dried for 48 h decreased by 1.50 log CFU/ml. When encapsulated with 0.5%, 0.25%, or 0.1% γ-PGA, *Lactobacillus plantarum* levels decreased by 0.19, 0.31, and 1.10 log CFU/ ml, respectively [16]. Another study was carried out with *Bifidobacterium* strains, and it was observed that the reduction in viability was around ~2.5 log CFU/ml. When10% γ-PGA alone was used, 1.24 - 1.26 log CFU/ml reduction in viability was observed [8].

In laboratories, we demonstrated prebiotic activity in vitro while observing the magnitude of growth of *Lactococcus lactis* growing in presence of this γ-PGA When such compounds are utilized by probiotic microorganisms, they produce short chain fatty acids that modulate both, the growth of lactic acid bacteria and overall gut health [4]. It was found that there was a significant increase in growth (32.14%) considering the effect of γ-PGA alone. Jin *et al.* [17] is the only study conducted for assessing the prebiotic potency of γ-PGA. In γ-PGA-treated mice, the relative abundance of *Lactobacillus* increased from 8% to 38%. However, various prebiotic studies like tolerance to bile salts, pH, and other gastrointestinal conditions can be carried out to check the efficacy of this biopolymer in the human gut.

Thus, this research mainly focused on obtaining a higher yield of biopolymer and its applications. Polyelectrolytes were also our main focus. This included incorporating the probiotic cultures within the γ-PGA-Gelatin complex that will efficiently protect them from the freeze-thaw process or other detrimental conditions. The role of γ-PGA as a prebiotic may be an additional mechanism to verify the beneficial effects of γ-PGA on human health and disease.

5. Conclusion

Taking into consideration the commercial importance of probiotics, this study focused on the microorganisms that have been found to naturally produce γ-PGA. Herein, we isolated a bacterial strain isolated from a fertilized soil which was able to produce γ-PGA at high levels. Vitek-2 Compact automated system identified this isolate as *Bacillus thuringiensis.* We also statistically (Plackett-Burman model*)* improved the yield of this biopolymer by analyzing various variables during the fermentation process. *Bacillus thuringiensis* is a widespread bacterium and is considered safe for humans. There are several studies that have found no evidence of sickness or infection as a result of exposure. Thus, this bacterium can be widely used for the biosynthesis of natural products like γ-PGA biopolymer. γ-PGA significantly improved the probiotic *Lactococcus lactis* survival during the various stages of preparation (freeze-drying) and storage at very low temperatures. Also, our laboratory-isolated γ-PGA showed a good prebiotic activity as well. However, various studies like tolerance to bile salts, pH, and other gastrointestinal conditions can be carried out to check the efficacy of this biopolymer in the human gut. Products are totally safe to be consumed if this biopolymer is used.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare. We have seen and agree with the contents of the manuscript and there is no financial

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