Characterization and statistical optimization of γ-PGA produced by *Bacillus megaterium* UP47 isolated from *Pentaclethra macrophylla*

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Abstract

Background. Gamma-polyglutamic acid (γ-PGA) is a microbially produced non-toxic peptide biopolymer which is gaining grounds in many biotechnological fields and has a wide range of applications.

Objectives. In this study, the characteristics of γ-PGA produced by *Bacillus megaterium* isolated from an oil seed were determined, while the nutritional requirements of the bacterium were optimized using a predictive 15 factor-16 run Plackett–Burman experimental design.

Materials and methods. The main effect of each factor, the interaction and quadratic effects of the factors on optimized production were determined from Box–Benkhen model using Dell Statistica v. 12 and 13 software. *Bacillus megaterium* UP47 produced the highest γ-PGA (16.33 g/L) out of 56 spore-forming *Bacillus* strains isolated from soil, water and fermented food samples.

Results. Hydrolysates of the produced γ-PGA had a retention factor which corresponded to the L-glutamic acid standard (retention factor (rf) 0.35), while high-definition fourier transform infrared (FT-IR) spectroscopic imaging showed characteristic peaks representative of the active bonds present in γ-PGA. The γ-PGA at a concentration as low as 50 mg/100 mL exerted antimicrobial inhibitions against test pathogens. A 2.00 w/v γ-PGA solution had 11 mm and 13 mm inhibition zones against *Staphylococcus aureus* and *Shigella dysenteriae*, respectively. A second order polynomial equation for prediction of γ-PGA was derived as:
γPGA yield = 3316.061 − 449.708A + 9.036A2 − 139.813B + 3.095B2 − 7.699C − 0.164C2 + 13.116AB − 0.087AB2 − 0.248A2B + 3.781AC − 0.076A2C − 0.394BC.
It showed an increase in γ-PGA yield with increasing L-glutamic acid and biotin, but a decrease with yeast extract.

Conclusions. *Bacillus megaterium* UP47 had a maximum γ-PGA yield of 54 g/L and 62 g/L, respectively, from the Plackett–Burman and Box–Benkhen design, thereby resulting in an appreciable increase in polymer yield after the optimization process with a 95% confidence level.

Key words: poly γ-glutamic acid, optimized production media, polymer characterization, precursor requirements, antimicrobial potentials

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Background

During the growth process of microorganisms, complex metabolic processes result in the production of natural polymers known as biopolymers. These materials include, among the others, exopolysaccharides and polyglutamic acid.[1,2] Poly-gamma-glutamic acid (γ-PGA) is a water-soluble, biodegradable, non-immunogenic, non-toxic, and unusual anionic homopolyamide and/or homopolypeptide made up of D- and L- α-amide-linked polymerized units of glutamic acid.[3–5] Figure 1A shows the structure of γ-PGA. The production of γ-PGA is influenced by different nutritional requirements of the γ-PGA-producing bacteria.[6,7] While some of these bacteria do not require glutamic acid, others grow only in its presence or require biotin.[7] The characteristics of γ-PGA biopolymer which have been exploited in its applications include its anionic, biodegradable and water-soluble attributes. What is of a great importance is the fact that γ-PGA biopolymer is edible and non-toxic to humans and the environment.[8,9] Other relevant applications of γ-PGA included their potential to thicken food, relieve bitterness in drugs, use as cryoprotective materials, in metal absorption and dye removal.[2] Reported to have a 'Generally Regarded as Safe' status (being a naturally derived food grade material),[10,11] γ-PGA is also documented to have antimicrobial characteristics.[12] Balogun-Agbaje et al. reported the antifungal activities of γ-PGA-based nanoparticles.[5] The antimicrobial potential of the γ-PGA was therefore investigated in this work to ascertain the possible suitability of the produced γ-PGA in applications such as the medical, agricultural, food, pharmaceutical, and wastewater treatment industries.

Experimental design techniques based on predictive models present more evenhanded alternative to one-variable-at-a-time (OVAT) approach for fermentation improvement in laboratory-based experimentations, such as γ-PGA production, considering the effects of time, cost and labor involved in the traditional approach.[13] In present-day biotechnology, experimental designs such as the Plackett–Burman design can be used if it is desired to screen a large number of factors (with as limited experimental run as possible) to reduce and/or identify the number of factors down to the key role-playing variables.[14–16] Furthermore, an economic factorial experimental design, the Box–Behnken model, can be used to determine the relationship between the response function and the experimental variables enlisted.[17]

Microbial γ-PGA production faces limitations due to a high production cost, low yield, diverse nature of media composition, and unique individual requirements of the producing microorganisms.[11] Since the use

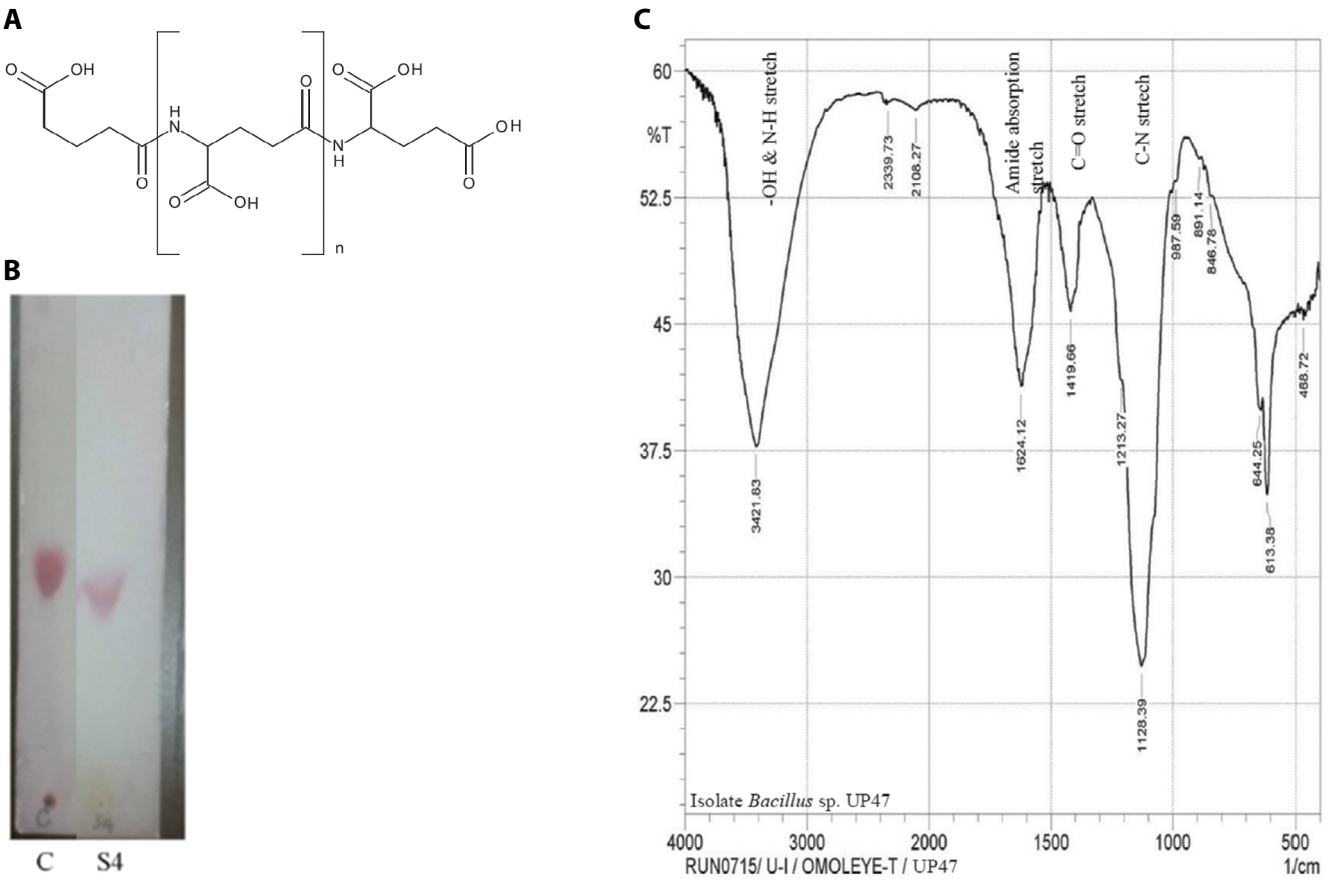

Fig. 1. Chemical structure of gamma-polyglutamic acid (γ-PGA) (A), and thin layer chromatographic (B) and spectroscopic characteristics (fourier transform infrared (FT-IR)) (C) of *Bacillus megaterium* UP47-produced γ-PGA

of predictive model provides a fast, reliable, labor-efficient, and relatively cost-effective way to obtain laboratory-based experimentation in product optimization,[18] it is expected that statistical models towards γ-PGA production could also lead to answers for cost-effective production of γ-PGA and other production problems of time consumption, unreliability and laboriousness. Hence, this work investigated the abilities of diversely sourced *Bacillus* species to produce γ-PGA, the characteristics and antimicrobial potentials of the produced polymer, and a statistical optimization of media components towards γ-PGA production.

Materials and methods

Media preparation and isolation of *Bacillus* species

All media, chemicals and reagents used were of analytical grade, and they were prepared and used according to the manufacturers' instructions. The enlisted γ-PGA production medium was the modified γ-PGA production medium (PPM) proposed by Bajaj and Singhal[18] and composed of (g/L): L-glutamic acid (20), NH_4Cl (6), citric acid (12), NaCl (25), KCl (0.66), $MgSO_4.7H_2O$ (6.8), $CaCl_2.2H_2O$ (0.2), $NaHCO_3$ (0.18), $MgCl_2.6H_2O$ (4.7), $MnSO_4.7H_2O$ (0.05), K_2HPO_4 (2), and glycerol (25 mL/L). Bacteria were isolated from samples of a laboratory fermented *Pentaclethra macrophylla* (*Ugba*) seeds, while soil and water samples were collected from 3 engine oil-contaminated sites, a maize rhizosphere, 2 domestic effluents, and 2 kettle/pot rinses. All samples (10 g or 10 mL) were heated in a boiling GFL 1083 shaker water bath (GFL Technology, Senden, Germany) for 15 min to kill off non-sporulating microbial cells and thus enhance the isolation of more *Bacillus* species, mixed with 90 mL sterile distilled water, vortexed vigorously and serially diluted. Different dilutions (1 mL) from each sample were aseptically transferred into 90 mm sterile disposable Petri dishes, plated out on nutrient agar and solidified PPM agar, and incubated at 30°C for 24–48 h.[19] Morphologically distinct bacteria from inoculated Petri dishes were subcultured onto nutrient agar plates to obtain pure cultures. The pure cultures were stored on nutrient agar slants in a refrigerator and in 25% glycerol nutrient broth at –20°C.

Screening and selection of *Bacillus* species for γ-PGA-producing potentials

The isolates obtained from the different samples were primarily screened for γ-PGA production by checking for slime-producing abilities using a sterile toothpick to touch the bacterial colony and then gently drawing upwards. The length of the stretched thread is regarded as an indication of an isolate's ability to produce more γ-PGA.[20,21]

Physiological and biochemical identification of *Bacillus* spp.

The identification of selected γ-PGA-producing strains was carried out using conventional methods of morphological and biochemical characterization, including Gram staining, spore staining, catalase reaction, oxidase test, starch hydrolysis, casein hydrolysis, citrate utilization, gelatin liquefaction, urease test, methyl red test, H_2S production, motility, growth at 6.5% and 10% NaCl, as well as carbohydrate fermentative abilities on sucrose, D-glucose, lactose, fructose, galactose, maltose, mannitol, xylose and sorbitol.[22,23]

γ-PGA production, extraction, purification, and quantification

The most promising γ-PGA producing bacterium was used in further γ-PGA production. The modified PPM medium (25 mL) was sterilized and inoculated with 10^8 CFU/mL of the selected bacterium. It was fermented at 30°C and 180 rpm for 72 h. After fermentation, the microbial cells were harvested by centrifuging the fermentation broth at 5000 rpm and 4°C for 15 min using an MSE High Speed 18 Centrifuge (MSE Ltd., Birmingham, UK). The γ-PGA was extracted from the supernatant using the ethanol precipitation method.[2] Briefly, the centrifuged cell-free broth was decanted into 3 volumes of cold ethanol, held at –4°C overnight and centrifuged again. The process was repeated 4 times to ensure increased purity of the extracted γ-PGA. The extracted γ-PGA was dried and weighed to determine the production in g/L.[24]

Characterization of the produced γ-PGA polymer

Ultraviolet spectroscopic analysis of the produced γ-PGA

The cell-free γ-PGA production broth was subjected to spectrophotometric analysis by reading the absorbance of the produced solutions between 190 nm and 900 nm, using Perkin Elmer Lambda 25 UV/Vis Spectrophotometer (Waltham, USA).[25] Isolates with the highest absorbance readings within 190 nm and 230 nm were expected to have higher γ-PGA producing abilities and were selected for further experiments.

Thin layer chromatographic analysis of the produced γ-PGA

The extracted and dried γ-PGA polymers were hydrolyzed in 7.5 M HCl and maintained in an autoclave at 121°C for 2 h 30 min. Thereafter, the acid-hydrolyzed mixtures were neutralized with 7.5 M NaOH and subjected to thin layer chromatography. The neutralized hydrolysates

were chromatographed against L-glutamic acid (Sigma-Aldrich, St. Louis, USA) as control, using ethanol-water (7:3) as the solvent system. After separation, the plates were left to dry and then, they were sprayed with 0.2% ninhydrin-acetone solution to detect the hydrolyzed amino acids bands. The retention factor (Rf) was determined using Equation 1 below.

$$Rf = \frac{\text{distance moved by spot}}{\text{distance moved by solvent front}} \quad (1)$$

The alignment of the emerging bands from the γ-PGA hydrolysates was compared with that of the L-glutamic acid standard.[21,26]

Fourier transform infrared spectroscopic analysis of the produced γ-PGA

The produced polymer was analyzed using a Shimadzu FT-IR Spectrophotometer (Shimadzu Corp., Kyoto, Japan) with 45 scans, 4 cm^{-1} resolution and Happ–Genzel apodization for its characteristic peaks and bonds. Distinctive strong and weak absorption peaks at different wavelengths characteristic of γ-PGA in the given samples were determined.[27,28]

Antimicrobial potentials of the produced γ-PGA using agar well diffusion assay

To determine the potential of the purified γ-PGA in industrial applications, the γ-PGA was used in the preparation of aqueous solutions at varying concentrations ranging from 0.5 mg/mL to 20.0 mg/mL. The appropriate concentrations of the γ-PGA biopolymer were dissolved in sterile water and their antimicrobial potential against selected laboratory stock cultures of indicator bacteria was determined. Ciprofloxacin (30 µL of a 5% concentration) was used as the control. The stock cultures of *Shigella dysenteriae*, *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli*, and *Bacillus cereus* were reactivated by subculturing from their agar slants and used as agar lawn inoculum for the agar well diffusion assay. Solidified Petri dishes containing Mueller–Hinton agar were swabbed with cell suspensions of 0.5 McFarland standard of each of the test pathogen preparations, and wells with 7 mm in diameter were bored into these swabbed plates with the aid of sterile cork borers. The different γ-PGA concentrations, as 30 µL volumes, were introduced into the agar wells and plates and were incubated overnight at 37°C. The diameter zones of inhibition exerted by the different concentrations on the test pathogens were measured and recorded in millilitres.[29] A positive inhibition was recorded as ≥2 mm inhibition zone.[30]

Optimization of γ-PGA production parameters

Media amendments and precursor requirement by selected isolate for γ-PGA production

The isolate with the best γ-PGA producing potential was cultivated in a fermentation medium supplemented with different precursors – biotin, monosodium glutamate and L-glutamic acid – using 5 media variations labelled A–E. Medium A was the γ-PGA production medium (PPM), medium B contained PPM with additional 100 µg/L biotin, medium C was PPM plus 15 g/L monosodium glutamate, while medium D was composed of PPM with biotin (100 µg/L) and monosodium glutamate (15 g/L). Medium E was composed of medium D devoid of L-glutamic acid. The fermentation setups were incubated at 30°C for 72 h at 180 rpm. The best precursors for γ-PGA production were thereafter selected.

Identification and optimization of significant factors for γ-PGA production using Plackett–Burman and Box–Behnken statistical models

An experimental design with 15 factors and 16 runs (Table 1) was set up to screen for the most important variable component that influenced γ-PGA production

Table 1. Base design for the 15 factor-16 run Plackett–Burman first order experimental design for gamma-polyglutamic acid (γ-PGA) production

Factor [units]	Code	Levels		Factor [units]	Code	Levels	
		–1 (low)	+1 (high)			–1 (low)	+1 (high)
Glycerol [mL/L]	X1	20	30	Biotin [µg/g]	X2	75	125
Glucose [g/L]	X3	25	75	L-glutamic acid [g/L]	X4	15	25
Ammonium chloride [g/L]	X5	4	8	Citric acid [g/L]	X6	9	15
NaCl [g/L]	X7	20	30	$MgSO_4.7H_2O$ [g/L]	X8	5.1	8.5
$CaCl_2.2H_2O$ [g/L]	X9	0.1	0.3	Yeast extract [g/L]	X10	2.5	7.5
Casein hydrolysate [g/L]	X11	15	25	Dummy*	X12	–	–
Corn steep liquor [mL/L]	X13	25	75	K_2HPO_4 [g/L]	X14	1	3
Inoculum size (McFarland)	X15	0.5	1.5	–	–	–	–

*X12 is an independent dummy factor; –1 indicates low value, whereas +1 indicates high value.

using Plackett–Burman experimental design[14,15] based on the first order model (Eq. 2):

$$Y = \beta_0 + \Sigma\beta_i X_i \quad (2)$$

where Y is the response (PGA yield), β_0 is the model intercept, β_i is the linear coefficient (both are constant coefficients), and X_i is the level of the independent variable.

Two factor levels (low (–) and high (+)) were considered for each independent nutritional variable in which the rows represented the trials and the columns represented the variable-independent factor. A dummy factor, whose effect on the experimental setup was not determined, was also included in the model, raising the number of trials under study from *n* to *n + 1*.

The factors that had an effect on γ-PGA yield at 95% confidence level were selected and analyzed at 3 levels (low, mid and high: –1, 0 and +1, respectively) in a Box–Behnken experimental design.[11,17] Additionally, some central points with factors fixed at their mid-levels were incorporated. The main effect of each factor, as well as the interaction and quadratic effects of the factors for the prediction of experimental yield on optimized production, were determined using Dell Statistica v. 12 and 13 (Dell Computer Corporation, Austin, USA). Based on the interaction analysis, the response surface graph of the interactions of factors against yield was plotted.[32] Some experimental trials were run with different concentrations of the factors in a scale-up experiment using increasing production volumes of 0.25 L, 0.5 L, 1.0 L and 3.0 L, to determine if the yield was commensurate with the predicted yield using the optimized medium derived for γ-PGA production. The predicted yield was calculated using the derived regression equation and compared with the laboratory experimental results. The confidence level was analyzed to determine the good fit of the equation.[17,31]

Results and discussion

From the 11 samples analyzed, 56 morphologically distinct isolates were obtained: 32.14% were isolated from the soil samples, 28.57% from the fermented food samples, 23.21% from domestic effluents, and 16.07% from the kettle/pot rinses (Table 2). Many γ-PGA producing bacteria have been recovered from diverse sources. Baxi reported the isolation of the bacteria from soils of various geographical locations, fermented flours/beans and industrial wastewater, domestic sewage, and sea water.[33]

Viscous colonies displayed mucoid consistencies which was an indication that a polymeric substance, possibly poly-γ-glutamic acid, had been produced.[34] The percentage of presumptive γ-PGA-producing isolates was low (16.1%), similarly to the reports of Baxi[33] and Ju et al.,[4] who recorded a 12.5% and 4.61%, respectively, of the isolates which possessed colonial characteristics and were similar to that of γ-PGA producers. The ultraviolet (UV) absorbance values of the cell-free γ-PGA solution ranged between 3.3 and 3.55, of which isolate UP47 had the highest absorbance, followed by isolates DS1, HE21 and EK31, respectively. The absorbance reading completely flattened out beyond 233 nm, and thus the isolate UP47 was selected for further experiments. Zeng et al. reported a UV spectrometric and high-performance liquid chromatography (HPLC) correlation of the quantification of γ-PGA at a best wavelength of 216 nm.[25]

Table 2. Colony count and screening of gamma-polyglutamic acid (γ-PGA)-producing bacteria from the analyzed samples

Sample	Sample code	CFU/mL* and number of presumptively positive γ-PGA-producing bacteria compared to selected distinct colonies		Isolate codes of selected γ-PGA producing bacteria and the corresponding absorbance for peak wavelength between 190 nm and 233 nm	
		CFU/mL	positive γ-PGA bacteria fraction	code	absorbance/γ-PGA [g/L]
Oil drainage soil site 1	DSI	1.3×10^6	1/3	DS01	3.540/11.67
Oil drainage soil site 2	DSII	1.5×10^6	1/4	DS05	3.445/7.63
Oil drainage soil site 3	DSIII	7.5×10^5	0/6	–	–
Maize farm soil	MFIV	2.4×10^7	1/5	MF10	3.435/8.67
Hostel domestic effluent	HEVI	5.4×10^6	1/3	HE21	3.502/12.00
Residential apartment domestic effluent	REIX	8.9×10^6	0/10	–	–
Pressure pot rinse water	PPVII	1.1×10^7	1/5	PP26	3.486/7.33
Electric kettle rinse water	EKVIII	7.1×10^5	1/4	EK31	3.496/12.33
Iru	IRV	2.8×10^6	2/6	IR15, IR18	3.372/8.00 3.464/10.00
Ugba fermented in plantain leaves	UPXI	1.1×10^7	1/5	UP47	3.547/16.33
Ugba fermented in aluminum foil	UAX	1.0×10^7	0/5	–	–
Bacteria ratio	–	–	9/56	–	–

* results/values are mean of triplicate readings.

Morphological and biochemical characteristics of selected isolates

Morphologically, the isolate UP47 colony was creamy, circular, mucoid, large, raised, opaque, Gram-positive rod, and a spore former. The bacterium was positive for catalase, oxidase, casein, gelatin, glucose and sucrose utilization, but negative for citrate utilization, starch hydrolysis, urease and gas production, and identified as *Bacillus megaterium* UP47. The bacterium was able to survive at high saline concentrations up to 10%. Choi et al. isolated slime-producing spore-forming bacilli from homemade Cheonggukjang for γ-PGA production,[20] and Hezayen et al. reported that most γ-PGA producing strains possessed the ability to grow in high saline conditions and thus survive hostile environments.[35]

Characterization of purified γ-PGA

The produced γ-PGA polymer was powdery and creamy-white. Thin layer chromatography of the *B. megaterium* UP47 γ-PGA hydrolysate revealed a single band with similar Rf values (0.32) as the L-glutamic acid standard (0.35), thus suggesting that the hydrolyzed polymer, reflecting a single spot, was probably composed of only glutamic acid subunits (Fig. 1B).

The active bonds of the γ-PGA polymer produced by *B. megaterium* UP47 corresponded with those found in γ-PGA (Fig. 1C). Produced γ-PGA had characteristic strong C–N group absorption peaks between 1085 cm^{-1} and 1165 cm^{-1}, weak carbonyl C=O absorption at ≈1390–1450 cm^{-1}, strong amide absorption at ≈1600–1660 cm^{-1}, characteristic aliphatic N–H stretching between 2800 cm^{-1} and 2900 cm^{-1}, and a strong hydroxyl OH absorption at ≈3400–3450 cm^{-1}. Similar findings were reported by Bhat et al.[3] and Khalil et al.[12]

The results of the bacterial inhibition exerted by the *B. megaterium* UP47 γ-PGA (Fig. 2) were an indication of its usefulness for possible applications in relevant fields. The *B. megaterium* UP47 γ-PGA showed the evidence of bacterial inhibitory activities against both Gram-positive and Gram-negative bacteria in the agar well diffusion assay. The γ-PGA concentration as low as 0.5 mg/mL was successful in exerting inhibitory activities against overnight cultures of *S. dysenteriae*, *S. aureus, P. aeruginosa* and *E. coli. Klebsiella pneumoniae* was inhibited by the γ-PGA concentration ≥1.0 mg/mL. Increasing concentrations of γ-PGA (≤20.0 mg/mL) also exerted inhibitory activity, which was most pronounced against *S. dysenteriae* (13 mm). The polymer at ≤20.0% was completely unable to exert any inhibitory effect on the *B. cereus* culture used in this study, while the effect of the control antibiotic on this bacterium was also low (3 mm). The microbial inhibitory activities reported in this work occurred with the use of very low γ-PGA concentrations. However, Lee et al. reported positive microbial inhibition by γ-PGA-adsorbed discs at 1% concentration.[36] In this study, there was a more pronounced inhibition of *S. aureus* and *S. dysenteriae* by the polymeric γ-PGA, albeit, lower than recorded using the control antibiotic. Su et al. also reported higher antibiotic activity on tested strains compared to the γ-PGA used.[37] Ajayeoba et al. reported inhibitory activities ranging between 16.6 mm and 22.5 mm on *S. aureus,* but higher concentrations of γ-PGA were used (150 mg/mL) in contrast to what was obtained with 20.0 mg/mL concentration of the *B. megaterium* UP47 γ-PGA in this work (10 mm).[38] The inhibitory activities of the γ-PGA on tested bacteria might be due to the nature and source of the specific microbes and the anionic nature of the γ-PGA, which allows it to possibly bind with materials on the bacterial cell wall and disrupt the cell content.[38,39] The bacterium could also induce stress responses to protect itself against the antimicrobial substance. Hence, increasing γ-PGA concentrations beyond 2.0% might give better antibacterial characteristics than those recorded in this research, especially against *S. dysenteriae* and *S. aureus* strains.[38]

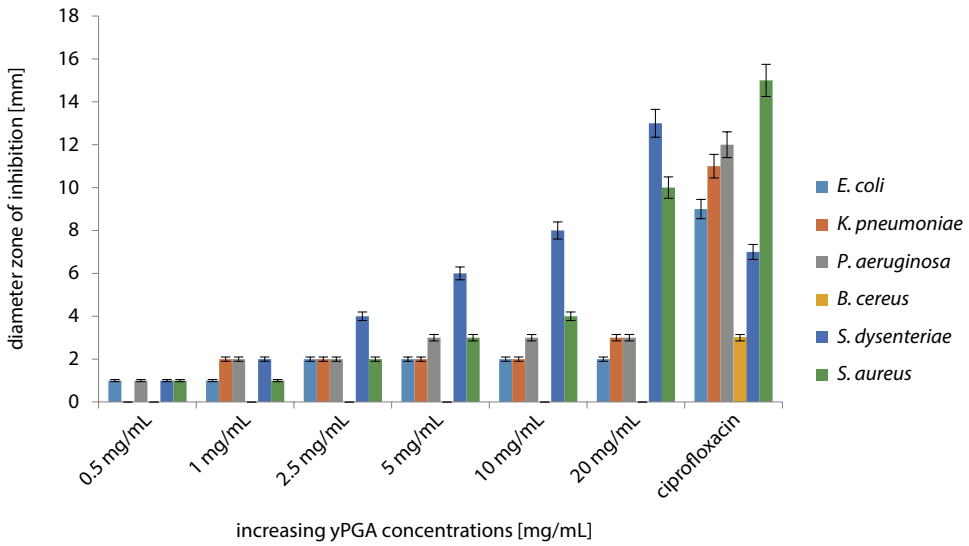

Fig. 2. Antibacterial potentials of 0.5–20 mg/mL concentrations of *Bacillus megaterium* UP47 gamma-polyglutamic acid (γ-PGA) against different test pathogens

The negligible effect of the *B. megaterium* UP47 γ-PGA observed against the tested *B. cereus* was similar to that reported by Ajayeoba et al.[38] and is suggested to be due to the biofilm-forming abilities of *B. cereus* through which it creates fibrous amyloid-like networks, which assemble structural proteins to form hydrophobic envelopes limiting the inhibitory potentials of the γ-PGA.[40]

B. megaterium UP47 precursor requirement for γ-PGA production

A precursor requirement for γ-PGA production is an important factor to be considered when screening for isolates suitable for use in a large scale production. The Randomized Complete Block Design (RCBD) showed that there was no significant difference in the production of γ-PGA by the selected *Bacillus* spp. ($p = 0.8276$), whereas in the treatment data a significant difference ($p = 5.1609$) was observed in the production of γ-PGA in 5 different media (data not shown). Analysis of variance (ANOVA) showed that the most significant difference in γ-PGA production was between media A and E and media B and E, implying that both media A and B contained components (L-glutamic acid and biotin, respectively) which greatly enhanced γ-PGA production. Thus, the *B. megaterium* UP47 was biotin- and glutamic acid-dependent. The production of γ-PGA by *Bacillus* spp. SW1-2 was enhanced by glutamic acid[13] while Goto and Kunioka reported a biotin-dependent γ-PGA producing *Bacillus subtilis* IFO 3335 which would not be produced in the absence of L-glutamic acid.[6]

Significant factors for γ-PGA production based on the Plackett–Burman analysis

The greater the coefficient, the greater the impact of a given factor on production; thus, a factor with a coefficient value close to 0 has very little impact on the production, while a factor with a high value coefficient has a large impact on the production. The optimum media composition for γ-PGA production with *B. megaterium* UP47 determined using the 15 factor-16 run Plackett–Burman analysis is shown in Table 3 and Table 4, respectively.

From the laboratory fermentation process, the highest γ-PGA yield in the experimental runs *B. megaterium* UP47 (54 g/L) was observed in run order D8 containing (g/L): glycerol (30), glucose (25), L-glutamic acid (25), ammonium chloride (4), citric acid (9), NaCl (20), $MgSO_4.7H_2O$ (8.5), $CaCl_2.2H_2O$ (0.3), yeast extract (7.5), casein hydrolysate (15), K_2HPO_4 (3), corn steep liquor (75 mL), inoculum size (0.5), and biotin (75 μg), respectively (Table 3). The lowest γ-PGA yield was observed in run order D16, which had all factors at their low levels.

The confidence level of all considered factors ranged between 80.94% and 98.00% for *B. megaterium* UP47 (Table 4). Factors X5, X6, X7, X13, X14 and X15 had negative effects on γ-PGA production by *B. megaterium* UP47; thus, a decrease in the concentration of these factors brought about an increase in production. The least yield recorded in run D16 agreed with the findings of Bajaj and Singhal.[18] Precursor factors (glucose, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and K_2HPO_4) included into our Plackett–Burman analysis were also considered by other authors.[13,15,18] While glucose had a positive effect on γ-PGA production by *B. megaterium* UP47, we also observed, in agreement with other authors,[13,15,18] its negative effect on γ-PGA yield. The $MgSO_4.7H_2O$ also had a positive effect on γ-PGA production by *B. megaterium* UP47, a finding corroborated by other publications.[13,15,18]

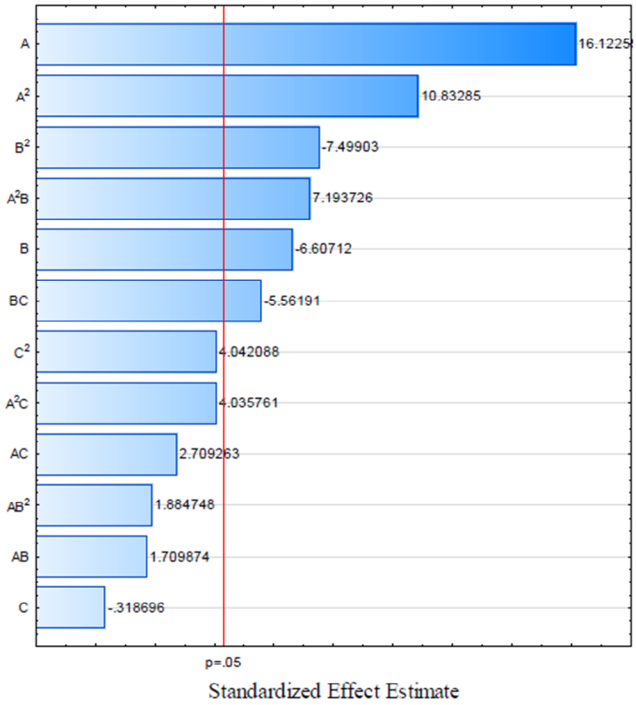

Fig. 3. Pareto chart of standardized effects of the Box–Behnken experimental analysis for *Bacillus thuringiensis* UP47-produced gamma-polyglutamic acid (γ-PGA)

Considering the effect of yeast extract on production of γ-PGA, results obtained in this work, as well as Mabrouk et al.[15] and Berekaa and Al-Otaibi,[13] showed that yeast extract had a positive effect on yield of γ-PGA. Tork et al.[34] recorded high γ-PGA yields when yeast extract and L-glutamic acid were used. For factors with a positive effect on γ-PGA production, an increase in the concentration brought about an increase in γ-PGA production. L-glutamic acid (X4), yeast extract (X10) and biotin (X2) impacted γ-PGA production by *B. megaterium* UP47 and were used in a 3 factor 1 block-15 run Box–Behnken design to determine the interaction of the factors with each other and with γ-PGA production (Table 5).

The effects of interactions of media components on γ-PGA production based on the Box–Behnken analysis showed (Fig. 3) that both the linear and quadratic relationship of L-glutamic acid and yeast extract had positive and negative effects on γ-PGA yield, respectively.

Table 3. Screening and optimization of significant variables for gamma-polyglutamic acid (γ-PGA) production by *Bacillus thuringiensis* UP47 using a 16-run Plackett–Burman design matrix

Run order	Variables															PGA* [g/L]
	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	
D1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	14.67 ±0.58
D2	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	51.33 ±2.42
D3	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	36.67 ±0.58
D4	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	44.00 ±0.58
D5	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	8.00 ±0.51
D6	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	−1	10.00 ±0.87
D7	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	+1	8.67 ±0.72
D8	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	−1	54.00 ±1.81
D9	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	+1	20.00 ±0.96
D10	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	+1	18.00 ±1.03
D11	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	+1	38.67 ±1.07
D12	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	−1	10.67 ±1.28
D13	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	+1	30.67 ±0.58
D14	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	+1	33.33 ±0.39
D15	+1	+1	−1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	−1	+1	42.67 ±1.58
D16	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	4.00 ±0.95

* observed values are the mean value of triplicates ± standard deviation (SD) at $p < 0.05$.

Table 4. Statistical analysis of the Plackett–Burman experiment for *Bacillus megaterium* UP47

Factor	Effect	Coefficient	t	F	p-value*
Mean/Intercept	–	26.584	–	–	–
X1	6.998	3.499	7.398	54.731	0.0855
X2	14.449	7.224	10.870	118.156	0.0584
X3	11.950	5.975	8.726	76.137	0.0726
X4	32.926	16.463	31.891	1017.040	0.0199
X5	−7.226	−3.613	−6.999	48.979	0.0903
X6	−12.701	−6.351	−9.274	86.007	0.0683
X7	−13.422	−6.711	−10.098	101.963	0.0628
X8	3.049	1.525	3.223	10.389	0.1915
X9	10.384	5.192	8.797	77.389	0.0720
X10	19.850	9.925	14.285	204.057	0.0444
X11	8.879	4.439	7.025	49.355	0.0900
X13	−8.856	−4.428	−7.007	49.104	0.0902
X14	−14.315	−7.157	−10.301	106.119	0.0616
X15	−7.211	−3.605	−6.109	37.322	0.1032

* significant p-value at $p \leq 0.05$.

However, the linear relationship of biotin had a negative effect on γ-PGA and its quadratic relationship had a positive effect on the yield of γ-PGA. There were positive and negative effects of both linear and quadratic relationships of the interactions of the 3 factors with each other (Table 5) on yield, whereas there were also some redundant effects (A^2B^2, AC^2, A^2C^2, BC^2, B^2C and B^2C^2), which were not estimated. All estimated effects ranged between 21.98% and 99.61% confidence level (Table 6).

Based on these results, response surface plots of the effects of L-glutamic acid and yeast extract against yield, L-glutamic acid and biotin against yield, and yeast extract and biotin against yield were generated (Fig. 4A,B,C). The plots showed that with an increase in L-glutamic acid and a decrease in yeast extract, there was an increase in the yield of γ-PGA. An increase in L-glutamic acid and biotin brought about an increase in γ-PGA yield (Fig. 4B) while, with an increase in biotin and a decrease in yeast extract, there was

Table 5. Design and run order of the 3-factor-1 block 15-run Box–Behnken design matrix with observed gamma-polyglutamic acid (γ-PGA) production results for *Bacillus megaterium* UP47

Run order	Values			PGA yield* [g/L]
	A	B	C	
1	–1	–1	0	39.33 ±0.31
2	1	–1	0	62.00 ±1.10
3	–1	1	0	14.67 ±0.89
4	1	1	0	45.33 ±0.45
5	–1	0	–1	16.00 ±0.85
6	1	0	–1	42.00 ±1.00
7	–1	0	1	4.67 ±0.22
8	1	0	1	43.33 ±1.20
9	0	–1	–1	36.00 ±0.41
10	0	1	–1	53.33 ±0.71
11	0	–1	1	57.33 ±0.22
12	0	1	1	48.67 ±0.75
13	0	0	0	47.67 ±0.32
14	0	0	0	43.33 ±0.53
15	0	0	0	44.00 ±0.10

* observed values are the mean value of triplicates ± standard deviation (SD); A – L-glutamic acid; B – yeast extract; C – biotin.

an increase in the yield of γ-PGA (Fig. 4C). A second order polynomial equation for prediction of γ-PGA from the Box–Behnken design using the regression coefficients of the estimated effects was derived as (Eq. 3):

$$\gamma\text{PGA yield} = \begin{matrix}(3316.061 - 449.708A + 9.036A2 \\ - 139.813B + 3.095B2 - 7.699C \\ - 0.164C2 + 13.116AB - 0.087AB2 \\ - 0.248A2B + 3.781AC \\ - 0.076A2C - 0.394BC)\end{matrix} \quad (3)$$

Four trials (at 0.25 L, 0.5 L, 1.0 L, and 3.0 L) using different levels of the 3 examined factors gave laboratory yields within 95% confidence levels of the predicted yields, obtained using the second order polynomial equation derived (Table 7). The adjusted R^2 (coefficient of regression) was calculated to be 0.97905, which indicated that 97.90% of the variability in the obtained response was explained by this model. Thus, it shows that the experimental model was of good fit for γ-PGA yield prediction. The regression value (R^2) was slightly higher than the 0.95 recorded by Berekaa and Al-Otaibi.[13] There was an increase in the yield of γ-PGA polymer from 16.33 g/L to 62 g/L by *B. megaterium* UP47 when the optimized media conditions were utilized. For the factors such as L-glutamic acid (A), yeast extract (B) and biotin (C), the second order polynomial equation derived for prediction of γ-PGA yield was (Eq. 4):

$$\gamma\text{PGA yield} = \begin{matrix}(3316.061 - 449.708A + 9.036A2 \\ - 139.813B + 3.095B2 - 7.699C \\ - 0.164C2 + 13.116AB - 0.087AB2 \\ - 0.248A2B + 3.781AC \\ - 0.076A2C - 0.394BC)\end{matrix} \quad (4)$$

Conclusion

Bacillus megaterium UP47 isolated from fermented seeds of *Pentaclethra macrophylla* (*Ugba)* produced an extracellular polymeric material which was characterized as γ-PGA. At low concentrations, the polymer possessed antibacterial activities. Through the Plackett–Burman and Box–Behnken studies, the bacterium required biotin and L-glutamic acid for optimized γ-PGA production. The experiments provided us with the knowledge of specific precursor factors that the particular strain under study would require to optimally produce γ-PGA and also for scaling up γ-PGA biosynthesis. *Bacillus megaterium* UP47 γ-PGA could therefore find application in food, agriculture, pharmaceutical, medical and wastewater treatment industries, among others.

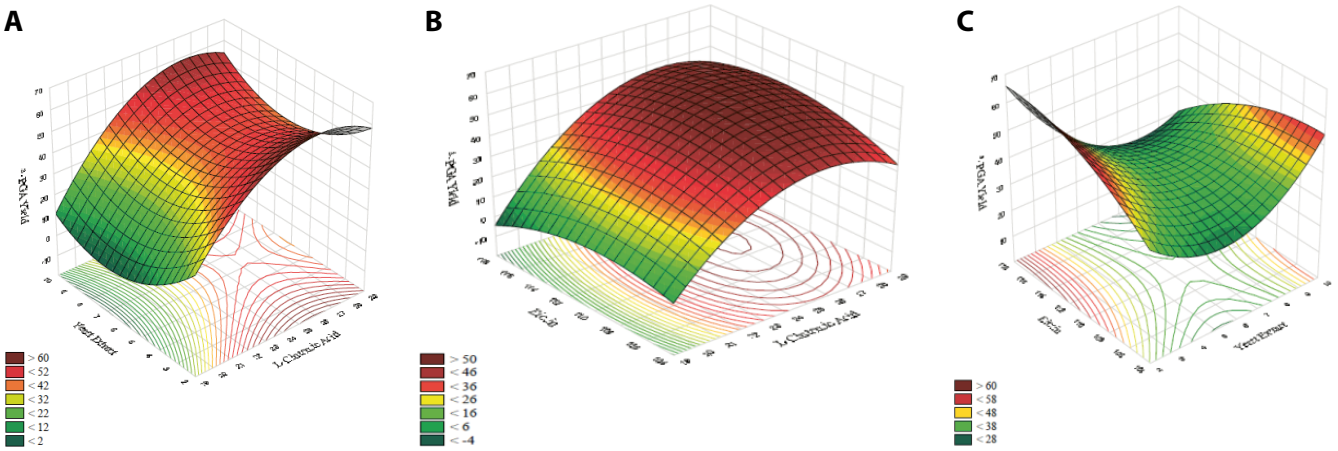

Fig. 4. A. 3D response surface plots showing the interaction of L-glutamic acid (x-axis) and yeast extract (y-axis) with their effect on the yield of gamma-polyglutamic acid (γ-PGA) (z-axis in g/L) for isolate *B. megaterium* UP47; B. 3D response surface plots showing the interaction of L-glutamic acid (x-axis) and biotin (y-axis) with their effect on the yield of γ-PGA (z-axis in g/L) for isolate *B. megaterium* UP47; C. 3D response surface plots showing the interaction of yeast extract (x-axis) and biotin (y-axis) with their effect on the yield of γ-PGA (z-axis in g/L) for isolate *B. megaterium* UP47

Table 6. Statistical analysis of the Box–Behnken experiment for gamma-polyglutamic acid (γ-PGA) production by *Bacillus megaterium* UP47

Factor	Effect	Coefficient	t	F	p-value
Mean/ Intercept	–	38.630	–	–	–
A	28.169	14.084	16.122	259.938	0.0038
A^2	13.196	6.598	10.832	117.350	0.0084
B	–11.544	–5.772	–6.607	43.654	0.0221
B^2	–9.135	–4.567	–7.499	56.235	0.0173
C	–0.555	–0.277	–0.318	0.101	0.7801
C^2	4.924	2.462	4.042	16.338	0.0561
AB	3.995	1.997	1.709	2.923	0.2294
AB^2	3.120	1.560	1.884	3.552	0.2001
A^2B	11.909	5.954	7.193	51.749	0.0187
AC	6.330	3.165	2.709	7.340	0.1135
A^2C	6.667	3.333	4.035	16.287	0.0562
BC	–12.995	–6.497	–5.561	30.934	0.0308

* significant p-value at $p \leq 0.05$; A – L-glutamic acid; B – yeast extract; C – biotin.

Table 7. Experimental and predicted gamma-polyglutamic acid (γ-PGA) yield of some production trials with the corresponding confidence levels

Trial	Laboratory experimental medium volume	Factor level*			Experimental yield**	Predicted yield	Confidence limit	
		A	B	C			–95%	+95%
1	0.25 L	20	3	105	36.00 ±0.72	33.50	19.68	47.32
2	0.50 L	28	9	116	44.00 ±0.43	35.10	22.10	48.10
3	1.0 L	28	9	105	50.67 ±0.97	46.80	32.90	60.60
4	3.0 L	20	3	116	38.67 ±0.95	35.16	22.20	48.12

* factors A, B and C indicate L-glutamic acid, yeast extract and biotin, respectively; ** the second order polynomial equation derived for prediction of γ-PGA yield is:
γPGA yield = 3316.061 – 449.708A + 9.036A2 – 139.813B + 3.095B2 – 7.699C – 0.164C2 + 13.116AB – 0.087AB2 – 0.248A2B + 3.781AC – 0.076A2C – 0.394BC.

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References

1. Adebayo-Tayo BC, Popoola AO. Biogenic synthesis and antimicrobial activity of silver nanoparticle using exopolysaccharides from lactic acid bacteria. *Int J Nano Dimens*. 2017;8:61–69. doi:10.22034/ijnd.2017.24377
2. Odeniyi OA, Avoseh DS. Effects of media components and agricultural residues on γ-polyglutamic acid production by *Bacillus toyonensis* AS8. *Polim Med*. 2018;48(2):91–97. doi:10.17219/pim/105555
3. Bhat A, Irorere VU, Bartlett T, et al. *Bacillus subtilis* natto: A non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *AMB Express*. 2013;3(1):36. doi:10.1186/2191-0855-3-36
4. Ju WT, Song YS, Jung WJ, Park RD. Enhanced production of poly-γ-glutamic acid by a newly isolated *Bacillus subtilis*. *Biotechnol Lett*. 2014;36(11):2319–2324. doi:10.1007/s10529-014-1613-3
5. Balogun-Agbaje OA, Odeniyi OA, Odeniyi MA. Drug delivery applications of poly-γ-glutamic acid. *Futur J Pharm Sci*. 2021;7:125. doi:10.1186/s43094-021-00280-w
6. Goto A, Kunioka M. Biosynthesis and hydrolysis of poly(γ- glutamic acid) from *Bacillus subtilis* IFO3335. *Biosci Biotechnol Biochem*. 1992;56(7):1031–1035. doi:10.1271/bbb.56.1031
7. Kunioka M. Biosynthesis and chemical reactions of poly-(amino acid)s from microorganisms. *Appl Microbiol Biotechnol*. 1997;47(5):469–475. doi:10.1007/s002530050958
8. Zhang D, Xu ZQ, Xu H, et al. Improvement of poly(γ-glutamic acid) biosynthesis and quantitative metabolic flux analysis of a two-stage strategy for agitation speed control in the culture of *Bacillus subtilis* NX-2. *Biotechnol Bioproc E*. 2011;16(6):1144–1151. doi:10.1007/s12257-011-0074-y
9. Moraes LP, Brito PN, Alegre RM. The existing studies on biosynthesis of poly(γ-glutamic acid) by fermentation. *Food and Public Health*. 2013;3(1):28–36. doi:10.5923/j.fph.20130301.04
10. Pan HM, Subramanian A, Ochs CJ, Dewavrin J, Beyer S, Trau DW. Edible polyelectrolyte microcapsules with water-soluble cargo assembled in organic phase. *RSC Adv*. 2014;4:35163–35166. doi:10.1039/x0xx00000x
11. Luo Z, Guo Y, Liu J, et al. Microbial synthesis of poly-γ-glutamic acid: Current progress, challenges, and future perspectives. *Biotechnol Biofuels*. 2016;9:134–145. doi:10.1186/s13068-016-0537-7
12. Khalil IR, Irorere VU, Radecka I, et al. Poly-glutamic acid: Biodegradable polymer for potential protection of beneficial viruses. *Materials (Basel)*. 2016;9(1):28–40. doi:10.3390/ma9010028
13. Berekaa MM, Al-Otaibi MS. Enhanced production of poly glutamic acid by *Bacillus* sp. SW1-2 using statistical experimental design. *Afr J Biotechnol*. 2013;12(5):481-490. doi:10.5897/AJB12.2947
14. Plackett RL, Burman JP. The design of optimum multifactorial experiments. *Biometrika*. 1946;33:305–325. doi:10.1093/biomet/33.4.305
15. Mabrouk M, Abou-Zeid DM, Sabra W. Application of Plackett–Burman experimental design to evaluate nutritional requirements for poly (γ-glutamic acid) production in batch fermentation by *Bacillus licheniformis* A13. *Afr J Microbiol Res*. 2012;1(2):6–18. http://damanhour.edu.eg/pdf/researches/Mabrouk%20et%20al.pdf. Accessed November 26, 2021.

16. Singh V, Haque S, Niwas R, Srivastava A, Pasupuleti M, Tripathi CKM. Strategies for fermentation medium optimization: An in-depth review. *Front Microbiol*. 2017;7:2087–2102. doi:10.3389/fmicb.2016.02087
17. Box GEP, Behnken DW. Some new three-level designs for the study of quantitative variables. *Technometrics*. 1960;2:455–475. doi:10.2307/1266454
18. Bajaj IB, Singhal RS. Sequential optimization approach for enhanced production of poly (γ-glutamic acid) from newly isolated *Bacillus subtilis*. *Fd Tech Biotechnol*. 2009;47(3):313–322. https://hrcak.srce.hr/39849. Accessed December 12, 2021.
19. Adedire OM, Odeniyi OA. Antimicrobial activities of bacteriocin-like extracellular metabolites produced by soil bacteria. *UK J Pharm Biosci*. 2017;5(4):47–54. doi:10.20510/ukjpb/5/i4/166545
20. Choi SH, Park JS, Whang KS, Yoon MH, Choi WY. Production of microbial biopolymer, poly(γ-glutamic acid) by *Bacillus subtilis* BS 62. *JACB*. 2004;47(2):60–64. https://www.koreascience.or.kr/article/JAKO200410103436471.page. Accessed December 12, 2021.
21. Yanglang C, Suppasil M. Screening of glutaminase-producing bacteria from Kung-Som. Paper presented at: 26th Annual Meeting of the Thai Society for Biotechnology and International Conference. 2014. https://tsb2014.mfu.ac.th/proceeding/03_PDF/(FF)%20Food%20biotech%20_%20Food%20safety/TSB2014%20Proceeding%20PR-FF-05.pdf. Accessed November 26, 2021.
22. Olutiola PO, Famurewa O, Sontag HG. *An Introduction to General Microbiology: A practical approach*. Heldelberg, Germany: Heidelberger Verlagsanstalt and Druckerei GmbH; 1991:42–44.
23. Barrow GI, Feltham RKA. *Cowan and Steel's Manual for Identification of Medical Bacteria*. 3rd ed. Cambridge, UK: Cambridge University Press; 1992.
24. Bang BH, Rhee MS, Kim KP, Yi DH. Influences of culture medium components on the production poly (γ-glutamic acid) by *Bacillus subtilis* GS-2 isolated *Chungkookjang*. *Kor J Food Nutr*. 2012;25(3):677–684. doi:10.9799/ksfan.2012.25.3.677
25. Zeng W, Chen G, Zhang Y, Wu K, Liang Z. Studies on the UV spectrum of poly(γ-glutamic acid) based on development of a simple quantitative method. *Int J Biol Macromol*. 2012;51:83–90. doi:10.9799/ksfan.2012.25.3.677
26. Qiu T, Li H, Cao Y. Pre-staining thin layer chromatography method for amino acid detection. *Afr J Biotechnol*. 2010;9:8679–8681. doi:10.5897/AJB10.817
27. Ho GH, Ho TI, Hsieh KH, et al. γ-polyglutamic acid produced by *Bacillus subtilis* (natto): Structural characteristics, chemical properties and biological functionalities. *JCCS*. 2013;53(6):1363–1384. doi:10.1002/jccs.200600182
28. Ogunleye A, Bhat A, Irorere VU, Hill D, Williams C, Radecka I. Poly-γ-glutamic acid: Production, properties and applications. *Microbiology (Reading)*. 2015;161:1–17. doi:10.1099/mic.0.081448-0
29. Balouiri B, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. 2016;6(2):71–79. doi:10.1016/j.jpha.2015.11.005
30. Motta AS, Brandelli A. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J Appl Microbiol*. 2002;92(1):63–70. doi:10.1046/j.1365-2672.2002.01490.x
31. Abdel-Fattah YR, Soliman NA, Berekaa MM. Application of Box–Behnken design for optimization of poly-γ-glutamic acid production by *Bacillus licheniformis* SAB-26. *Res J Microbiol*. 2007;2(9):664–670. doi:10.3923/jm.2007.664.670
32. Dell Statistica [computer software]. Version 12 and 13. Round Rock, USA: Dell Incorporated; 2015.
33. Baxi NN. Use of nonspecific, glutamic acid-free, media and high glycerol or high amylase as inducing parameters for screening *Bacillus* isolates having high yield of polyglutamic acid. *Int Sch Res Notices*. 2014:608739. doi:10.1155/2014/60873
34. Tork SE, Aly MM, Alakilli SY, Al-Seeni MN. Purification and characterization of gamma poly glutamic acid from newly *Bacillus licheniformis* NRC20. *Int J Biol Macromol*. 2015;74:382–391. doi:10.1016/j.ijbiomac.2014.12.017
35. Hezayen FF, Rehm BH, Tindall BJ, Steinbüchel ER. Transfer of *Natrialba asiatica* B1T to *Natrialba taiwanensis* sp. nov. and description of *Natrialba aegyptiaca* sp. nov., a novel extremely halophilic, aerobic, non-pigmented member of the *Archaea* from Egypt that produces extracellular poly-(glutamic acid). *Int J Syst Evol Microbiol*. 2001;51(3):1133–1142. doi:10.1099/00207713-51-3-1133
36. Lee NR, Go TH, Lee SM, et al. In vitro evaluation of new functional properties of poly-γ-glutamic acid produced by *Bacillus subtilis* D7. *Saudi J Biol Sci*. 2014;21:153–158. doi:10.1016/j.sjbs.2013.09.004
37. Su, CY, Chen CC, Chen HY, Lin CP, Lin FH, Fang HW. Characteristics of an alternative antibacterial biomaterial for mouthwash in the absence of alcohol. *J Dent Sci*. 2019;14(2):192–197. doi:10.1016/j.jds.2019.01.002
38. Ajayeoba TA, Dula S, Ijabadeniyi OA. Properties of poly-glutamic acid producing-*Bacillus* species isolated from ogi liquor and lemon-ogi liquor. *Front Microbiol*. 2019;10:771. doi:10.3389/fmicb.2019.00771
39. Bajestani MI, Mousavi SM, Mousavi SB, Jafari A, Shojaosadati SA. Purification of extra cellular poly-g-glutamic acid as an antibacterial agent using anion exchange chromatography. *Int J Biol Macromol*. 2018;113:142–149. doi:10.1016/j.ijbiomac.2018.02.082
40. Majed R, Faille C, Kallassy M, Gohar M. Bacillus cereus biofilms: Same, only different. *Front Microbiol*. 2016;7:1054. doi:10.3389/fmicb.2016.01054