Poly (γ-glutamic acid) Production Enhancement in Submerged Fermentation of *Bacillus Licheniformis* ATCC 9945[a] Using Optimization of Operating Variables and Glutamate Feeding

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ABSTRACT: *Poly (γ-glutamic acid) is a versatile biopolymer that can be used on an industrial scale if efficient methods are developed to increase production. In this study, first, based on the central composite design method of the response surface module, the effect of operational variables including temperature in the range of 30-44 °C, pH 4.5-8.5, and stirring in the range of 600-1000 rpm on poly (γ-glutamic acid) production was investigated in the batch fermentation of Bacillus licheniformis ATCC 9945[a] for the first time. Under optimal conditions viz. T of 37.4 °C, pH of 6.6, and agitation rate of 784.2 rpm, 15.5 g/L γ-PGA was obtained. According to the statistical analyses, adjusted R^2 was 0.9572, and analysis of variance explicated that T-T, pH-pH, and agitation-agitation effects indicated the lowest p-values and had the most significant influence on biopolymer synthesis. Under the optimal conditions, glutamate (a novel feed) pulse feeding (as poly (γ-glutamic acid)-based monomer) was optimized, for the first time, using the one-factorial method to achieve a maximum of 42.13 g/L of biopolymer production (highest in comparison with others' studies of this strain) by the two-pulsed feeding method. The chemical confirmation and novel physical characterization of the powdered product indicated a pure poly (γ-glutamic acid) sample suitable for biological, biomedical, and biopharmaceutical applications.*

KEYWORDS: *γ-PGA biopolymer; Central composite design optimization; Bacillus licheniformis; Pulse feeding; One-factorial.*

INTRODUCTION

Poly (γ-glutamic acid) (γ-PGA) is a versatile biopolymer that constitutes the main portion of capsules obtained from many *Bacillus* strains during fermentation. It contains one or both units of D-glutamic acid and L-glutamic acid, which are linked by amide bonds formed between alpha-amino and gamma-carboxyl groups. γ-PGA is an anionic, biocompatible, biodegradable, non-toxic, water-soluble, environmentally friendly, and healthy to a human polypeptide that can be preferably used in the medical, pharmaceutical, food, agricultural, and

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1021-9986/2023/6/2018-2035 *18/$/6.08*

cosmetics industries. Applications such as food additives (viz. for value addition, long time preserving, protection against freezing, drinking thickening, bitterness relieving, better forming of bakery products, etc.), drug release, drug delivery, encapsulation, hydrogel, water retaining and releasing, wastewater treatment, humectant, fertilizer improvement and so forth are among the very known focused fields that have attracted the attention of many to this polyamide. The applications of γ-PGA are spreading increasingly. Thus, a high demand exists for simple, significant, and efficient methods that improve γ-PGA production [1-6].

Past research on γ-PGA synthesis using *Bacillus licheniformis* ATCC 9945[a] has suggested the E culture medium for proper production of γ-PGA. An investigation of the effects of different additives and pH in several studies has shown that adjusting the values of $MnSO_4$ and ferric ions, and pH of 6.5 of the culture medium can increase γ-PGA production. Also, other effective parameters in batch culture performance such as conditions of aeration and inoculum, temperature, and nutrients, have also been examined and set independently in separate studies [7-10]. It has also been shown that glutamate as a γ-PGA monomer, electron receptor, and source of carbon and nitrogen has the highest effect on improving γ-PGA production [11-15]. However, to further increase γ-PGA production and reduce costs, effective process variables such as agitation rate, pH, and temperature need to be optimized for each bacterial strain. Additionally, the effects of different carbon sources such as citrate, glutamate, glucose, glycerol, fructose, and sorbitol, should be evaluated for each type of γ-PGA producing microbial strain. In a fed-batch culture, feeding strategy, composition, amount, and concentration of feed have significant effects on increasing production. Prior research based on fed-batch fermentation proposed pulsed feeding as an efficient strategy for enhancing γ-PGA production by *Bacillus licheniformis* ATCC 9945[a]. In these studies, the effect of pulse number, time, amount, and strategy (constant rate, sudden, etc.) of feeding on increasing production of γ-PGA has been investigated. However, each of these studies has used limited research to increase production and has not focused exclusively on optimizing the parameters of the pulse feeding strategy [15-17].

The need to introduce more specialized methods for the production of valuable materials such as γ-PGA from the industrial viewpoint is currently expanding. As observed in recent research, up to 50% of the raw material cost is saved using industrial waste to feed Bacillus subtilis for agricultural γ-PGA production [18]. Also, recently new bacterial strains have been introduced that can lead to higher production of γ-PGA and the industrial development of the corresponding fermentation process [19]. Environmental considerations of γ-PGA production are an essential issue in its synthesis process, especially on an industrial scale. Many studies have used metal ions, especially copper ions, to extract γ-PGA from its culture medium, which causes environmental pollution. Therefore, in recent research, the alcohol precipitation method has been used instead of the above technique to end in an environmentally friendly process [20, 21].

Also, previous research has used trial and error methods using randomized experimental designs on specific operating parameters to increase γ-PGA production [1-4]. Some of these studies have used a limited range of a parameter to find the preferred conditions for increasing production [7-17]. This type of investigation is mainly based on linear optimization techniques [7-17], while production increase is caused by the interaction of factors affecting it. Hence, it cannot be expected to achieve maximum production of γ-PGA using these techniques [7-17]. In addition, no study has been reported on the simultaneous effect of several operational parameters on increasing γ-PGA production by *B. licheniformis* ATCC 9945a.

The statistical Design of Experiments (DOE) by providing the possibility of the simultaneous study of factors affecting production and consideration of the interaction between them in addition to further increasing production, significantly reduces the time and cost of performing experiments [22]. In these series of methods, more commonly applied in the two forms of Taguchi and especially Response Surface Methodology (RSM) as Central Composite Design (CCD) nowadays, the number of required experiments for optimization is reduced based on defined levels of variables and statistical methods [23-25]. DOE is a set of predefined settings for the process variables of interest, providing an efficient way to schedule experiments to optimize production. This statistical and applied method is a basic technique to design the experiments to be performed, analyze the results, interpret the results based on experiment conditions, and determine the significance of the role of all

covered variables in achieving a defined value or purpose of a target or a group of target response parameters [26]. As mentioned, among the various methodologies of statistical design of experiments, response surface methods with two algorithms, Box-Behnken, and especially central-composite design and Taguchi statistical method are the most common methods for optimizing biological processes. The reason for this can be the simple and user-friendly entity of the relevant procedures of these techniques, their efficiency, and the economic way of the design of experiments [22-26]. The response surface method (especially the CCD algorithm) is usually preferred to the Taguchi method because it gives a mathematical predictive model of the influencing factors along with their interactions for the objective (response) function which is based on experimental data [27, 28]. Both the methods (Taguchi and CCD) are capable of optimizing the process factors for an optimization purpose. However, the model proposed by CCD is capable of predicting similar process results in the defined ranges of variables and finding the optimum points of each and all factors for the optimal response (while considering the interaction of basic variables) [27, 28].

The most effective process variables of fermentation (T, pH, and agitation rate) have not been optimized for *Bacillus licheniformis* ATCC 9945a strain (and should be optimized for each strain to increase γ-PGA production and reduce production costs). Therefore, first, for γ-PGA production enhancement, the operating parameters of the submerged fermentation of *Bacillus licheniformis* ATCC 9945[a] were optimized by a response surface methodology (RSM) technique with a central composite design (CCD) algorithm as the first novelty of this research. On the other hand, pulse feeding was used in previous studies. But, pulse feeding's most effective strategy parameters were not optimized for this strain (investigated as another novelty of this research) which can further open the way for commercial development of γ-PGA production with *Bacillus licheniformis* ATCC 9945a. One more point in the novelty of the current research is choosing a new and effective component for feeding in the fed-batch fermentation. As mentioned earlier, L-glutamic acid is the constructional monomer of γ-PGA polymer and one of the most effective carbon and nitrogen sources for *Bacillus licheniformis* ATCC 9945a strain that has not previously been used as a feed. Therefore, in the second stage, for further production increase, a simple glutamate (a novel feed) pulsed-feeding method was developed and optimized by a one-factorial experimental design as another novelty of this research. The produced γ-PGA was characterized both chemically (using FT-IR and NMR) to verify its identity by distinguishing the involved chemical bonds, and physically (using SEM to investigate the powdered sample for the first time) to determine the size, shape, and structure of γ-PGA particles.

EXPERIMENTAL SECTION

The experiments and analysis results of this research were reported by mean amounts of triplicates with a maximum 5% error.

Microorganism, culture medium, and submerged fermentation

Bacillus licheniformis ATCC 9945[a], a gram-positive, aerobic, and glutamate-dependent bacterial strain with high γ-PGA production capacity [29], stocked by freezing at -80 °C, was inoculated by 6% volumetric ratio (3 mL) into a 500 mL Erlenmeyer flask constituting 50 mL working volume comprised of sterilized (at 121°C for 20 minutes) inoculum culture medium (including (in g/L) glycerol: 40, glutamate: 30, citrate: 12, ammonium chloride: 7, $MgSO_4.7H_2O$: 0.5, $FeCl_2.6H_2O$: 0.04 and K_2HPO_4: 0.5) and grown at initial pH 7.4 (by 6M NaOH), 37°C and 200 rpm inside a shaking incubator for 16 hours. This seed culture was then added as the starter of fermentation with a 10% portion (v/v) to sterilized (at 121 °C for 20 minutes) E culture medium (including (in g/L) glycerol: 80, glutamate: 20, citrate: 12, ammonium chloride: 7, K_2HPO_4: 0.5, $MgSO_4.7H_2O$: 0.5, $FeCl_2.6H_2O$: 0.04, $MnSO_4.H_2O$: 0.104 and $CaCl_2.2H_2O$: 0.15 [7]) constituting an overall 500 mL working volume of an Infors AG bioreactor (with 1-liter total volume). Dissolved oxygen (DO) was maintained above 30% of air saturation by 2 vvm aeration and applying pure oxygen instead of air when required.

Basics of optimization of the operating parameters by Central Composite Design (CCD)

For the optimization of γ-PGA production, the effect of three effective operating variables of agitation rate in the range of 600-1000 rpm, pH from 4.5-8.5, and temperature

Table 1: The experiments proposed by central composite design with three operating variables and the achieved mean responses of triplicates (n=3) with less than five percent standard error

Runs	Real values (coded levels)			Response: γ-PGA production (g/L)
	Factor 1 A: Temperature (°C)	Factor 2 B: pH	Factor 3 C: Agitation (rpm)	
1	37.0 (0)	8.5 (+1.682)	800.0 (0)	10.5±0.46
2	37.0 (0)	6.5 (0)	800.0 (0)	15.1±0.61
3	32.8 (-1)	7.7 (+1)	681.1 (-1)	10.1±0.44
4	32.8 (-1)	7.7 (+1)	918.9 (+1)	9.22±0.41
5	37.0 (0)	6.5 (0)	800.0 (0)	15.73±0.64
6	41.2 (+1)	5.3 (-1)	681.1 (-1)	10.7±0.47
7	32.8 (-1)	5.3 (-1)	918.9 (+1)	8.59±0.39
8	41.2 (+1)	5.3 (-1)	918.9 (+1)	8.9±0.4
9	44.0 (+1.682)	6.5 (0)	800.0 (0)	11.93±0.51
10	37.0 (0)	6.5 (0)	800.0 (0)	15.6±0.63
11	41.2 (+1)	7.7 (+1)	918.9 (+1)	9.79±0.43
12	37.0 (0)	4.5 (-1.682)	800.0 (0)	8.335±0.37
13	30.0 (-1.682)	6.5 (0)	800.0 (0)	11.57±0.5
14	41.2 (+1)	7.7 (+1)	681.1 (-1)	10.975±0.48
15	37.0 (0)	6.5 (0)	1000.0 (+1.682)	10.3±0.45
16	37.0 (0)	6.5 (0)	800.0 (0)	14.75±0.6
17	32.8 (-1)	5.3 (-1)	681.1 (-1)	9.5±0.42
18	37.0 (0)	6.5 (0)	800.0 (0)	15.6±0.63
19	37.0 (0)	6.5 (0)	600.0 (-1.682)	11.22±0.49
20	37.0 (0)	6.5 (0)	800.0 (0)	15.5±0.62

of 30-44 °C was investigated by CCD experimental design. The logical ranges proposed were based on the bacterial strain growth characteristics and mesophile bacterial optimum growth temperatures [1, 3, 8, 13, 17]. The design of experiments (DOE) and analysis of variance of the obtained results by the CCD approach were performed using design-expert software version 13. Table 1 displays the designed experimental conditions in the two forms of real values and coded levels with the real responses by less than 5% error. A quadratic polynomial relation (Eq. (1)) was used to fit the data, explain the roles of all factors and their interactions, as well as analyze and predict the desired responses [30-32].

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \quad (1)$$

Where Y represents the predicted response. X_i and X_j indicate operating parameters. β_i and β_{ij} show the linear and interaction coefficients, respectively. β_{ii} represents a square coefficient and β_0 indicates a constant. CCD as an RSM module defines a certain number of factor levels based on model features to search for points or ranges of desired variables with respect to certain target response(s). The introduced levels by the software include factorial, center, and star points that account for curves. The factorial level points are usually determined ±1 unit away from the design center point defined for all coded variables. In this situation, the distance unit ± α for star points with an absolute value greater than 1 is determined based on variable numbers and design specifications [30-32].

When the considerations about the fit statistics of the quadratic model represents a coefficient of determination (R^2) and adjusted R^2 of close to unity (at least with 0.05 tolerance), it is verified that the second-order polynomial equation applied for predictive modeling of the response in the determined space of the based operating variables is significant. The p-values of any of these variables or other model terms, which were determined to be lower than 0.05 in the analysis of variance (ANOVA) table, were chosen to be significant based on the software

pre-sets. The other model terms including the variables, their interactions, or squared terms that were not in range according to this criterion (or were overall far away from the limit of less than 0.1 of p-value considering some exceptions), can be omitted from the model in order to reduce it. Three-dimensional (3D surface) plots of the interactive variations of two variables at a fixed value of the others can be generated in the graphical section of design expert software, which performs the analysis of regression to depict the model [30-39]. The CCD matrix of 20 experiments with five levels for each operating variable generated by the software has been displayed i n Table 1 along with the attributed γ-PGA production values.

Pulsed-feeding methods of glutamate scheduled based on one-factorial experimental design

In addition to relying on optimization of operating parameters using CCD, it was planned to feed L-glutamic acid monomer due to its principal role in increasing γ-PGA biopolymer production [1, 3, 7, 17, 40]. The effect of four factors, namely amount, concentration, pulse numbers, and timing of feeding on γ-PGA production was investigated using the one-factor at a time method. First, the effect of glutamate feeding in 5, 10, 15, 20, and 25 g (per liter of working volume) at the ninth hour of the fermentation process γ-PGA production was investigated. Then, the effect of feeding time on production was evaluated in the 9^{th}, 24^{th}, and 48^{th} hours of the process at the best feeding amount of the one-pulse feeding strategy. Then, the effect of pulse feeding frequency in 2, 4, and 8 pulse feedings on increasing γ-PGA production was investigated. Finally, by studying the effect of glutamate feed concentration in the range of 250 to 400 g/L, its optimal value for further production of γ-PGA was determined.

Extraction, purification, and concentration determination of γ-PGA

To extract γ-PGA, first, the cells were removed by centrifugation of the culture medium at 11440 × g for 20 min and 4 °C. The cooled supernatant and ethanol were mixed together with a volumetric ratio of 1 to 4, respectively, and centrifuged at 4 °C and 14480 × g for 30 min to precipitate the γ-PGA and separate it from the culture medium components. The extracted biopolymer was then subjected to a purification process by dialysis against 1 L of distilled water lasting for 12 h with three times of water exchange and 3500 Daltons molecular weight cut-off. The concentration of γ-PGA was determined by the Gel Permeation Chromatography (GPC) method and a GMPW column and TSK gel using a 50-mM sodium nitrate mobile phase [17].

The methods for the determination of time profiles of culture medium ingredients

The Optical Density (OD) of the fermented culture medium was determined by measuring the absorbance of samples at 600 nm by a spectrophotometer (UV-Vis 6310-JENWAY) against the fresh culture medium. Due to the effects of the γ-PGA product on the turbidity of the culture medium solution, OD_{600} measurements could not be representative of cell growth, and instead, Dry Cell Weight (DCW) was measured. DCW measurements were started by removing 5 mL aliquots from the culture medium, followed by a centrifugation step at 11440 × g for 15 min in a tube with a specific mass. Finally, the cells were washed with distilled water and dried in an oven at a temperature of about 100 °C to reach a constant weight [17]. Finally, by multiplying the difference between the final weight and the weight of the tube by 200, the dry cell weight per liter was obtained.

The concentration of glutamate was measured using a Boehringer Mannheim enzyme kit. The trend of changes in citric acid and glycerol concentration was determined by the high-performance liquid chromatography (HPLC) method using a Hitachi L-3300 RI monitor HPLC device equipped with an Aminex HPX-87H ion-exchange column with sulfuric acid at a concentration of 0.02 M as the mobile phase [17].

Chemical and surface characterization of γ-PGA

The purified γ-PGA by dialysis was subjected to freeze-drying for two days before being ground into a fine powder, to be prepared for chemical and surface characterizations. The chemical characteristics of the γ-PGA powder, as KBr pellets formed by mixing 2 mg sample powder with 200 mg KBr and pressing it into a disk, were identified by Nicolet 800 (USA) Fourier-Transform InfraRed (FT-IR) spectroscopy equipment using MS/DOS operating system. The resultant pellet sample was scanned at 200 × from 400 to 4000 cm^{-1} wave numbers with 4 cm^{-1} resolutions.

Nuclear Magnetic Resonance (NMR) spectroscopy is generally applied to determine the structure and purity

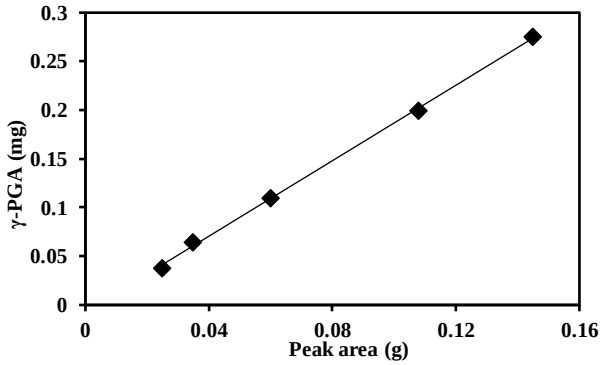

Fig. 1: Calibration curve for determining the concentration of γ-PGA in production medium samples

of γ-PGA. This spectrometry technique investigates the magnetic properties of atom nuclei to determine the structure of a compound. In the current research, the HNMR spectra of a γ-PGA sample were obtained by applying an NMR device (Bruker, Germany). The sample was dissolved in D_2O. Chemical shifts were displayed on the basis of signal in units of parts per million (ppm). The frequency used was 300 to 130,000 MHz and other conditions applied in the analysis were UXNMR, Bruker Analytische Messtechnik GmbH source; 1 H nucleus; D_2O solvent; the temperature of 380 degrees Kelvin; the number of 30 scans; pulse width of 14.9 seconds; the frequency of the spectrometer of 300.13 MHz; spectrum width of 7812.5 Hz; and the lowest frequency of 1507.6 Hz. The data obtained were analyzed by MestReNova software. Finally, the results were compared with others' works.

Surface characterization of γ-PGA powder, after subjecting its surface to sputter-coating with gold, was done by scanning electron microscopy (SEM) analysis. VEGA3 TESCAN SEM equipment (Czech Republic) was applied to investigate the surface morphology and size of the coated powder product (powder sample SEM is novel).

Molecular weight determination of γ-PGA by GPC

GPC is a proper analytical technique to distinguish natural and synthetic proteins and biopolymers. It uses porous gel particles to separate biopolymers in solution. GPC separates analytes based on their dimension. The analytes are passed through porous particles in a packed column. The smaller analytes can easily enter the pores and therefore have a longer residence time in the column. However, the analytes with bigger molecular weights cannot enter the pores. Thus, they have a short residence time and exit the column faster. Thus, GPC works as an effective technique to determine the molecular weight of γ-PGA.

The GPC analysis was applied using an Agilent1100 device equipped with Agilent-PL aquagel-OH Mixed-H columns to determine the molecular weight of the biopolymer using the refractometer index detector. γ-PGA was dissolved in distilled water. The distilled water of pH=7 with a flow rate of 1 mL/min, at 30°C, was used for washing. First, the device was graded with a range of Pullulan polymers, and then the samples were injected into the device after preparation. According to the obtained data, the molecular weight of the biopolymer was obtained. The following method was used to prepare the samples. First, 20-mg biopolymer was added to 10 mL of detergent solution. 12 h were given for the sample to dissolve completely. Then, the sample was maintained for 30 min at a temperature of 50°C until it was completely dissolved in the solvent.

RESULTS AND DISCUSSION

Calibration curve of γ-PGA quantification

Similar to the method developed by *Birrer et al*. [7], a very analogous calibration curve was obtained to determine γ-PGA concentration as described therein. The calibration curve of the prepared sample (as explained in this article's methods section: free of cells and pure) is displayed in Fig. 1.

The peak area data of GPC is related to the injected weight of γ-PGA by this calibration curve (Fig. 1). This facilitates the measurement of the concentration of γ-PGA in the culture broth samples of submerged fermentation with *Bacillus licheniformis* ATCC 9945a. This calibration curve displayed a correlation coefficient close to unity (more than 0.99) which explicates that it is fit for this type of evaluation. The sample may need dilution for the linear determinations of the diagram depicted in Fig. 1.

Analysis of variance, proposed model, and statistical details

A three-variable CCD design created twenty experiments that included two levels for axial points, two-level factorial point calculations, and six repetitions coverage at the center point, mainly to consider the response tolerance into account. Stochastic combinations of experimental variables designed as a matrix were organized based on five-level factors, to determine their overall effects on the concentration-response of the biopolymer obtained

Table 2: Analysis of variance (ANOVA) of the proposed quadratic equation of response surface technique obtained for γ-PGA concentrations (g/L)

Factor	Sum of squares	DOF*	Mean square	F-value	p-value	Considerations
Model	130.22	9	14.47	48.23	< 0.0001	significant
A: Temperature	0.9282	1	0.9282	3.09	0.1091	
B: pH	2.67	1	2.67	8.89	0.0138	
C: Agitation	2.93	1	2.93	9.76	0.0108	
AB	0.0005	1	0.0005	0.0018	0.9674	
AC	0.1785	1	0.1785	0.5951	0.4583	
BC	0.0520	1	0.0520	0.1734	0.6859	
A^2	28.94	1	28.94	96.46	< 0.0001	
B^2	72.42	1	72.42	241.41	< 0.0001	
C^2	45.00	1	45.00	150.01	< 0.0001	
Residual	3.00	10	0.3000			
Lack of fit	2.29	5	0.4582	3.23	0.1119	not significant
Pure error	0.7090	5	0.1418			

* Degree-of-freedom.

after the experiments were performed (as shown in Table 1).

The statistical significance of model factors can be determined by the ANOVA table created by the software for the quadratic model equation. According to the estimated p-values of the terms of this model (Table 2), by determining a 95% confidence level, only the variables pH and agitation, and all the quadratic terms of temperature, pH, and agitation became significant (terms B, C, A^2, B^2, and C^2). The reason for temperature variable term (A) not being in the range of 0 to 0.05 p-value can be attributed to the strain type, originating from the mesophile entity of *B. licheniformis* ATCC 9945[a], which has made it possible for the bacteria to grow and be productive over a wide range of moderate temperatures [8, 13, 17, 41, 42].

Table 2 represents the sum of squares, degree of freedom (DOF), and mean square relevant to the quadratic model, its terms, residual, and lack of fit and pure error. The table also displays the F-values and p-values of the model, its terms, and lack of fit. These criteria demonstrated the model's significance and that lack of fit was not significant, which is in favor of the robustness and fit of the predictive quadratic equation [43, 44]. The proposed models in terms of coded and actual factors are respectively displayed as Equations (2) and (3).

$$\gamma\text{-PGA}=15.4+0.2607*A+0.442*B-0.4629*C-0.0081*AB-0.1494*AC+0.0806*BC-1.42*A^2-2.24B^2-1.77C^2 \quad (2)$$

$$\gamma\text{-PGA}=-251.49785+6.36758*T+20.58282*pH+0.203486*Agitation-0.001641*T*pH-0.000302*T*Agitation+0.00057*pH*Agitation-0.081795*T^2-1.58512*pH^2-0.000125*Agitation^2 \quad (3)$$

Comparison factor coefficients of the coded Eq. (2) identify the relative effects of factors on the response. In contrast, the actual Eq. (3) cannot be used for this purpose, because its coefficients correspond to a scale based on each operating unit. Both equations predict the amount of γ-PGA production based on arbitrary levels of each factor. In Eq. (2), the levels of the factors are coded as -α, -1, 0, 1, and α. Eq. (3) is suitable for determining levels based on the real units of each factor [45]. Both model equations can be reduced by removing factor terms that were not significant according to the ANOVA table (Table 2) [46].

Fit statistics table to check the adequacy of the model

The statistics data related to the fit of the model and its adequacy have been depicted in Table 3. The squared correlation coefficient (R^2) depends on the process entity and the correct detection of the error sources [47]. The coefficient of determination (R-squared) represents the fitness quality of empirical data by the model. R^2 should be in the vicinity of 1 and preferably higher than 0.9. Unlike R^2, Adjusted R^2 is not sensitive to a number of factors. This means that adjusted R^2 resolves the deficiency of addition to R^2 values by the appended insignificant factors [43, 47]. The amount of Predicted R^2

Table 3: Model adequacy based on the statistics for γ-PGA production

The considered statistical component	The statistics amount for γ-PGA Production
R^2	0.9775
Adjusted R^2	0.9572
Predicted R^2	0.8617
Adequate precision	18.2909
Standard deviation	0.5477
Mean	11.7
Coefficient of variation %	4.68

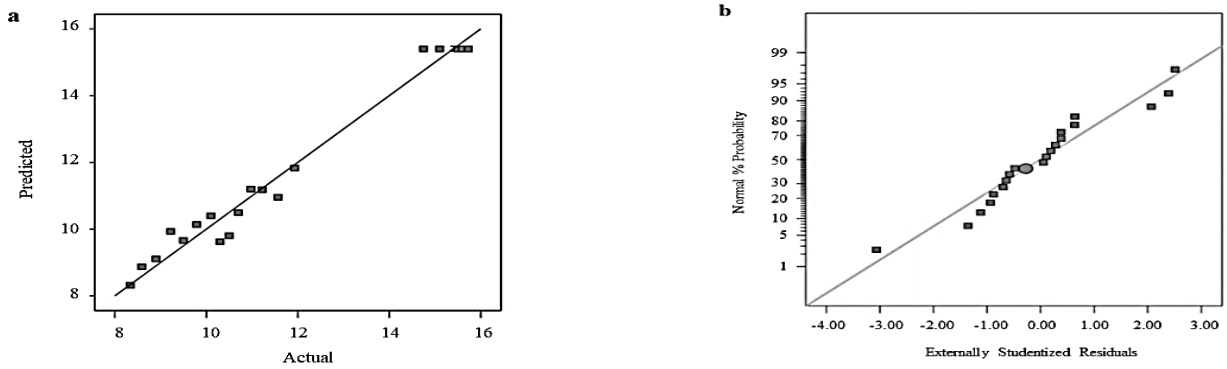

Fig. 2: γ-PGA production predicted vs. actual values (a) and residuals normal % of probability (b)

is indicative of the presence of an acceptable, reasonable, and logical agreement with the Adjusted R^2 value; because it complies with the general rule of lower than 0.2 difference [43, 47, 48]. The value of Adequate Precision measures the signal-to-noise ratio. The ratios higher than four are desirable. Its amount is much higher than this quantity in the present research, which shows the signal is quite proper [49]. Also, the coefficient of variation, which shows the ratio of standard deviation value to the mean amount, is so much lower than the maximum desired value of 10% [50, 51]. Thus, this model is very efficient for strategic use in the design space, based on its entity.

Comparing the model predictions and the experimental results in Fig. 2(a, b) shows a good distribution of all points in the vicinity of the 45° line and a normal straight line. This indicates that the model is accurate the experimental data are well distributed and the factors, levels, and responses are excellent [52, 53].

Two-factor interaction effects

Three-dimensional surface plots (3D) in Fig. 3 show the simultaneous effect of two factors at a constant level of the third factor on γ-PGA production. The increase of each of the three parameters leads to an increase in the γ-PGA production at the beginning of the study range, but it decreases with a further increase in each of them. Fig. 3(a) shows the γ-PGA concentration as a function of pH and temperature changes at a constant agitation rate of 800 rpm. It can be seen from this Figure that the temperature range of 35-39 °C leads to a high product concentration. Fig. 3(a) shows that increasing pH from 5.3 to 6.5 is quite effective in increasing γ-PGA production. However, when the stirring rate is increased to the midpoint threshold or about 800 rpm, the biopolymer synthesis is enhanced significantly. A higher stirring rate reduced production (Fig. 3 (b)), possibly due to the occurrence of cells damaged by the high-speed stirrer [54]. Fig. 3(c) shows the interaction of agitation rate and pH variables on a 3D response surface plot of γ-PGA concentration at a constant level of 37°C. The maximum production of γ-PGA can be obtained at about 750-820 rpm and pH 6-7.

The 3D plots of Fig. 3 demonstrate that among operating variables, agitation had the most significant effect on production, considering the slope gradients. In this regard, pH explicated a comparable significant effect, too. The parabolic investigation of the 3D plots shows that all the triple quadratic terms were equally the most significant terms in the model affecting γ-PGA production.

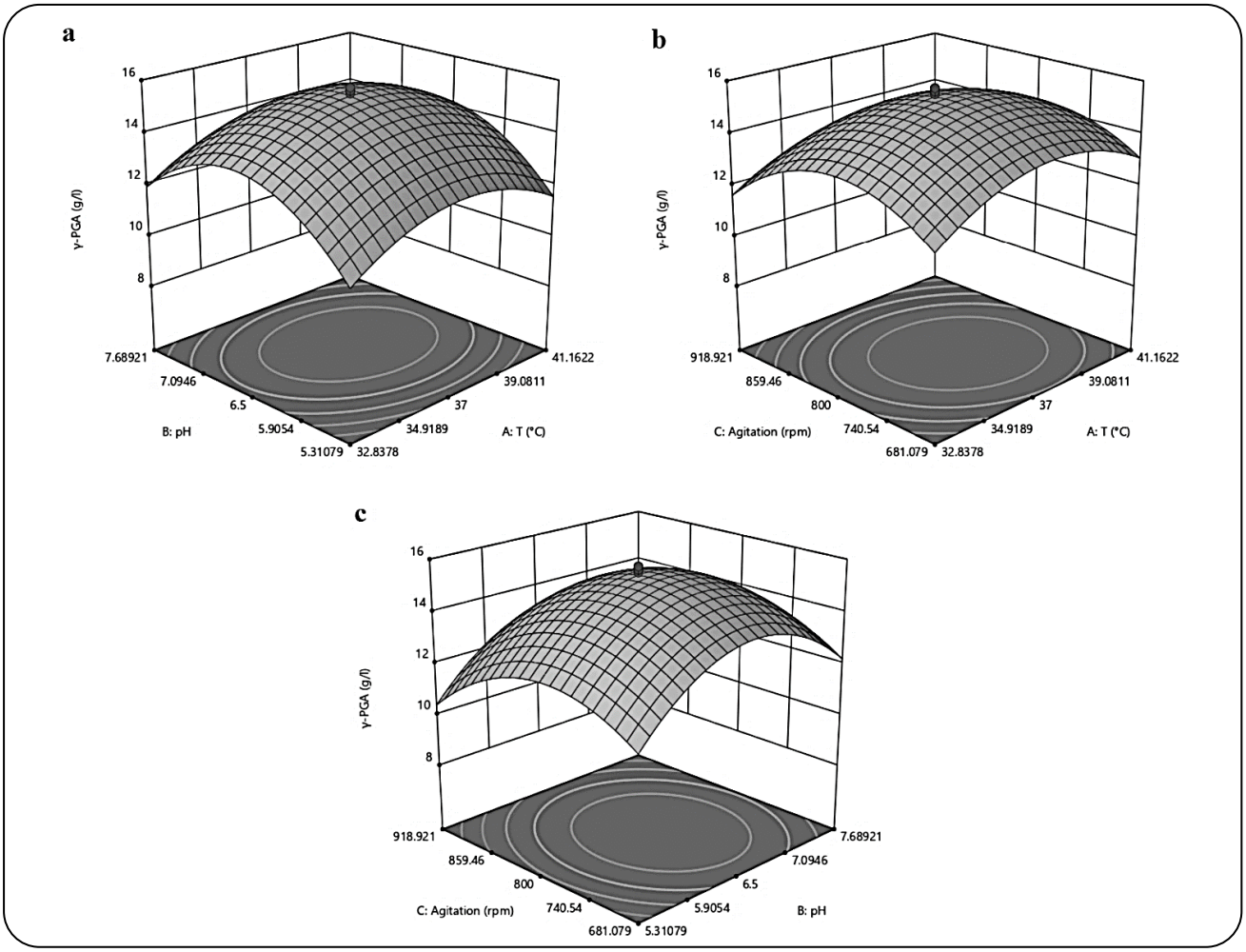

Fig. 3: 3D response surface plots of γ-PGA concentration as a function of: (a) pH and temperature at agitation=800 rpm, (b) agitation and temperature at pH=6.5, and (c) agitation and pH at temperature=37 °C

Microorganism type has a decisive influence on the optimum temperature range for achieving more γ-PGA. The proposed moderate temperature range (35-39 °C) was proper for production enhancement with Bacillus *licheniformis* ATCC 9945a, which is a mesophile microorganism. Higher or lower temperatures reduce the microorganism's activity and consequently decrease biopolymer production [55]. The pH values in the range of 6-7 were also beneficial for better metabolism of the cells, beyond which production diminishes. An acidic environment or very high pH can be detrimental to cells [1, 3, 56, 57]. Agitation in the range of 750-820 rpm increased γ-PGA concentration. Agitation beyond this range cannot show positive effects on biopolymer synthesis. This indicates that sufficient oxygen is provided in this area and higher agitation disrupts the cells [55]. Paying attention to the observed effects of the variations of the variables on production, the most contributions can be attributed to agitation and pH, respectively. γ-PGA synthesis amount showed the most sensitivity to the agitation rate, so that by enhancing agitation speed from 682 to 800 rpm, production increased from 10 to about 15 g/L (Fig. 3 (c)). At a higher agitation rate, biopolymer production diminished. At the constant temperature of about 37 °C, the most increments of γ-PGA synthesis with rpm variations were noticed (Fig. 3 (b)). A very acidic or alkaline culture medium represses cell activity and biopolymer secretion due to cell membrane damage (Fig. 3 (a, c)) [1, 3, 56].

Optimization of the base operating variables for obtaining the highest γ-PGA concentration

The studied values of agitation rate, pH, and temperature were selected to increase the γ-PGA production according

Table 4: One-factorial experimental design experiments for optimizing glutamate feeding. The experiments and relevant mean γ-PGA production of triplicates (n=3) with less than five percent standard error

Experiment No.	Feed Conc. (g/L)	No. of feeding pulses	Feeding amount (g)	Feeding time (h)	γ-PGA yield (g/L)
1	250	1	5	9	11.46±0.49
2	250	1	10	9	12.59±0.51
3	250	1	15	9	13.93±0.54
4	250	1	20	9	16.04±0.64
5	250	1	25	9	10.97±0.48
6	250	1	20	24	15.01±0.57
7	250	1	20	48	12.13±0.5
8	250	2	10	9, 24	25.1±0.99
9	250	2	15	9, 24	29±1.1
10	250	2	20	9, 24	36.42±1.4
11	250	2	25	9, 24	30.9±1.2
12	250	4	20	9, 24, 36, 48	34.44±1.35
13	250	8	20	5, 9, 24, 29, 36, 48, 53, 57	16.41±0.65
14	300	2	20	9, 24	38.86±1.49
15	350	2	20	9, 24	40.04±1.53
16	385	2	20	9, 24	42.13±1.74
17	400	2	20	9, 24	39.3±1.51

to the same procedure proposed by *Zhou et al*. in 2018 [57]. Under optimal conditions, including a stirring speed of 784 rpm, pH 6.6, and 37.4 °C, 15.5 g/L γ-PGA was obtained. The importance of optimizing the operating variables becomes more apparent when the optimized production is compared with the worst conditions of the defined ranges, i.e. stirring speed of 1000 rpm, pH=4.5, and 33°C, which produces about 6.8 g/L γ-PGA. This shows that by optimizing the stirring speed, pH, and temperature, the production is more than doubled. In the study by *Park et al*., 2001 [58] was concluded that a pH of approximately 6 is suitable for maximum biopolymer production. Due to the high difference in the applied strain characteristics compared to the present research, a very different optimal temperature of 20°C was reported for production enhancement. A temperature of 25°C and a pH of 5 were optimal for biopolymer synthesis with *Auricularia polytricha* [59]. The xanthan biopolymer production by *Xanthomonas campestris* showed similar optimum conditions as the current research at 500 rpm and 30 °C [60].

Glutamate feeding experiments design developed based on the one-factorial method

Optimizing operational variables was a big step in increasing biopolymer production. Combining the optimal conditions with effective and simple methods, such as pulse-feeding techniques, can improve γ-PGA synthesis and reduce production costs. The base glutamate feed concentration was 250 g/L. One pulse feeding had little influence on production improvement after 72 hours. Increasing the feed amount from 5 to 10, 15 and 20 g enhanced production but decreased with its increase to 25 g (due to overfeeding). Change of the feeding time from 9 to 24 and 48 hours with the preferred amount of 20 g glutamate reduced production. It may be due to the reduction of substrate uptake, cell death, accumulation of toxic metabolites, and the cells' undesirable conversions in late-stage feed [1-4, 7, 17]. Rows 1 to 7 of Table 4 display these experiments. One-pulsed feeding of glutamic acid was best done by 20 g of feed at the 9th hour of the fermentation process. The next proper feeding time (to achieve high production) was the 24th hour. Based on this information, two pulsed-feeding was done by feeding 20 g glutamic acid at the 9th and 24th hours. The effect of the glutamic acid amount of the two-pulsed feed on γ-PGA production was investigated as shown in rows 8 to 11 of Table 4.

The next step was increasing the feeding pulses to 4 and 8 pulses. Table 4 (rows 10, 12, and 13) indicates the effect of feeding pulse numbers on γ-PGA production at the preferred total feed amount of 20 g glutamate of 250 g/L

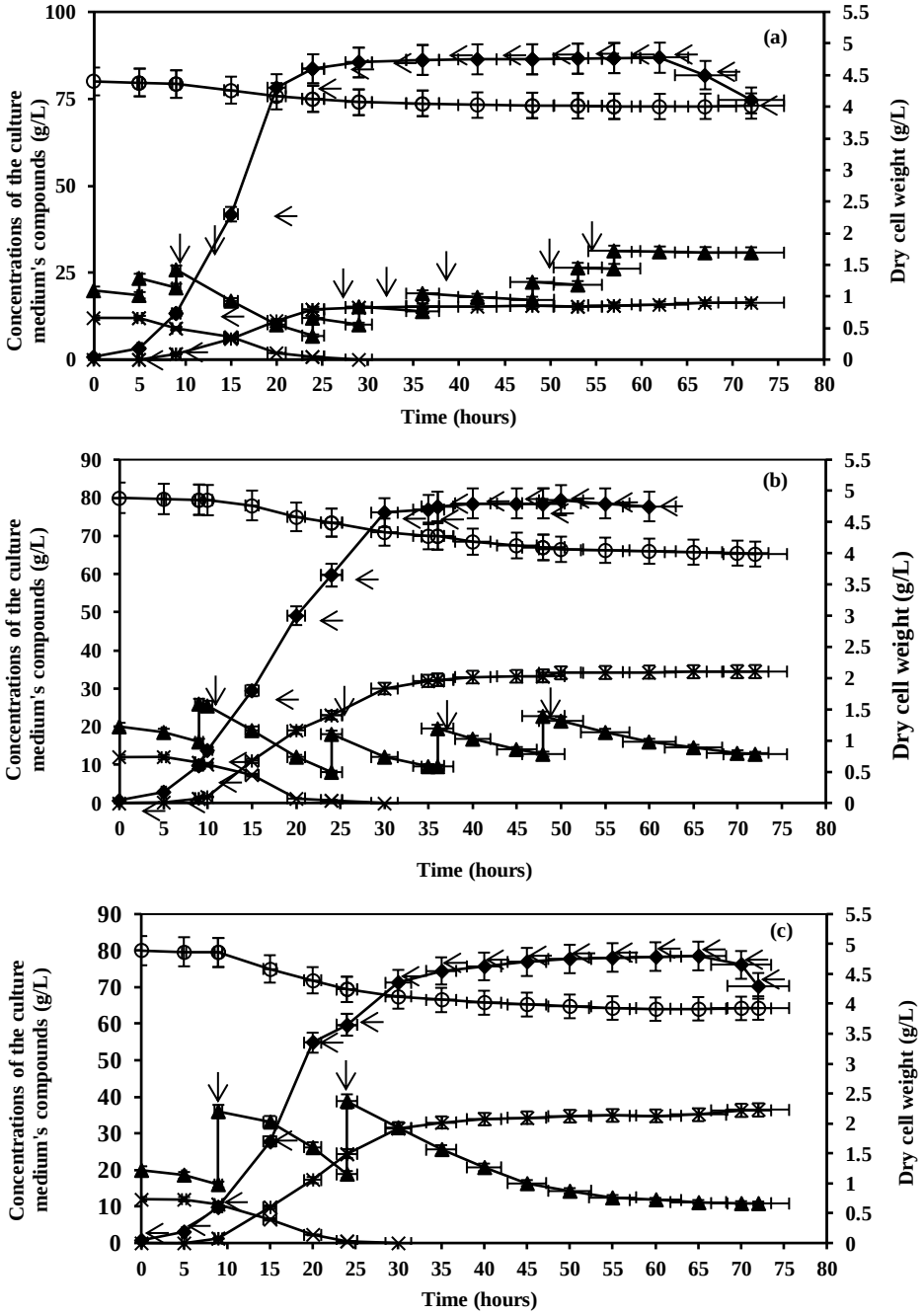

Fig. 4: Production of γ-PGA in the fed-batch culture with 20 g multi-pulsed glutamate feeding (eight pulses: Fig. 4 (a), four pulses: Fig. 4 (b) and two pulses: Fig. 4 (c)) with a concentration of 250 g/L. Three replicates mean time profiles of concentrations of γ-PGA (stars, *), dry cell weight (diamonds, ♦), glutamate (triangles, ▲), citrate (crosses, ×), and glycerol (circles, ○) with standard error bars of less than five percent tolerance (the vertical arrows indicate glutamate feeding times and the horizontal arrows indicate the data related to 2nd Y-axis (dry cell weight))

concentration. Fig. 4 depicts the attributed time profiles of culture medium ingredients of these three experiments. The feeding times were chosen based on the growth characteristics of the microorganism cells [1-4, 7, 17]. For the three fed-batch cultures in Fig. 4 (a-c), the value of growth yield or X respectively was 4.2 for Fig. 4 (a) between 0 and 20 h, 4.6 for Fig. 4 (b) between 0 and 30 h, and 4.3 g/L for Fig. 4 (c) between 0 and 30 h. Furthermore, the amount of yield coefficient or Y based on the consumption quantity of L-glutamic acid substrate was

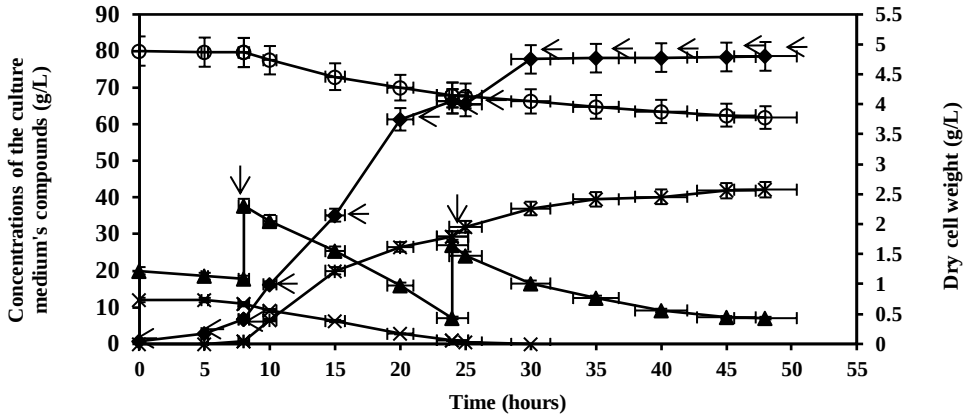

Fig. 5: Production of γ-PGA in the fed-batch culture with 20 g two-pulsed glutamate feeding of 385 g/L concentration. Three replicates mean time profiles of concentrations of γ-PGA (stars, *), dry cell weight (diamonds, ♦), glutamate (triangles, ▲), citrate (crosses, ×), and glycerol (circles, ○) with standard error bars of less than five percent tolerance (the vertical arrows indicate glutamate feeding times and the horizontal arrows indicate the data related to 2nd Y-axis (dry cell weight))

equal to 0.21 for Fig. 4 (a) between 0 and 20 h, 0.16 for Fig. 4 (b) between 0 and 30 h, and 0.15 for Fig. 4 (c), between 0 and 30 h, respectively.

Feeding L-glutamic acid, as the main monomer directly associated with the polymerization of the product, increased the γ-PGA production dramatically. According to previous research, the sudden shock of glutamate saturated concentration, preferably in 2 pulses, can better push forward the reaction of producing γ-PGA (from accumulating glutamate in the gamma-aminobutyric acid shunt) and act as a more robust amino acid stimulus. On the other hand, sudden feeding of a large amount of this acidic feed could release the trapped metallic ions stuck to the chelating biopolymer product, which worked as secondary limiting substrates [1, 3, 61, 62]. Using the preferred two-pulsed feeding for higher γ-PGA production chosen from different pulses numbers is also consistence with the reported data by *Abdel-Fattah et al*. in 2007 [63].

In the final stage of γ-PGA production enhancement, the glutamate concentration of feed in the range of 250 to 400 g/L including 250, 300, 350, and 385 g/L were investigated using the one-factorial at a time approach. Regarding this matter, the experimental rows 10, 14, 15, and 16 of table 4 show production increasing, respectively. However, a further increase in feed concentration led to a γ-PGA production decrease (Table 4, experiment row 17). Increasing glutamate feed concentration from 250 to 385 g/L enhanced γ-PGA production and productivity and decreased fermentation duration time from 72 h in other cases to 48 h (Table 4 experiment row 16). Further increasing the feed concentration beyond this limit illustrated adverse effects on the production amount and productivity of γ-PGA. L-glutamic acid concentration of feed can change the metabolism and physiology of the cells by affecting pH, enhancing or reducing biopolymer secretion, creating changes in cell membrane structure, and the cells' production of effective components and their exchanges [1-4]. Fig. 5 indicates the time profiles of the culture medium ingredients in the experiment with the best results (Table 4, experiment row 16).

Fig.s 4 and 5 indicate the changes in concentration of DCW, γ-PGA production, and substrates. The DCW and γ-PGA plots initiated with a lag phase at the start of fermentation (zero-order reaction). For the major and important part of the biological process, which is the high production phase, growth and production profiles are exponential (first-order kinetics). When the growth and production curves reach a plateau in the final stage (stationary phase), zero-order kinetics is the dominant phenomenon.

The productivity of γ-PGA was 0.88 g/ (L h) (Fig. 5). Cellular growth efficiency (X) was 4 g/L between 0 and 24 h of the fermentation process (in Fig. 5). Additionally, the yield coefficient (Y) based on the consumed quantity of glutamate substrate was 0.12 g/g (between 0 and 24 h). Fig. 5

shows the kinetics of γ-PGA production, cell growth, and uptake of carbon and nitrogen sources including citrate, glutamate, and glycerol under the optimum conditions for the highest γ-PGA production. Citrate is one of the main carbon sources and precursor of γ-PGA production with *Bacillus licheniformis* ATCC 9945a [15-17].

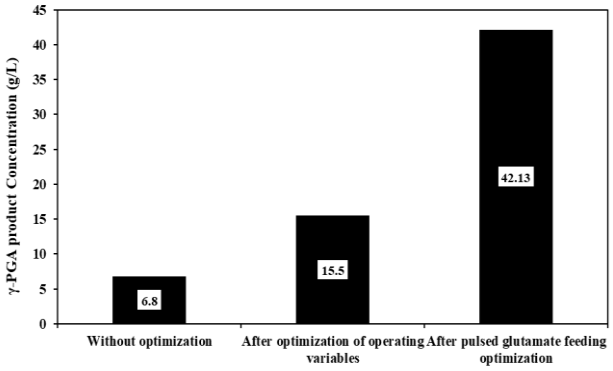

Fig. 6: The influence of applied optimizations on mean γ-PGA concentration of triplicates (n=3) with less than five percent standard error

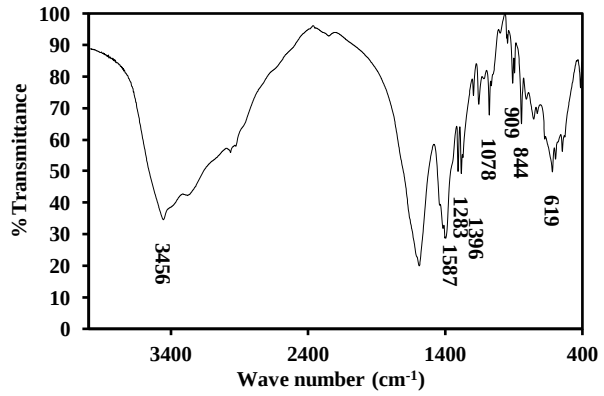

Fig. 7: FTIR pattern of the produced γ-PGA powder

The first pulse of glutamate caused a sudden increase in output. The second feeding in lower dimensions had the same effect. The first feeding was associated with a high increase in cell growth rate, indicating high uptake of nutrients (as was the second feeding). This proves that the increase in production kinetics depends on the cell growth kinetics. This increase in production was also attributed to the theory of stimulation of production by the amino acid [62] and the release of metal ions that were attached to the chelate prior to glutamic acid feeding. However, especially the first feeding shows that a large part of the improved kinetics of γ-PGA production, as a primary metabolite, is attributed to increased cell growth kinetics during the exponential phase of fermentation [1-4].

The importance of the main introduced techniques for improving production

Optimization of physicochemical variables and pulse feeding strategy factors were useful methods applied for increasing γ-PGA production in the current research. Fig. 6 displays the importance of optimization of operating conditions and glutamate-pulsed feeding techniques. It is clear that biopolymer production is enhanced to about 228% for the fermentation with optimization of operating variables in comparison with the fermentation without operating variables optimization.

Techniques for improving pulsed glutamate feeding parameters using a one-factorial optimization method also increased γ-PGA concentration. Fig. 6 shows a 272% increase in biopolymer production after optimizing the parameters of a glutamate feeding strategy using a one-factorial technique compared to optimal conditions without feeding. This is about 620% more than the case without optimization.

FT-IR and HNMR analysis results for the chemical characterization of γ-PGA

Fig. 7 shows the FT-IR pattern of γ-PGA powder produced in this study as a suitable technique for detection of component chemical bonds. This template is used to identify biopolymers as well as their functional groups. O-H stretching band at wave number 3456 cm^{-1}, N-H stretching band at 1587 cm^{-1}, minimum flexural C=O at 1396 cm^{-1}, C-N stretching band at 1283 cm^{-1}, and COOH stretching band at 1078 cm^{-1} is shown. In addition to these chemical groups, stretching vibration bands from 1000 cm^{-1} to 500 cm^{-1} indicated the presence of $–CH_2$, $–CH_3$, and $–(CH_2)_n–$ with n higher than 4, respectively (as shown in Fig. 7). These results are consistent with previous research in the field of biomedical applications of interest [64-66].

The structure of the γ-PGA sample was confirmed by the NMR. Fig. 8 displays the obtained HNMR spectrum. It represents a high signal-to-noise ratio, which can be due to the sample's high viscosity. Three types of hydrogen nuclei were distinguished, named alpha, beta, and gamma. As shown in the Figure, D_2O has a peak at 4.69 ppm concentration, α-CH at 4.082 ppm, β-CH_2 at 1.857 and 1.45 ppm, and γ-CH_2 at 2.237 ppm.

This pattern is comparable with other NMR analyses in previous studies [67, 68], that confirm the purity and good structural accordance of the entity of γ-PGA.

SEM analysis results

Scanning electron microscopy determines γ-PGA surface properties such as particle shape and dimensions.

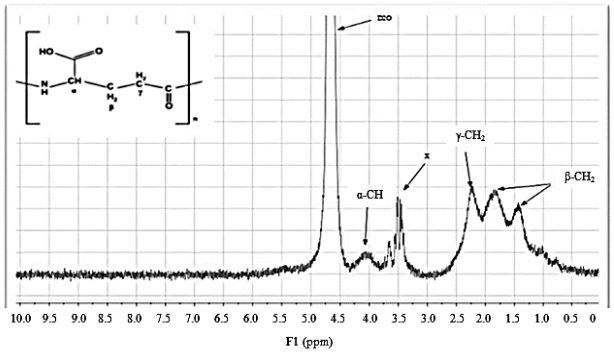

Fig. 8: HNMR pattern of the produced γ-PGA

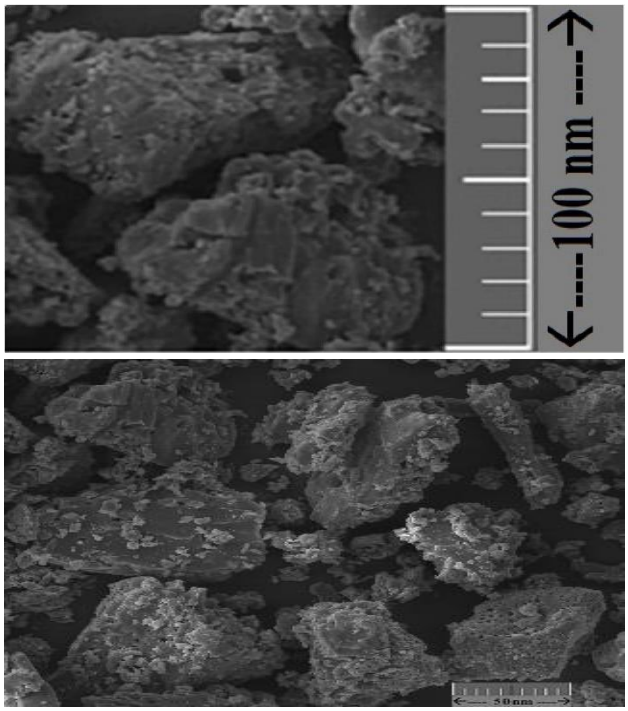

Fig. 9: SEM images of γ-PGA powder

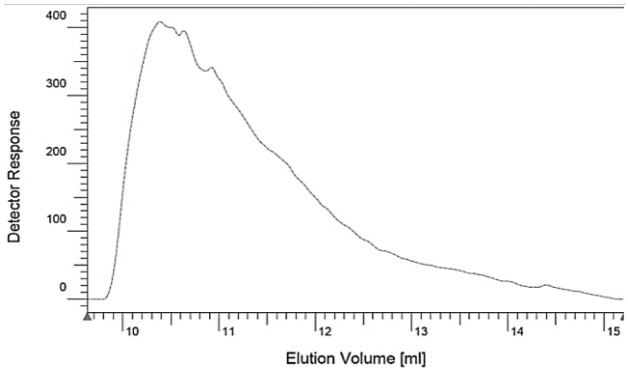

Fig. 10: GPC spectrum of γ-PGA produced by Bacillus licheniformis

The uniform shape of the particles can facilitate the prediction of the overall properties of the product. For example, when used for drug delivery purposes, the drug release rate pattern of the γ-PGA product can be predicted as a case-sensitive effective carrier. Particle size is another important issue in the morphological examination of the product. Smaller particle size provides more contact surface area to increase mass transfer, which can facilitate drug delivery efficiency to biological organs [69]. Fig. 9 shows SEM images of γ-PGA nanostructured powder. Crystal-like particles have an approximately uniform shape and size, which is a good factor for biomedical and biopharmaceutical applications (such as invariable drug release). Their dimensions' range was less than 50 nm to a maximum of less than 100 nm, which is suitable for biological purposes (because of the low dimensions required to facilitate biological applications). The nanometer size of powder particles facilitates the mass transfer and efficacy of the drugs delivered by these carriers by creating an improved contact surface and biotransformation [69].

Molecular weight of γ-PGA assessed by GPC

The molecular weight of γ-PGA, determined by GPC, can be analyzed based on the chromatograph displayed in Fig. 10.

The numerical average molecular weight (Mn) was 7041600 g/mol. The average molecular weight (Mw) of γ-PGA was equal to 28261000 g/mol. This Mw range makes the product particularly useful in food, agriculture, and also some biological fields [1-4]. The procedure that a technician follows for GPC molecular weight determination starts with using a known molecular weight marker. The relevant calibration curve with the vertical axis of "log (molecular weight)" and the horizontal axis of "log (elution amount)" that the technician prepares at this stage helps to compare and calculate the desired biopolymer sample molecular weight. The average molecular weight is also determined based on the summation of the all-chains' weights divided by the number of chains. The general formulae of $Mn=\Sigma (N_iM_i)/\Sigma N_i$ and $Mw=\Sigma (N_iM_i^2)/\Sigma N_iM_i$ can be used to determine the principal characteristics of the biopolymer (especially Mw is important).

CONCLUSIONS

Bacillus licheniformis ATCC 9945[a], as a mesophile bacterial strain could grow and be productive over an extensive range of moderate temperatures and operating variables, allowing for achieving enough high cell density for adequate γ-PGA production. Biopolymer synthesis was proved to be totally sensitive to and influenced by variations in the three parameters of agitation, pH, and temperature.

The central composite design RSM technique was used to investigate the important factors, analyze their mutual effects, and optimize all these effects to achieve improved γ-PGA production. This research ends with the obvious results mentioned in the following paragraphs:

1) Optimization of operational variables for maximum γ-PGA production (using CCD as a novel method for this bacterial strain) showed that the optimum point (T=37°C, pH=6.6, and agitation=784 rpm) resulted in a concentration of 15.5 g/L γ-PGA (and a novel accurate model was developed for the prediction of γ-PGA production in the defined ranges of operating variables, accounting for the parameters' interactions). Using this optimum point, two pulsed feeding of glutamate (based on the optimization of one-factor at a time method as a novel feed and optimization trend for this strain) resulted in a concentration of more than 42 g/L γ-PGA (the highest amount reported for this strain among the others' studies).

2) The chemical, morphological, and molecular weight properties determination analyses of the product powder were done by FTIR, HNMR, SEM, and GPC, respectively. The chemical FTIR and HNMR analyses confirmed the entity of a pure γ-PGA sample. Therefore, it is most proper for sensitive applications like biological and biopharmaceutical (drug delivery and drug release) fields, where the purity of the biopolymer is of particular priority. The nano-structure and uniformity of the product, determined by SEM (as a novel trend for powdered γ-PGA), make it especially effective in uniform (because of the uniform structure) and effective (because of the higher contact surface) drug release in small dimensions as in biological organs. Overall, and also based on the determined molecular weight range of the product, it is anticipated to be acceptable for food, agricultural, and biological industries' applications.

Putting all this together, the current research findings lead the way through the development of commercially valuable bioprocesses and the large-scale industrial production of metabolites and biological products, such as the γ-PGA biopolymer of *Bacillus licheniformis*, which could be very useful for drug delivery applications.

Acknowledgments

The authors wish to thank the Malek Ashtar University of Technology for providing all the necessary materials and equipment for this research.

Received : Jul.21, 2022 ; Accepted : Oct.24, 2022

REFERENCES

[1] Luo Z., Guo Y., Liu J., Qiu H., Zhao M., Zou W., Li S., Microbial Synthesis of Poly-γ-Glutamic Acid: Current Progress, Challenges, and Future Perspectives, *Biotechnol. Biofuels*, **9(1)**: 1-12 (2016).

[2] Bajaj I., Singhal R., Poly (Glutamic Acid)–An Emerging Biopolymer of Commercial Interest, *Bioresour. Technol.*, **102(10)**: 5551-5561 (2011).

[3] Ogunleye A., Bhat A., Irorere V.U., Hill D., Williams C., Radecka I., Poly-γ-Glutamic Acid: Production, Properties and Applications, *Microbiology*, **161(1)**: 1-17 (2015).

[4] Shih L., Van Y.T., The Production of Poly-(γ-Glutamic Acid) from Microorganisms and its Various Applications, *Bioresour. Technol.*, **79(3)**: 207-225 (2001).

[5] Ebrahimzadeh Kouchesfahani M., Bahrami A., Babaeipour V., Enhanced Production of Poly-γ-Glutamic Acid by *Bacillus Licheniformis* ATCC 9945[a] Using Simultaneous Pulse-Feedings of Citrate and Glutamate, *Prep. Biochem. Biotechnol.*, (2022).

[6] Ebrahimzadeh Kouchesfahani M., Bahrami A., Babaeipour V., Improving Poly-γ-Glutamic Acid Production by *Bacillus Licheniformis* ATCC 9945a Strain under Citrate and Glutamate Pulsed Feedings and Biopolymer Characteristic Evaluation, *Polym. Bull.*, (2022).

[7] Birrer G.A., Cromwick A.M., Gross R.A., γ-Poly (Glutamic Acid) Formation by *Bacillus Licheniformis* 9945a: Physiological and Biochemical Studies, *Int. J. Biol. Macromol.*, **16(5)**: 265-275 (1994).

[8] Cromwick A.M., Gross R.A., Effects of Manganese (II) on *Bacillus Licheniformis* ATCC 9945A Physiology and γ-Poly (Glutamic Acid) Formation, *Int. J. Biol. Macromol.*, **17(5)**: 259-267 (1995).

[9] Cromwick A.M., Birrer G.A., Gross R.A., Effects of pH and Aeration on γ-Poly (Glutamic Acid) Formation by *Bacillus Licheniformis* in Controlled Batch Fermentor Cultures, *Biotechnol. Bioeng.*, **50(2)**: 222-227 (1996).

[10] Feng J., Shi Q., Zhou G., Wang L., Chen A., Xie X., Huang X., Hu W., Improved Production of Poly-γ-Glutamic Acid with Low Molecular Weight under High Ferric Ion Concentration Stress in *Bacillus Licheniformis* ATCC 9945a, *Process Biochem.*, **56**: 30-36 (2017).

[11] Troy F.A., chemistry and Biosynthesis of the Poly (γ-d-Glutamyl) Capsule in *Bacillus Licheniformis*, i. Properties of the Membrane-Mediated Biosynthetic Reaction, *J. Biol. Chem.*, **248(1)**: 305-315 (1973).

[12] Troy F.A., Chemistry and Biosynthesis of the Poly (γ-d-glutamyl) Capsule in *Bacillus Licheniformis* II. Characterization and Structural Properties of the Enzymatically Synthesized Polymer, *J. Biol. Chem.*, **248(1)**: 316-324 (1973).

[13] Ko Y.H., Gross R.A., Effects of Glucose and Glycerol on γ-poly (glutamic acid) Formation by *Bacillus Licheniformis* ATCC 9945a, *Biotechnol. Bioeng.*, **57(4)**: 430-437 (1998).

[14] Mitsunaga H., Meissner L., Büchs J., Fukusaki E., Branched Chain Amino Acids Maintain the Molecular Weight of Poly (γ-glutamic acid) of *Bacillus Licheniformis* ATCC 9945 During the Fermentation, *J. Biosci. Bioeng.*, **122(4)**: 400-405 (2016).

[15] Mitsunaga H., Meissner L., Palmen T., Bamba T., Büchs J., Fukusaki E., Metabolome Analysis Reveals the Effect of Carbon Catabolite Control on the Poly (γ-glutamic acid) Biosynthesis of *Bacillus Licheniformis* ATCC 9945, *J. Biosci. Bioeng.*, **121(4)**: 413-419 (2016).

[16] Giannos S.A., Shah D., Gross R.A., Kaplan D.L., Mayer J.M., "Poly (glutamic acid) Produced by Bacterial Fermentation", In: "Novel Biodegradable Microbial Polymers", 457-460, Springer, Dordrecht (1990).

[17] Yoon S.H., Do J.H., Lee S.Y., Chang H.N., Production of Poly-γ-Glutamic Acid by Fed-Batch Culture of *Bacillus Licheniformis*, *Biotechnol. Lett.*, **22(7)**: 585-588 (2000).

[18] Zhang C., Wu D., Ren H., Economical Production of Agricultural γ-Polyglutamic Acid Using Industrial Wastes by *Bacillus Subtilis*, *Biochem. Eng. J.*, **146**: 117-123 (2019).

[19] Wang D., Hwang J. S., Kim D.H., Lee S., Kim D.H., Joe M.H., A Newly Isolated *Bacillus Siamensis* SB1001 for Mass Production of Poly-γ-glutamic acid, *Process Biochem*, **92**: 164-173 (2020).

[20] Zhiyi Y., Ran Q., Chang Z., Gao H., Jia C., Recovery of Low-Molecular-Weight γ-PGA by Metal Cation from the Fermentation Broth, *Process Biochem*, **82**: 215-221 (2019).

[21] Li M., He Y., Ma X., Separation and Quantitative Detection of Fermentation γ-polyglutamic Acid, *J. Future Foods*, **2(1)**: 42-48 (2022).

[22] Morowvat M. H., Babaeipour V., Rajabi Memari H., Vahidi H., Optimization of Fermentation Conditions for Recombinant Human Interferon Beta Production by *Escherichia coli* Using the Response Surface Methodology, *Jundishapur J. Microbiol.*, **8(4)**: e16236 (2015).

[23] Rao R.S., Kumar C.G., Prakasham R.S., Hobbs P.J., The Taguchi Methodology as a Statistical Tool for Biotechnological Applications: A Critical Appraisal, *Biotechnol. J.*, **3(4)**: 510-523 (2008).

[24] Khuri A.I., Mukhopadhyay S., Response Surface Methodology, *Wiley Interdiscip. Rev. Comput. Stat.*, **2(2)**: 128-149 (2010).

[25] Bagherinia M., Babaeipour V., Soleimani A., Optimization of Bacterial Nano-Cellulose Production in Bench-Scale Rotating Biological Contact Bioreactor by Response Surface Methodology, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **40 (2)**: 407-416, (2021).

[26] Kasemiire A., Avohou H.T., Bleye C.D., Sacre P.Y., Dumont E., Hubert P., Ziemons E., Design of Experiments and Design Space Approaches in the Pharmaceutical Bioprocess Optimization, *Eur. J. Pharm. Biopharm.*, **166**: 144-154 (2021).

[27] Narenderan S.T., Meyyanathan S.N., Karri V.V.S.R., Experimental Design in Pesticide Extraction Methods: A Review, *Food Chem.*, **289**: 384-395 (2019).

[28] Asghar A., Raman A.A.A., Daud W.M.A.W., A Comparison of Central Composite Design and Taguchi Method for Optimizing Fenton Process, *Sci. World J.*, (2014).

[29] Candela T., Fouet A., poly-Gamma-Glutamate in Bacteria, *Mol. Microbiol.*, **60(5)**: 1091-1098 (2006).

[30] Zakaria A., Haouti R.E., Lhanafi S., Benafqir M., Azougarh Y., Alem N.E., Treated Digested Residue During Anaerobic Co-Digestion of Agri-Food Organic Waste: Methylene Blue Adsorption, Mechanism and CCD-RSM Design, *J. Environ. Chem. Eng.*, **5(6)**: 5857-5867 (2017).

[31] Bhattacharya S., "Central Composite Design for Response Surface Methodology and its Application in Pharmacy", In: "Response Surface Methodology in Engineering Science", IntechOpen (2021).

[32] Zhang Z., Xiaofeng B., Comparison about the Three Central Composite Designs with Simulation, *2009 Int. Conf. Adv. Comput. Control. (ICACC)*, **IEEE 2009**: 163-167 (2009).

[33] Diaconu M., Pavel L.V., Hlihor R.M., Rosca M., Fertu D.I., Lenz M., Corvini P.X., Gavrilescu M., Characterization of Heavy Metal Toxicity in Some Plants and Microorganisms—A Preliminary Approach for Environmental Bioremediation, *N. Biotechnol*, **56**: 130-139 (2020).

[34] Krishnan S., Suzana B.N., Wahid Z.A., Nasrullah M., Munaim M.S.A., Din M.F.B.M., Taib S.M., Li. Y.Y., Optimization of Operating Parameters for Xylose Reductase Separation through Ultrafiltration Membrane Using Response Surface Methodology, *Biotechnol. Rep.*, **27**: e00498 (2020).

[35] Kiruthika J., Murugesan S., Studies on optimization of L-glutaminase Production under Submerged Fermentation from Marine *Bacillus Subtilis* JK-79, *Afr. J. Microbiol. Res.*, **14(1)**: 16-24 (2020).

[36] Sharma R., Ghoshal G., Optimization of Carotenoids Production by *Rhodotorula Mucilaginosa* (MTCC-1403) Using Agro-Industrial Waste in Bioreactor: A Statistical Approach, *Biotechnol. Rep.*, **25**: e00407 (2020).

[37] Haaland P.D., "Experimental Design in Biotechnology", CRC press (2020).

[38] Ranganadh R.A., Vidya P.K., Venkateswarulu T.C., Krupanidh S., Bobby M.N., Abraham P.K., Sudhakar P., Vijetha P., Statistical Optimization of PolyHydroxy Butyrate (PHB) Production by Novel *Acinetobacter Nosocomialis* RR20 Strain Using Response Surface Methodology, *Curr. Trends Biotechnol. Pharm.*, **14(1)**: 62-69 (2020).

[39] Goukanapalle P.K.R., Kanderi D.K., Rajoji G., Kumari B.S.S., Bontha R.R., Optimization of Cellulase Production by a Novel Endophytic Fungus *Pestalotiopsis Microspora* TKBRR Isolated from Thalakona Forest, *Cellulose*, **27**: 6299-6316 (2020).

[40] Du G., Yang G., Qu Y., Chen J., Lun S., Effects of Glycerol on the Production of Poly (γ-glutamic acid) by *Bacillus Licheniformis*, *Process Biochem.*, **40(6)**: 2143-2147 (2005).

[41] Yusuf H.A., Hossain S.Z., Khamis A.A., Radhi H.T., Jaafar A.S., Optimization of CO_2 Biofixation Rate by Microalgae in a Hybrid Microfluidic Differential Carbonator Using Response Surface Methodology and Desirability Function, *J. CO_2 Util.*, **42**: 101291 (2020).

[42] Rahimi T., Kahrizi D., Feyzi M., Ahmadvandi H.R., Mostafaei M., Catalytic Performance of MgO/Fe_2O_3-SiO_2 Core-Shell Magnetic Nanocatalyst for Biodiesel Production of Camelina Sativa Seed Oil: Optimization by RSM-CCD Method, *Ind. Crops Prod.*, **159**: 113065 (2021).

[43] Yahya H.S.M., Abbas T., Amin N.A.S., Optimization of Hydrogen Production via Toluene Steam Reforming over Ni–Co Supported Modified-Activated Carbon Using ANN Coupled GA and RSM, *Int. J. Hydrog. Energy*, **46(48)**: 24632-24651 (2021).

[44] Lazzari E., dos Santos Polidoro A., Onorevoli B., Schena T., Silva A.N., Scapin E., Jacques R.A., Caramão E.B., Production of Rice Husk Bio-Oil and Comprehensive Characterization (Qualitative and Quantitative) by HPLC/PDA and GC × GC/qMS, *Renew. Energy*, **135**: 554-565 (2019).

[45] Santhosh A.J., Tura A.D., Jiregna I.T., Gemechu W.F., Ashok N., Ponnusamy M., Optimization of CNC Turning Parameters Using Face Centred CCD Approach in RSM and ANN-Genetic Algorithm for AISI 4340 Alloy Steel, *Results Eng.*, **11**: 100251 (2021).

[46] Sharma J., A.S. Chadha, V. Pruthi, P. Anand, J. Bhatia, B.S. Kaith., Sequestration of Dyes from Artificially Prepared Textile Effluent Using RSM-CCD Optimized Hybrid Backbone Based Adsorbent-Kinetic and Equilibrium Studies, *J. Environ. Manage.*, **190**: 176-187 (2017).

[47] Foroutan R., Mohammadi R., Razeghi J., Ramavandi B., Biodiesel Production from Edible Oils Using Algal Biochar/CaO/K_2CO_3 as a Heterogeneous and Recyclable Catalyst, *Renew. Energy*, **168**: 1207-1216 (2021).

[48] Ince O.K., Aydogdu B., Alp H., Ince M., Experimental Design Approach for Ultra-Fast Nickel Removal by Novel Bio-Nanocomposite Material, *Adv. Nano Res.*, **10(1)**: 77-90 (2021).

[49] Igwegbe C.A., Onukwuli O.D., Ighalo J.O., Menkiti M.C., Bio-coagulation-flocculation (BCF) of Municipal Solid Waste Leachate Using Picralima Nitida Extract: RSM and ANN Modeling, *Curr. Res. Green Sustain. Chem., (CRGSC)*, **4**: 100078 (2021).

[50] Muniyasamy A., Sivaporul G., Gopinath A., Lakshmanan R., Altaee A., Achary A., Chellam P.V., Process development for the Degradation of Textile Azo Dyes (mono-, di-, poly-) by Advanced Oxidation Process-Ozonation: Experimental & Partial Derivative Modelling Approach, *J. Environ. Manage.*, **265**: 110397 (2020).

[51] Amin M., Chetpattananondh P., Ratanawilai S., Application of Extracted Marine Chlorella Sp. Residue for Bio-Oil Production as the Biomass Feedstock and Microwave Absorber, *Energy Convers. Manag.*, **195**: 819-829 (2019).

[52] Raheem A., Zhao M., Dastyar W., Channa A.Q., Ji G., Zhang Y., Parametric Gasification Process of Sugarcane Bagasse for Syngas Production, *Int. J. Hydrog. Energy*, **44(31)**: 16234-16247 (2019).

[53] Anfar Z., Amedlous A., Ait El Fakir A., Ahsaine H.A., Zbair M., Lhanafi S., El Haouti R., Jada A., El Alem N., Combined Methane Energy Recovery and Toxic Dye Removal by Porous Carbon Derived from Anaerobically Modified Digestate, *ACS Omega*, **4(5)**: 9434-9445 (2019).

[54] Ndikubwimana T., Zeng X., Liu Y., Chang J.S., Lu Y., Harvesting of Microalgae Desmodesmus sp. F51 by Bioflocculation with Bacterial Bioflocculant, *Algal Res.*, **6**: 186-193 (2014).

[55] Yong X., Cui Y., Chen L., Ran W., Shen Q., Yang X., Dynamics of Bacterial Communities During Solid-State Fermentation using Agro-Industrial Wastes to Produce poly-γ-glutamic acid, revealed by Real-Time PCR and Denaturing Gradient Gel Electrophoresis (DGGE), *Appl. Microbiol. Biotechnol.*, **92(4)**: 717-725 (2011).

[56] Sirisansaneeyakul S., Cao M., Kongklom N., Chuensangjun C., Shi Z., Chisti Y., Microbial Production of poly-γ-glutamic acid, *World J. Microbiol. Biotechnol.*, **33(9)**: 1-8 (2017).

[57] Zhu R., Ma X., Liu J., Optimization of γ-polyglutamic acid Synthesis Using Response Surface Methodology of a Newly Isolated Glutamate Dependent Bacillus Velezensis Z3, *Int. Microbiol.*, **21(3)**: 143-152 (2018).

[58] Park J.P., Kim S.W., Hwang H.J., Yun J.W., Optimization of Submerged Culture Conditions for the Mycelial Growth and Exo-Biopolymer Production by Cordyceps Militaris, *Lett. Appl. Microbiol.*, **33(1)**: 76-81 (2001).

[59] Xu C.P., Yun J.W., Optimization of Submerged-Culture Conditions for Mycelial Growth and Exo-Biopolymer Production by *Auricularia Polytricha* (Wood Ears Fungus) Using the Methods of Uniform Design and Regression Analysis, *Biotechnol. Appl. Biochem.*, **38(2)**: 193-199 (2003).

[60] Zakeri A., Pazouki M., Vossoughi M., Use of Response Surface Methodology Analysis for Xanthan Biopolymer Production by Xanthomonas Campestris: Focus on Agitation Rate, Carbon Source, and Temperature, *Iran. J. Chem. Chem. Eng. (IJCCE)*, **36(1)**: 173-183 (2017).

[61] Cromwick A.M., Gross R.A., Investigation by NMR of Metabolic Routes to Bacterial γ-poly (Glutamic Acid) Using 13C-Labeled Citrate and Glutamate as Media Carbon Sources, *Can. J. Microbiol.*, **41(10)**: 902-909 (1995).

[62] Zhang C., Wu D., Qiu X., Stimulatory Effects of Amino Acids on γ-polyglutamic acid Production by *Bacillus Subtilis*, *Sci. Rep.*, **8(1)**: 1-9 (2018).

[63] Abdel-Fattah Y., Soliman N., Berekaa M., Application of Box-Behnken Design for Optimization of Poly-γ-glutamic Acid Production by *Bacillus Licheniformis* SAB-26, *Res. J. Microbiol.*, **2(9)**: 664-670 (2007).

[64] Pereira C.L., Antunes J.C., Gonçalves R.M., Ferreira-da-Silva F., Barbosa M.A., Biosynthesis of Highly Pure Poly-γ-Glutamic Acid for Biomedical Applications, *J Mater Sci Mater Med*, **23(7)**: 1583-1591 (2012).

[65] Lin B., Li Z., Zhang H., Wu J., Luo M., Cloning and Expression of the γ-Polyglutamic Acid Synthetase Gene pgsBCA in *Bacillus subtilis* WB600, *Biomed Res. Int.*, **2016**: 3073949 (2016).

[66] Wang F., Liang J., Wang W., Fu D., Xiao W., A New and Efficient Method for Purification of Poly-γ-Glutamic Acid from High-Viscosity Fermentation Broth, *Trop. J. Pharm. Res.*, **16(6)**: 1267-1275 (2017).

[67] Xavier J.R., Kumarr M.M.M., Natarajan G., Ramana K.V., Semwal A.D., Optimized Production of Poly (γ-Glutamic Acid)(γ-PGA) Using Bacillus Licheniformis and its Application as Cryoprotectant for Probiotics, *Biotechnol. Appl. Biochem.*, **67(6)**: 892-902 (2020).

[68] Cao M., Geng W., Zhang W., Sun J., Wang S., Feng J., Zheng P., Jiang A., Song C., Engineering of Recombinant E Scherichia Coli Cells Co-Expressing Poly-γ-Glutamic Acid (γ-PGA) Synthetase and Glutamate Racemase for Differential Yielding of γ-PGA, *Microb. Biotechnol.*, **6(6)**: 675-684 (2013).

[69] Patra J.K., Das G., Fraceto L.F., Campos E.V.R., Rodriguez-Torres M.D.P., Acosta-Torres L.S., Diaz-Torres L.A., Grillo R., Swamy M.K., Sharma S., Habtemariam S., Shin H.S., Nano Based Drug Delivery Systems: Recent Developments and Future Prospects, *J. Nanobiotechnology*, **16(1)**: 1-33 (2018).