Biocatalysis and Agricultural Biotechnology 22 (2019) 101413

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Optimized production of gamma poly glutamic acid (γ-PGA) using sago

Rajamanickam Mohanraj [a], Balasubramanian Mythili Gnanamangai [a,*], Kanthasamy Ramesh [b], Paneerselvam Priya [a], Ravichandran Srisunmathi [a], Shanmugam Poornima [a], Ponnusamy Ponmurugan [c], Jayachandran Philip Robinson [a]

[a] Department of Biotechnology, K. S. Rangasamy College of Technology, Tiruchengode 637215, Namakkal District, Tamil Nadu, India
[b] Tamilnadu Water Investment Company Limited, Tiruppur 641602, Tamil Nadu, India
[c] Department of Botany, Barathiyar University, Coimbatore 641046, Tamil Nadu, India

ARTICLE INFO

Keywords:
Sago
Bacillus subtilis
γ-PGA

Polyglutamic acid is a unique biodegradable and anionic homopolyamide biopolymer which is made up of D and L-glutamic acid units. γ-PGA is the principal component of a traditional Japanese food, Natto. Present study compares the γ-PGA production from substrate like soybean (SB) and sago (S) with glutamate dependent and independent media (GDM and GIM) by *Bacillus subtilis*. γ-PGA was purified from fourth day of fermented medium by precipitation method. Dry weight of purified and lyophilized γ-PGA was estimated to screen and select the potential media (From 1 L of GDM-S, GDM-SB, GIM-S and GIM-SB respectively 39.8 g, 20.3 g, 25 g and 14 g). The presence of γ-PGA was confirmed by using UV spectrum at 216 nm in GDM-S and GDM-SB with high absorption range. The presence of γ-PGA functional groups were identified from FTIR peaks (C═O and C–N). The molecular weight of γ-PGA was estimated as 150 kDa by SDS-PAGE. The concentration of three media components such as L-glutamic acid, yeast extract and sodium chloride were optimized using RSM. The quadratic model was utilized to generate 3D response plots model and was found significant. γ-PGA production was increased to ~7% from optimized media when compared with non-optimized media.

1. Introduction

Biopolymers are defined as polymers that are produced from living organisms like, plants, animals and microorganisms. In these days, biopolymer plays a very important role in our day to day life. Vast amount of carbohydrate based biopolymers are majorly produced by microbes and their applications mainly rely in food, medical and pharmaceutical industries. Although, poly glutamic acid (PGA) is a degradable biopolymer that is made up of D-glutamic acid and L-glutamic acid units. Depending on the attachment of the amino group to the carboxyl group, PGA is classified into two isoforms, namely poly-α-glutamic acid (α-PGA) and poly-γ-glutamic acid (γ-PGA). γ-PGA is a form where the peptide bonds are between the amino group of glutamic acid and the carboxyl group at the end of the glutamic acid side chain (Fig. 1) (Alsaheb et al., 2016). In the recent studies, there exist four different methods for γ–PGA production such as chemical synthesis, peptide synthesis, biotransformation and microbial fermentation (Sanda et al., 2001). Comparing with other methods, microbial fermentation is the simple and more cost effective method. γ-PGA can be synthesised by *Bacillus* species, *Fusobacterium nucleatum*, archaea and few eukaryotes (Candela et al., 2009), but *Bacillus* species are used most widely used for the production.

A biosynthetic pathway of γ–PGA production has been shown in Fig. 2. The microbial biosynthetic pathway of γ–PGA required L-glutamic acid units which was derived by exogenous or endogenous methods with α-ketoglutaric acid as a direct precursor (Ko and Gross, 1998). In endogenous method, L-glutamic acid requires conversion of a carbon source via glycolysis and TCA cycle intermediates with glutamate dehydrogenase and glutamate 2-oxoglutarate aminotransferase. L-glutamic acid is converted to L-glutamine with the help of the enzyme glutamine synthetase in exogenous method. The synthesised L-glutamic acid is converted to L-alanine with L-glutamic acid pyruvate aminotransferase and then D-alanine form with alanine racemase and further conversion of D-glutamic acid form by D-glutamic acid pyruvate aminotransferase. γ-PGA is synthesised from L-glutamic acid and D-glutamic acid by PGA synthetase. After this process γ-PGA was freely secreted into the fermented medium (Bovarnick, 1942).

Microbial synthesis of γ-PGA is done by four following steps, racemization, polymerization, regulation and degradation. The above steps have a major role in the PGA production with specific enzymes that are

* Corresponding author.
E-mail address: mythumithras@gmail.com (B.M. Gnanamangai).

https://doi.org/10.1016/j.bcab.2019.101413
Received 20 July 2019; Received in revised form 24 October 2019; Accepted 30 October 2019
Available online 31 October 2019

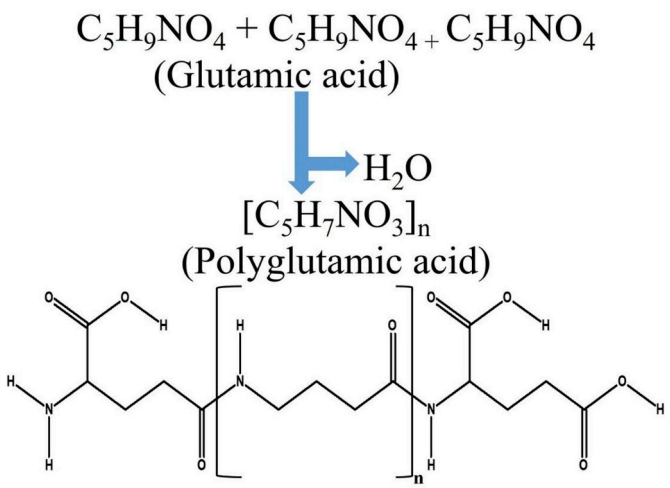


Fig. 1. Linkage mechanism of γ–PGA.

coded by respective genetic materials such as, *racE/glr*, *yrpC, pgsB, C, A, E, ComP-ComA* regulator system, *DegS-DegU, DegQ*, *SwrA* systems, *ywtD, dep* or *pgdS* and *ggt* (Luo et al., 2016).

Sago is a processed starch food, which is marketed in the form of small globules or pearls. Sago is manufactured from the starchy core of the stem of several palms, the principal being the sago palm (*Metroxylon sagu and M. rumphii*). In India, Sago is manufactured from the starch obtained from the tubers of Tapioca (*Manihot utilissima*). Sago starch consists of oval granules with diameters in the range of 20–40 μm. The apparent total amylose contents (lipid free starch) in sago starch was in the range of 24%–31% (Ahmad et al., 1999).

In recent studies, some techniques were used to screen, identify, determine and validate γ–PGA. Ultra violet (UV) Spectrum analysis was performed to detect the organic and inorganic substances in the biological samples by absorption range due to their co-responding resonance spectral peaks. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method to detect and screen the molecular weight of the synthesised biomolecules. Hence, γ–polyglutamic acid produced was detected by SDS-PAGE and basic dye staining (Yamaguchi et al., 1996). Fourier Transform Infrared Spectroscopy (FTIR) was used to identify chemical bonds and functional groups in a molecule by producing an infrared absorption spectrum. Response surface methodology (RSM) is used in biotechnology to determine the optimal values of media parameters as nutrients value, pH, temperature, degree of aeration and feeding rates. This optimization process involves three major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model.

PGA has various industrial applications like cryoprotectant, bitterness relieving agent, surgical adhesive, biodegradable fibers, heavy metal absorbers and biopolymer flocculants. Bio flocculants are composed of high molecular weight biopolymers such as proteins, glycoproteins, and polysaccharides and they are biodegradable in nature secreted by microorganisms (Suh et al., 1997; Takeda et al., 1992).

The present study aim to synthesis γ-PGA from glutamate dependent and independent media with sago and soybean as a carbohydrate and nitrogen sources by *Bacillus subtilis* 2756. Screening of high yield producing media from the four different media compositions by dry weight assay method. The synthesised γ-PGA were identified and characterized by UV-spectrum analysis, FTIR and SDS-PAGE. This study also deals with optimization of γ-PGA production by RSM to improve the product yield with an aim to produce a low cost and high purity product of γ-PGA for industrial applications.

2. Materials and methods

2.1. Culture preparation

Bacillus subtilis 2756 was procured from Microbial Type Culture

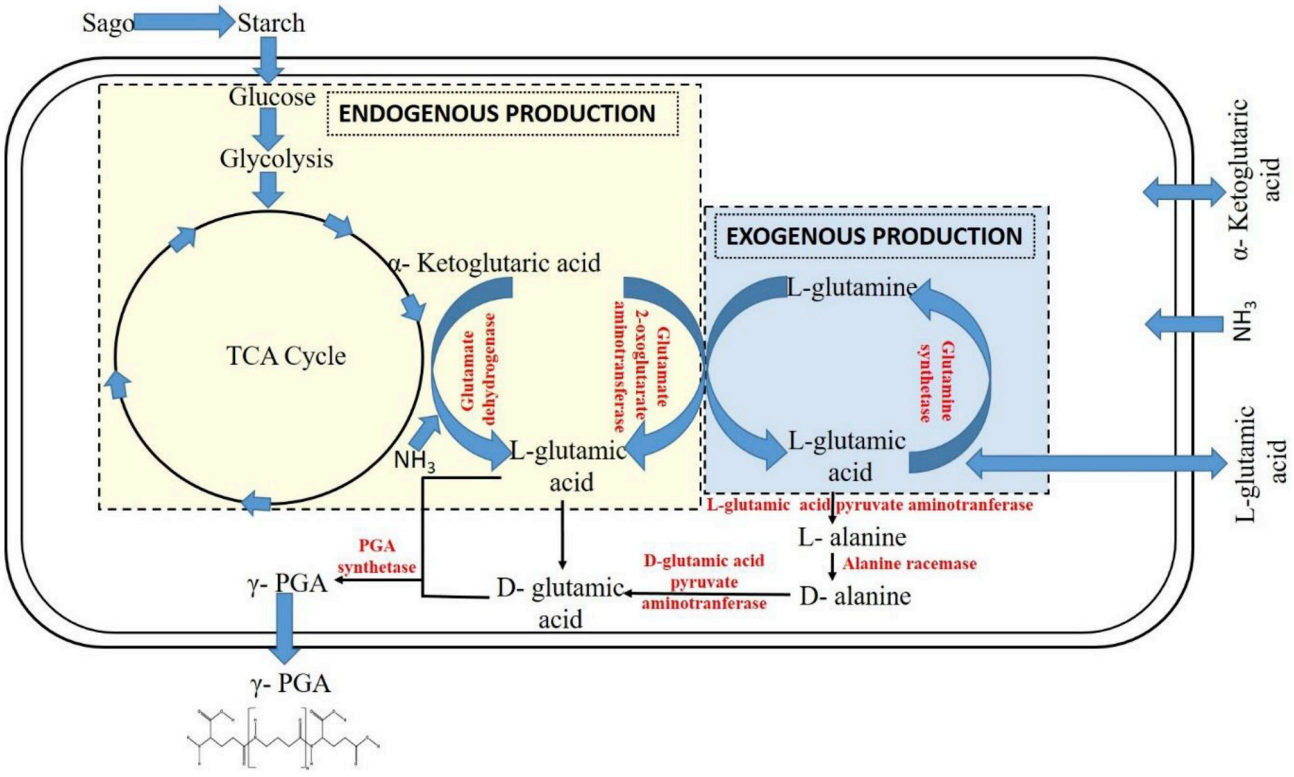


Fig. 2. Process of γ–PGA synthesis.

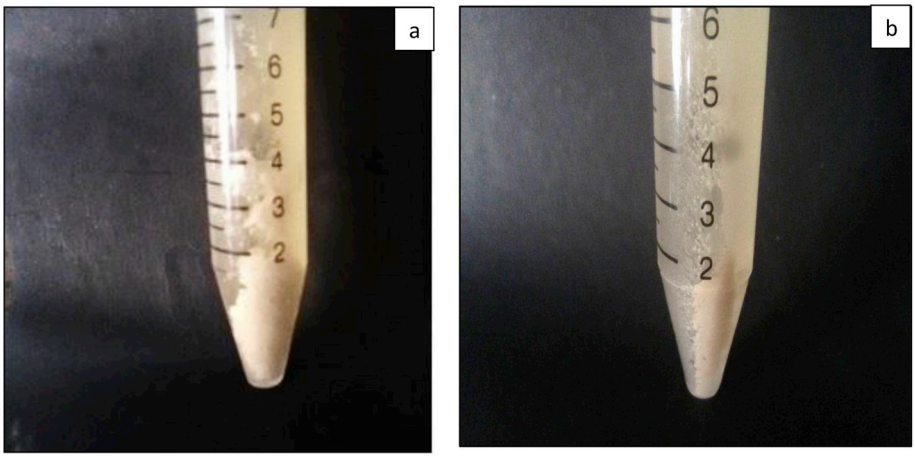


Fig. 3. Precipitation of γ-PGA a) PGA from sago and b) PGA from soybean.

Fig. 4. Lyophilized PGA a) Lyophilized sago-PGA and b) Lyophilized soybean-PGA.

Collection (MTCC), Chandigarh, India. Active culture was maintained by subculturing in nutrient agar at specific time intervals to maintain a pure strain. Six hours old liquid culture was used as a seed culture to prepare γ-PGA.

2.2. Growth media

Four different types of media were evaluated for the production of γ-PGA such as glutamate dependent media with sago (GDM-S), glutamate dependent media with soy bean (GDM-SB), glutamate independent media with sago (GIM-S) and glutamate independent media with soy bean (GIM-SB). The components of the growth media GDM-S and GDM-SB are (g/L); Pre-treated Sago/Soybean, 90; L-glutamic acid, 80; Yeast extract, 40; Sodium chloride, 15; Calcium chloride, 1.5; Magnesium sulphate, 1.5 and Manganese sulphate, 1.5. GIM-S and GIM-SB containing (g/L); Pre-treated Sago/Soybean, 75; Ammonium chloride, 18;

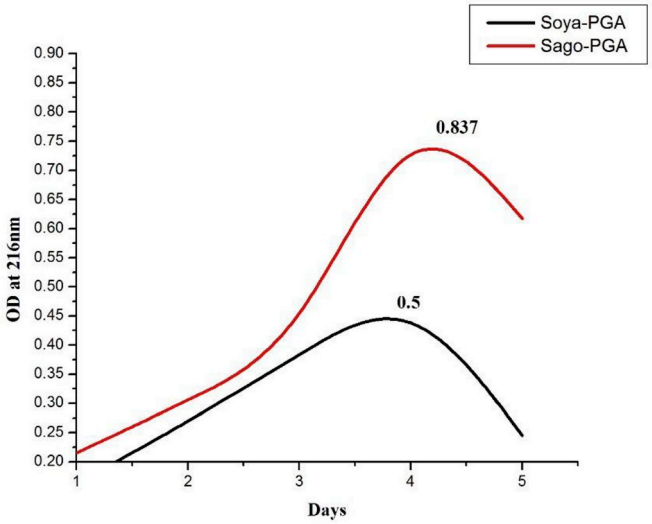


Fig. 5. UV spectral Analysis.

Di-potassium hydrogen phosphate, 1.5; Magnesium sulphate, 0.35; Manganese sulphate, 0.5 and Calcium carbonate, 30. Sago and soybean were pre-treated by soaking in water for 2 h (Sago) and 12 h (Soybean) before media preparation. Then soaked sago or soybeans were dried by nylon filter and were used in media preparation.

2.3. Synthesis of γ-PGA

Bacillus subtilis was subjected to produce γ-PGA in the presence and absence of glutamate. The GDM-S, GDM-SB, GIM-S and GIM-SB were prepared and sterilized at 121 °C with 15 psi for 15 min. After the sterilization, media flasks were inoculated with 1:10 ratio of seed culture (Moraes et al., 2013). The inoculated media were placed in a shaker at 37 °C with 80 rpm for four days till the viscous nature was obtained.

2.4. Purification of γ-PGA

Biomass and unutilized ingredients were separated from fermented media by centrifugation at 20,000 rpm for 30 min at 4 °C. Supernatant was mixed with four volumes of ethanol and was again centrifuged at 20,000 rpm for 30 min at 4 °C (Goto and Kunioka, 1992). The resultant precipitate was dissolved with distilled water (Fig. 3) and were lyophilized. The weight of lyophilized γ-PGA was measured by simple weighing method (BS223S, Sartorius) to screen the high yield media.

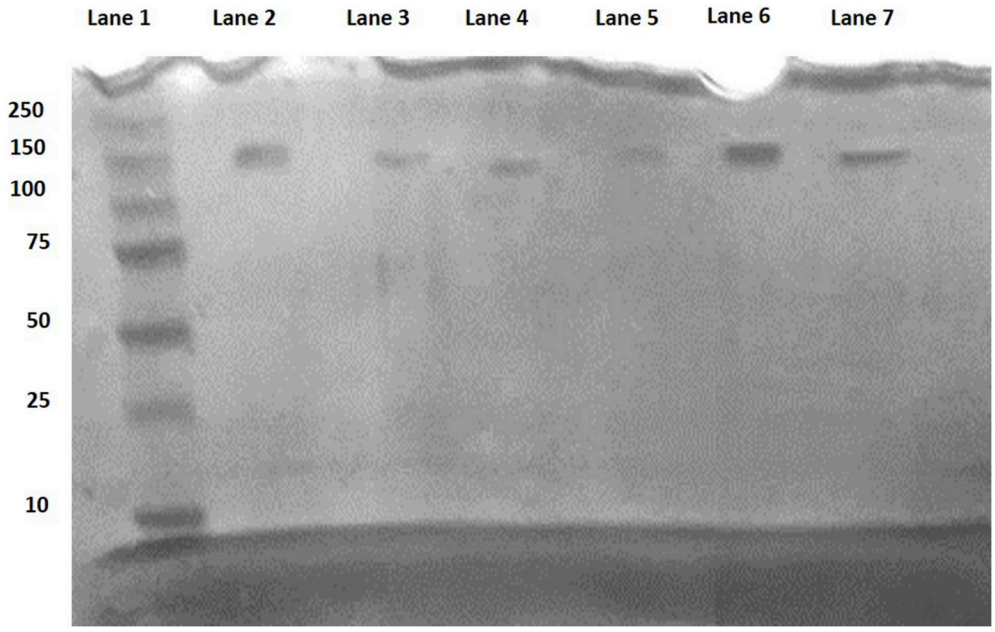


Fig. 6. Molecular size of γ-PGA obtained from Sago and Soybean with marker. Lane 1) Marker (250-10 kDa), Lane 2) Soybean PGA, Lane 3–7) Sago PGA (1–5 days).

2.5. Detection of γ-PGA by UV-Spectrometric analysis

UV spectrophotometric method (Zeng et al., 2012) was used to determine γ-PGA content in biological samples. Two ml of GDM-S and GDM-SB fermented media (Day 0, Day 1, Day 2, Day 3, Day 4, and Day 5) was collected frequently and absorbance values were measured at 216 nm (U-2900E, Model.no-2JI-0003, Hitachi, Tokyo, Japan) against deionized water.

2.6. Detection of γ -PGA by SDS PAGE

SDS-PAGE was performed by Laemmli's method to separate biological macromolecules from samples. Soybean-PGA and sago-PGA samples were mixed with sample buffer and loaded in the appropriate lanes. After the electrophoretic run, the gel was stained by 250 mg of Coomassie brilliant blue with 40 ml of acetic acid for overnight and destained with acetic acid, methanol and water (10:40:50) (Yamaguchi et al., 1996). Protein molecular marker having molecular weight ranges from 10 to 250 (kDa) was used for the determination of molecular weight of the unknown sample.

2.7. FTIR analysis

FTIR spectroscopy (IR Prestige 21, Shimadzu) was a measurement technique used for recording IR-spectra (Gomaa, 2016). FTIR interferogram detects the functional groups in the synthesised polyglutamic acid samples that can be determined by identifying the respective peak values in the graphical results of FTIR.

2.8. Optimization of γ-PGA using response surface methodology (RSM)

RSM is a method to detect the relationship between several explanatory variables and one or more response variables. RSM is widely used to determine the optimal conditions like pH, temperature, degree of aeration and feeding rates. In this study, Batch mode of feeding strategy was followed for the entire fermentation period of γ-PGA production. Dissolved oxygen (DO) was observed for the fermentation period, using DO probe (Systronics water analyser 371) to screen the oxygen uptake rate of *Bacillus subtilis*. Design–Expert version 11 was used to perform design of experiments (DOE) with central composite design (CCD) (Shi et al., 2006). Design-Expert version 11 offers comparative tests, screening, characterization, optimization, robust parameter design, mixture designs and combined designs. CCD was carried out to obtain a quadratic model consisting of trials plus a star configuration to estimate quadratic effects and central points to estimate pure process variability and to reassess the gross curvature with maximization of γ-PGA production being the expected response. Concentration of three ingredients (L-glutamic acid, yeast extract and sodium chloride) were optimized. A series of experiments were performed by using runs generated by design expert. Low and high factor settings were coded as -1 and +1 respectively and midpoint setting was coded as zero.

$$Y = \beta 0 + \sum_{i=1}^{k} \beta i\, xi + \sum_{i=1}^{k} \beta ii\, xi\, xj + \sum\sum_{i<j} \beta ij\, xi\, xj$$

Y was termed as the predicted response; β_0, β_i, β_{ii}, β_{ij} were termed as constant coefficients and x_i, x_j were the coded independent variables or factors. *t*-test was used to test the significance of the regression coefficients. F-test was used to determine statistical significance.

3. Results and discussion

3.1. Yield of γ-PGA

Purified γ-PGA product was lyophilized and stored in airtight container for further process (Fig. 4). γ-PGA was obtained from 1 L of GDM-S, GDM-SB, GIM-S and GIM-SB respectively 39.8, 20.3, 25 g and 14 g. It was confirmed that, *Bacillus subtilis* 2756 were able to produce γ-PGA from both glutamate dependent and independent media but high yield was obtained from GD based medium. GIM-S media consist of both nitrogen and carbohydrate components. Here, Ammonium chloride serves as a nitrogen source and sago provides the carbohydrate source. γ–PGA production is aided by glucose in the endogenous production method with the activation of TCA cycle and glutamic acid racemization supported by ammonium supplemented in media. This process enhance the γ–PGA yield from the GIM-S when compared to GDM-SB. In the GDM-SB media, it consist of glutamic acid and soybean that serves as the nitrogen source with minimal carbon source which in turn doesn't activates the endogenous system of γ–PGA production that might have led to the low yield. Glutamic acid dependent bacteria have potential for

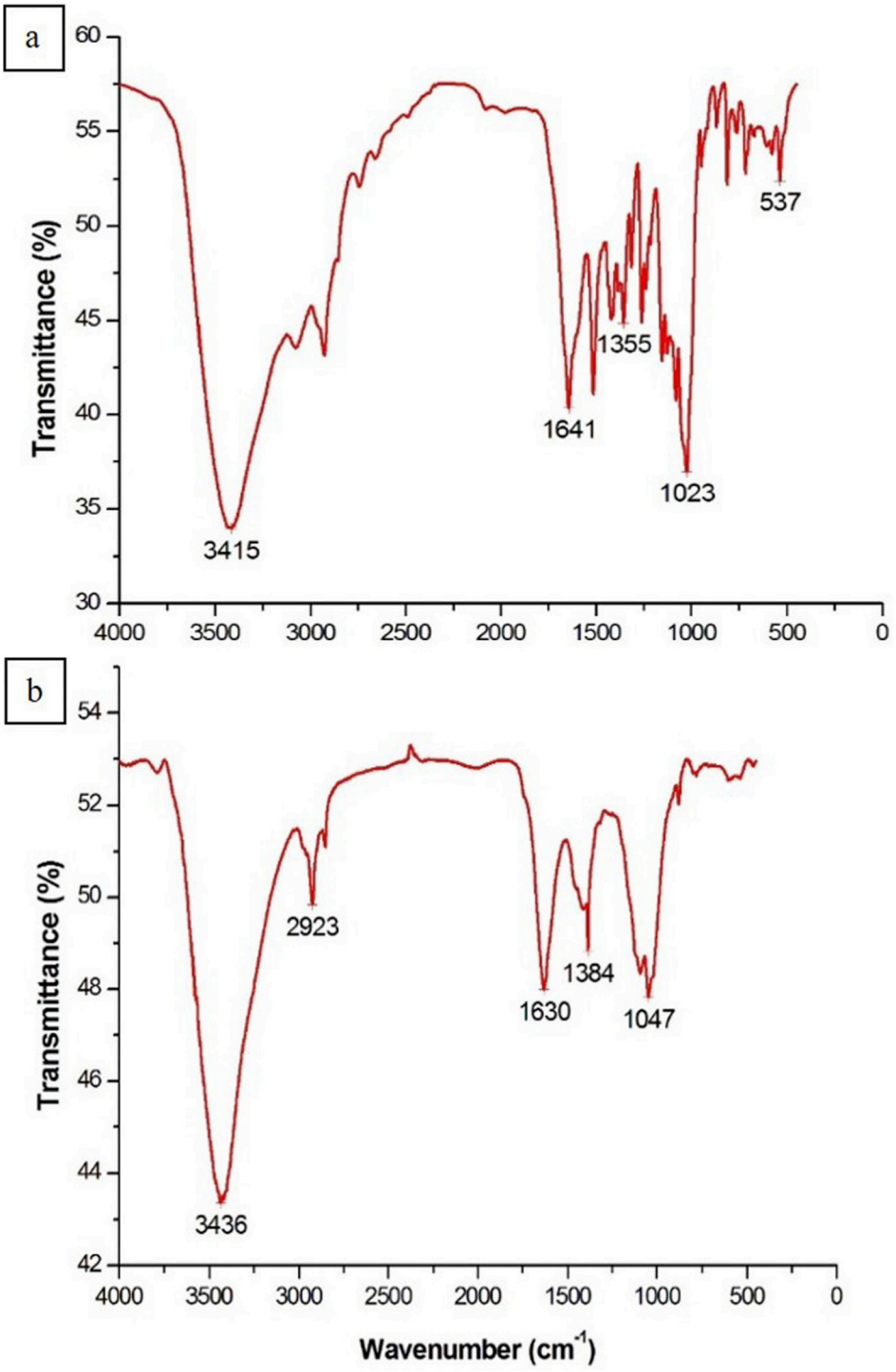


Fig. 7. FTIR spectrum a) γ-PGA obtained from sago b) γ-PGA obtained from soybean.

γ-PGA production higher than those without glutamic acid. *Bacillus* strain depends greatly on the composition of the medium which can be divided into two groups; glutamic acid dependent and independent bacteria (Feng et al., 2014). So further process and characterization were done in GDM-S and GDM-SB.

In the previous study, γ-PGA was synthesised by *bacillus licheniformis* TISTR 100 from B medium which doesn't have glutamic acid as a nitrogen source. B medium consist of high carbon (glucose and citric acid) and nitrogen sources (ammonium chloride). Glucose and citric acids play a vital role in the TCA cycle to form α-ketoglutaric acid and the ammonium serve as a key in endogenous process to form an L-glutamic acid from α-ketoglutaric acid with glutamate dehydrogenase. The quantitative analysis of γ-PGA production clearly described low amount of product obtained from glutamate independent medium when compared to the glutamate medium as L-glutamic acid served as a direct precursors of γ-PGA (Kongklom et al., 2015).

The synthesis of PGA was repressed by exogenous glutamate with *Bacillus licheniformis* but not in the *Bacillus subtilis*, it's indicating a clear difference in the regulation of synthesis of capsular slime in these two species (Kambourova et al., 2001). The isolation and characterization of a new γ-polyglutamic acid producer, *Bacillus mesentericus* MJM1, from Korean domestic chungkukjang bean paste. It produced γ-PGA at the

Table 1
Runs generated by Design Expert version 11.

Run	Factor 1 A: L-glutamate	Factor 2 B:Yeast extract	Factor 3 C: Sodium chloride	Response R1
1	0	0	0	0.837
2	1	0	0	1.076
3	1	1	1	1.152
4	1	-1	-1	1.259
5	1	1	-1	1.168
6	0	0	0	0.837
7	-1	1	-1	1.112
8	0	0	0	0.837
9	1	-1	1	1.175
10	0	1	0	1.035
11	-1	0	0	0.966
12	-1	-1	-1	0.906
13	0	0	0	0.837
14	0	0	0	0.837
15	0	0	1	1.02
16	0	0	0	0.837
17	-1	1	1	1.045
18	0	0	-1	1.05
19	0	-1	0	1.021
20	-1	-1	1	1.119

Table 2
Two way ANOVA table for quadratic model.

Source	Sum of squares	Df	Mean Square	F-value	p-value	
Model	0.2312	9	0.0257	5.57	0.0065	significant
A-L-glutamate	0.0612	1	0.0612	13.27	0.0045	
B-Yeast extract	0.0016	1	0.0016	0.3555	0.5643	
C–NaCl	0.0062	1	0.0062	1.33	0.2749	
AB	0.0123	1	0.0123	2.67	0.1330	
AC	0.0568	1	0.0568	12.32	0.0056	
BC	0.0013	1	0.0013	0.2822	0.6069	
A^2	0.0012	1	0.0012	0.2631	0.6191	
B^2	0.0185	1	0.0185	4.01	0.0730	
C^2	0.0074	1	0.0074	1.61	0.2328	
Residual	0.0461	10	0.0046			
Lack of Fit	0.0461	5	0.0092			
Pure Error	0.0000	5	0.0000			
Cor Total	0.2773	19				

level of 10 g/l in suitable media (Zhao et al., 2005).

The existing studies on biosynthesis of γ-PGA by fermentation reveals that enzymes are involved in the production of γ–PGA. It was concluded that enzymatic processes synthesised γ–PGA not by ribosomal protein synthesis. In nutritional requirements, the concentration of Mn^{+2} can influence the configuration of the glutamic acid in γ-PGA produced by *Bacillus subtilis*. The other residues used in γ–PGA production include soybean and sweet potato residues, which are rich in proteins and carbohydrates (Moraes et al., 2013).

The γ-PGA producing *Bacillus* species were isolated from Kinema. Kinema is an ethnic fermented, non-salted and sticky soybean food. Stickiness is one of the best qualities, which is due to the production of γ-PGA (Chettri et al., 2016).

The co-production showed that the size of soybean particle and rice husk significantly improved the co-production of nattokinase and γ-PGA, yielding 2503.4 mg and 320 mg, respectively in the improved culture medium that composed of 16.7% soybean flour and 13.3% rice husk with 70% water content (Nie et al., 2015).

3.2. Characterization of γ-PGA

3.2.1. UV spectrum analysis

The concentration of γ-PGA was determined by UV spectrum analysis to determine the optimal time for higher γ-PGA production. Both GDM-S and GDM-SB fermented broth (Day, 1, 2, 3, 4, and 5) were collected in sterile condition and absorbance were measured at 216 nm. The absorption range was found at 216 nm (for GDM-S the peak were found at 0.215, 0.306, 0.396, 0.837, and 0.617 likewise for GDM-SB 0.162, 0.269, 0.383, 0.5 and 0.245 respective to day one to five). From UV analysis, it was concluded that γ-PGA production showed higher absorption on day 4 in GDM-S compare to GDM-SB with increased surface plasmon resonance at its corresponding absorption spectrum (Fig. 5).

UV spectrophotometry is used extensively for various determinations of inorganic and organic substances and is available easily in most laboratories. It has the advantages of significant precision, accuracy, low cost and easy handling. Furthermore, it doesn't need any additional reagent and don't consume the sample during the determination process. In addition, there are few reports about the UV absorption spectrum of γ-PGA relative to that of infrared spectrum (IR). Absorption spectrum for γ-PGA were analysed within the range 190–340 nm and the maximum absorption at 216 nm for γ–PGA in distilled water is validated as detection spectrum for γ-PGA (Zeng et al., 2012).

3.2.2. Detection of γ-PGA by SDS-PAGE

The molecular weight of sago-PGA and soybean-PGA were estimated by SDS-PAGE. Soybean-PGA (4th day) was considered as standard. The Lane 1 was loaded with marker (250-10 kDa), Lane 2 for soybean-PGA (4th day) and Lane 3–7 for sago-PGA. Lane (3–7) represents the γ-PGA yield on day 1–5 for the fermentation of sago medium for respective days. The marker was used to identify the molecular weight of product. The molecular weight of γ-PGA was estimated as 150 kDa by the band's nature. Lane 6 have the band is quite dark and distinct indicating the presence of significant sago PGA on Day 4. This showed that γ-PGA was a soluble protein. It is also concluded that the production was efficient on day 4 (Fig. 6).

Halmschlag et al. (2019) screened the response of genetically modified *Bacillus subtilis* strain in γ-PGA production. γ-PGA synthetase gene was derived from *Bacillus amyloliquefaciens* and glutamate racemase gene was derived from *Bacillus anthracis*. The derived genes are expressed in the *Bacillus subtilis* as a host organism. This genes modification proved *Bacillus subtilis* can produced lower to higher molecular weight (40–8000 kDa) of γ-PGA with 3–60% of D glutamate content. The produced γ-PGA polymer consisted of 2000 glutamic acid residues with even proportion of L and D types with molecular mass of about 200–300 kDa (Zhao et al., 2005). Molecular weight of bacteria based PGA vary from lower to higher range like 10 kDa and 100–1000 kDa. This range was dependent on different parameters such as bacterial species, media composition and incubated conditions (Alsaheb et al., 2016). Molecular weight of γ-PGA was estimated as 275 kDa by comparison with molecular markers (Yamaguchi et al., 1996).

3.2.3. FTIR analysis

FTIR was performed in the range of 4000–400 cm^{-1} to detect the functional groups of γ-PGA. FTIR spectra of γ-PGA obtained from sago was most similar to the γ-PGA obtained from soybean. The infrared spectra of sago γ-PGA and soybean γ-PGA samples showed characteristic strong amide absorption at 1641 and 1630 cm^{-1} respectively. The absorption peaks at 1355 and 1384 cm^{-1} are characteristic of C═O groups. The absorption peaks observed at 1023 and 1047 cm^{-1} are characteristic of C–N groups. The strong hydroxyl absorption at 3415 and 3436 cm^{-1} was observed. The strong amine absorption at 2905 and 2923 cm^{-1}. The strong halide absorption was observed at 537 and 539 cm^{-1} (Fig. 7). The results were in agreement to previous reports of γ-PGA.

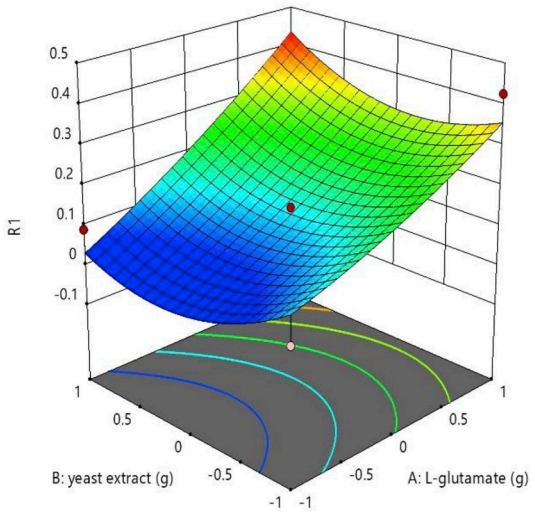
FTIR spectrum of γ-PGA showed strong amide absorption peak at 1634 and 1627 cm^{-1}, carbonyl absorption peak at 1432 and 1455 cm^{-1}. The absorption peaks at 1304 and 1368 are characteristic of C═O groups. The absorption peaks at 1194 and 1198 cm^{-1} are characteristic of C–N groups. This functional groups were compared with commercially available γ-PGA (Gomaa, 2016).

a) **Design-Expert® Software**
Factor Coding: Actual

R1
Design points above predicted value
Design points below predicted value
0.033 0.425

X1 = A: L-glutamate
X2 = B: yeast extract

Actual Factor
C: NaCl = -1

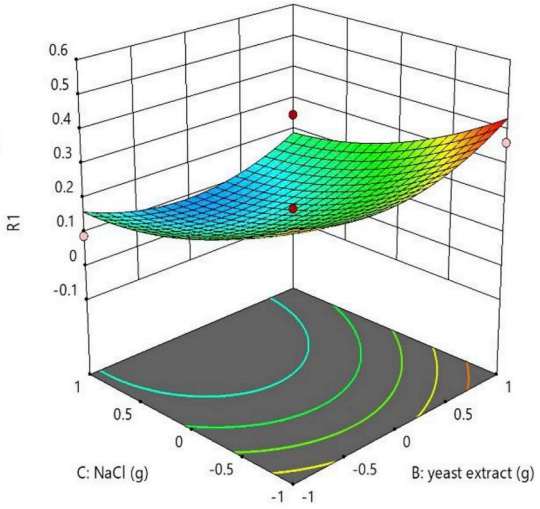


b) **Design-Expert® Software**
Factor Coding: Actual

R1
Design points above predicted value
Design points below predicted value
0.033 0.425

X1 = B: yeast extract
X2 = C: NaCl

Actual Factor
A: L-glutamate = 1



c) **Design-Expert® Software**
Factor Coding: Actual

R1
Design points above predicted value
Design points below predicted value
0.033 0.425

X1 = C: NaCl
X2 = A: L-glutamate

Actual Factor
B: yeast extract = 1

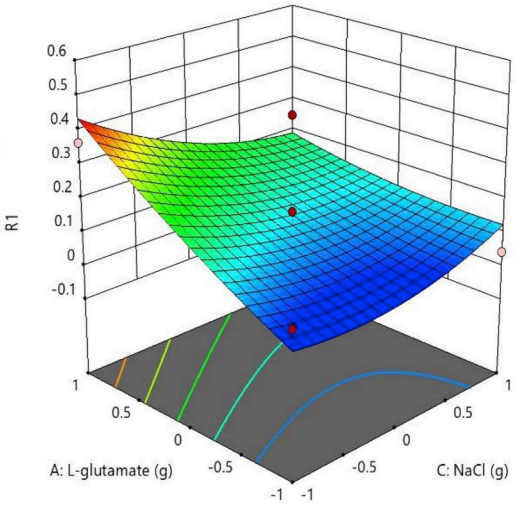


Fig. 8. Effects of L-glutamate and yeast extract b) Effects of yeast extract and sodium chloride c) Effects of L-glutamate and sodium chloride on γ-PGA production.

The structural characteristics of the salts of γ-polyglutamates (Na^+, K^+, NH^{4+}, Ca^{2+} and Mg^{2+}) with 1H- and 13C NMR spectroscopy and FTIR spectroscopy. The conformational states, hydrogen bonding and polyanionic nature make γ-PGA a versatile multi-functional biopolymer possessing many useful biological functionalities (Ho et al., 2006).

3.3. Optimized production of γ-PGA by response surface methodology

Twenty experiments were performed with various ranges of dependent variables. Responses were analysed and validated by means of measuring OD value at 216 nm. The designs and responses were tabulated in Table 1. The results revealed that, quadratic model of analysis was fitted to the F value 5.57 which was implied the model was significant. R^2 value was predicted as 0.9548 implies the model was more significant. The lack of fit was observed as 0.009 indicated that lack of fit was not significant relative to the pure error (Table 2).

3.4. Coded factors

R1 = 0.051 + 0.064*A +1.100E-003*B -0.039*C+ 0.022*A*B -0.067*A*C -0.030*B*C +0.027*A^2 +0.088*B^2 +0.058*C^2

3.5. Actual factors

R1 = 0.050873 + 0.064300* L-glutamate +1.1000E-003* Yeast extract-0.038700 *Sodium chloride+ 0.021875* L-glutamate* Yeast extract -0.066875 *L-glutamate* Sodium chloride - 0.030125* Yeast extract* Sodium chloride +0.027318 *L-glutamate^2 +0.088318 *Yeast extract^2 +0.058318 *Sodium chloride^2.

The contour model was represented in Fig. 8. Desirability factors were computed based upon the response values corresponds to the criteria of design. The response and dependent factors with their desirability was mentioned in Fig. 9. The desirability was achieved as 0.938 which indicates the optimum level of the components. L-glutamate (90 g/L), yeast extract (30 g/L) and sodium chloride (5 g/L) were the optimum level for higher production of PGA. DO concentration was 35% initially and it was decreased to 10% at the end of fourth day. The decreasing scenario of DO concentration indicated the effective oxygen uptake rate by *Bacillus subtilis*.

RSM were earlier used to optimize culture medium for production of γ-PGA by *Bacillus licheniformis*. The effects of four medium components (L-glutamic acid, citric acid, glycerol and NH_4Cl) on the production of γ-PGA in flask cultures by *Bacillus licheniformis* CCRC 12826 (ATCC 9945) with initial medium pH 6.5. The PGA yield (35.34 g/L) was optimized using glutamic acid, citric acid, glycerol and NH_4Cl where in the optimized medium resulted insignificant increase of the γ-PGA yield by *B. licheniformis* CCRC 12826 in shake-flask cultivation without any feeding process at 35.52 g/L. (Lu et al., 2004).

Optimized production of γ-PGA by *Bacillus subtilis* ZJU-7 using response surface methodology with carbon and nitrogen sources. Out of seven carbon sources, sucrose influenced the highest rate of γ–PGA productivity. Among nitrogen sources, tryptone was repeated to be the best effect. The factors were screened using a fractional factorial design. Finally, the optimal medium was formulated by using central composite design. The production was increased up to two fold when using an optimized medium (Shi et al., 2006).

In a previous study, Marine based *Bacillus subtilis* was isolated and has been identified by 16S rRNA sequencing (Bajaj and Singhal, 2009). The identified strain showed γ-PGA production. The PGA production was studied by one factor-at-a-time method to screen the effect of carbon sources, nitrogen sources and pH. The most important nutrients influencing the PGA yield was selected by Plackett-Burman design. The production of PGA was further improved by supporting the medium with α-ketoglutaric acid. By using sequential optimization methods, the production increased from 7.64 to 25.38 g/L. This study revealed that, *Bacillus subtilis* has a potential to produce and also optimization method as an effective way to increase PGA production (Bajaj and Singhal, 2009).

4. Conclusion

In economic and efficiency perspective present study concludes that, *Bacillus subtilis* produced high yield of γ-PGA from carbohydrate based glutamate dependent media when compared to nitrogen based glutamate dependents media, carbohydrate and nitrogen based glutamate independent media. Since, Tamil Nadu is famous for tapioca cultivation especially in Salem, Namakkal, Erode, Dharmapuri and Villupuram districts was rich in sago source so it was selected as substrate. Presence of γ-PGA was detected by UV spectroscopy technique at 216 nm with high adsorption peak on fourth day of fermented media, this peak indicated that presence of high concentration of γ-PGA. FTIR peaks were confirmed that, functional groups of γ-PGA with specific absorption ranges. Molecular weight of γ-PGA was detected as 150 kDa by SDS-PAGE with quite dark and distinct nature. The media components were optimized by using Response surface methodology. The desirability was achieved as 0.938 which indicate that the model was

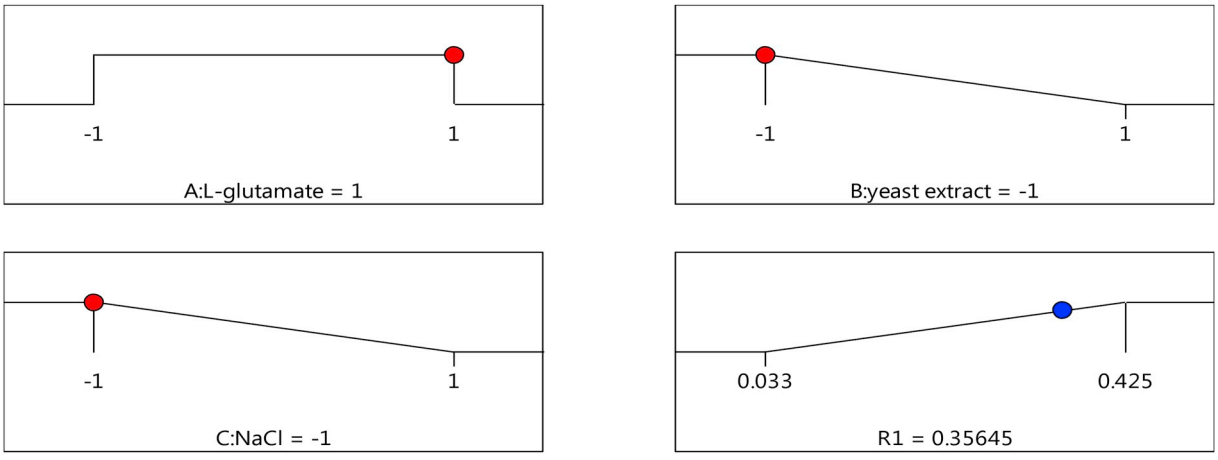


Fig. 9. Desirability of the model.

desirable and significant. From this study, it was conclude that, sago (Carbohydrate) was an effective substrate for the production of γ-PGA when compare to the soybean (nitrogen).

Acknowledgement

The authors are thankful to, the Management and Principal of K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu for their enormous support and providing the necessary facilities to carry out this research work. The project is catalysed and financially supported by Tamil Nadu state council for science and technology, Department of higher education, Government of Tamil Nadu, under student project scheme (TNSCST-ES-031). The authors also acknowledge DBT–STAR (BT/HRD/11/09/2018) and DST-FIST (SR/FST/College-235/2014).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101413.

References

Ahmad, F.B., Williams, P.A., Doublier, J.L., Durand, S., Buleon, A., 1999. Physico-chemical characterisation of sago starch. Carbohydr. Polym. 38, 361–370.

Alsaheb, R.A.A., Othman, N.Z., Malek, R.A., Leng, O.M., Aziz, R., Enshasy, H.A.E., 2016. Polyglutamic acid applications in pharmaceutical and biomedical industries. Der Pharm. Lett. 8 (9), 217–225.

Bajaj, I.B., Singhal, R.S., 2009. Sequential optimization approach for enhanced production of poly(g-glutamic acid) from newly isolated *bacillus subtilis*. Food Technol. Biotechnol. 47 (3), 313–322.

Bovarnick, M., 1942. The formation of extracellular d(-)-glutamicacid polypeptide by *bacillus subtilis*. J. Biol. Chem. 145, 415–424.

Candela, T., Moya, M., Haustant, M., Fouet, A., 2009. Fusobacterium nucleatum, the first Gram negative bacterium demonstrated to produce polyglutamate. Can. J. Microbiol. 55, 627–632. https://doi.org/10.1139/W09-003.

Chettri, R., Bhutia, M.O., Tamang, J.P., 2016. Poly- -glutamic acid (PGA)-producing bacillus species isolated from kinema, Indian fermented soybean food. Front. Microbiol. 7 (971) https://doi.org/10.3389/fmicb.2016.00971.

Feng, J., Gu, Y., Sun, Y., Han, L., Yang, C., Zhang, W., Cao, M., Song, C., Gao, W., Wang, S., 2014. Metabolic engineering of *bacillus amyloliquefaciens* for poly-gamma-glutamic acid (γ-PGA) overproduction. Microb. Biotechnol. 7, 446–455.

Gomaa, E.Z., 2016. Cryoprotection of probiotic bacteria with poly-c-glutamic acid produced by *Bacillus subtilis* and *Bacillus licheniformis*. J. Genet. Eng. Biotechnol. 14, 269–279. https://doi.org/10.1016/j.jgeb.2016.10.001.

Goto, A., Kunioka, M., 1992. Biosynthesis and hydrolysis of poly(γ- glutamic acid) from *bacillus subtilis* IF03335. Biosci. Biotechnol. Biochem. 56 (7), 1031–1035. https://doi.org/10.1271/bbb.56.1031.

Halmschlag, B., Steurer, X., Putri, S.P., Fukusaki, E., Blank, L.M., 2019. Tailormade Poly-γ-Glutamic Acid Production. Metabolic Eng.

Ho, G.H., Ho, T.I., Hsieh, K.H., Su, Y.C., Lin, P.Y., Yang, J., Yang, K.H., Yang, S.C., 2006. γ -Polyglutamic acid produced by *bacillus subtilis* (natto): structural characteristics, chemical properties and biological functionalities. J. Chin. Chem. Soc. 53, 1363–1384.

Kambourova, M., Tangney, M., Priest, F.G., 2001. Regulation of polyglutamic acid synthesis by glutamate in *bacillus licheniformis* and *bacillus subtilis*, Applied and environment. Microbiology 67 (2), 1004–1007. https://doi.org/10.1128/AEM.67.2.1004–1007.2001.

Ko, Y.H., Gross, R.A., 1998. Effects of glucose and glycerol on γ-poly (glutamic acid) formation by *bacillus licheniformis* ATCC 9945a. Biotechnol. Bioeng. 57 (4), 430–437.

Kongklom, N., Luo, H., Shi, Z., Pechyen, C., Chisti, Y., Sirisansaneeyakul, S., 2015. Production of poly-γ-glutamic acid by glutamic acid-independent *Bacillus licheniformis* TISTR 1010 using different feeding strategies. Biochem. Eng. J. https://doi.org/10.1016/j.bej.2015.04.007.

Lu, W.K., Chiu, T.Y., Hung, S.H., Shih, I.L., Chang, Y.N., 2004. Use of response surface methodology to optimize culture medium for production of poly-γ-glutamic acid by *bacillus licheniformis*. Int. J. Appl. Sci. Eng. 2 (1), 49–58.

Luo, Z., Guo, Y., Liu, J., Qiu, H., Zhao, M., Zou, W., Li, S., 2016. Microbial synthesis of poly-γ-glutamic acid: current progress, challenges, and future perspectives. Biotechnol. Biofuels 9 (134). https://doi.org/10.1186/s13068-016-0537-7.

Moraes, L.P., Brito, P.N., Alegre, R.M., 2013. The existing studies on biosynthesis of poly (γ-glutamic acid) by fermentation. Food Public Health 3 (1), 28–36. https://doi.org/10.5923/j.fph.20130301.04.

Nie, G., Zhu, Z., Liu, F., Nie, Z., Ye, Y., Yue, W., 2015. Co-production of nattokinase and poly (γ-glutamic acid) under solid-state fermentation using soybean and rice husk. Braz. Arch. Biol. Technol. 58 (5), 718–724. https://doi.org/10.1590/S1516-89132015050172.

Sanda, F., Fujiyama, T., Endo, T., 2001. Chemical synthesis of poly-g-glutamic acid by polycondensation of g-glutamic acid dimer: synthesis and reaction of poly-g-glutamic acid methyl ester. J. Polym. Sci. Part A: Polymer Chem. 39, 732–741.

Shi, F., Xu, Z., Cen, P., 2006. Optimization of γ-polyglutamic acid production by *bacillus subtilis* ZJU-7 using a surface–response methodology. Biotechnol. Bioproc. Eng. 11 (3), 251–257.

Suh, H.H., Kwon, G.S., Lee, C.H., Kim, H.S., Oh, H.M., Yoon, B.D., 1997. Characterization of bioflocculant produced by *bacillus sp.* DP-152. J. Ferment. Bioeng. 84 (2), 108–112.

Takeda, M., Koizumi, J.I., Matsuoka, H., Hikuma, M., 1992. Factors affecting the activity of a protein bioflocculant produced by *nocardia amarae*. J. Ferment. Bioeng. 74 (6), 408–409.

Yamaguchi, F., Ogawa, Y., Kikuchi, M., Yuasa, K., Motai, H., 1996. Detection of γ-polyglutamic acid (γ-PGA) by SDS-Page. Biosci. Biotechnol. Biochem. 60 (2), 255–258. https://doi.org/10.1271/bbb.60.255.

Zeng, W., Chen, G., Zhang, Y., Wu, K., Liang, Z., 2012. Studies on the UV spectrum of poly(γ-glutamic acid) based on development of a simple quantitative method. Int. J. Biol. Macromol. 51, 83–90. https://doi.org/10.1016/j.ijbiomac.2012.04.005.

Zhao, X. Qing, Park, K.H., Jin, Y.Y., Lee, I.H., Yang, Y.Y., Suh, J.W., 2005. Isolation and characterization of a new γ-Polyglutamic acid producer, *bacillus mesentericus* MJM1, from Korean domestic chungkukjang bean paste. J. Microbiol. Biotechnol. 15 (1), 59–65.