Poly (γ-) Glutamic Acid : A Promising Biopolymer

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ABSTRACT

Poly (γ-) glutamic acid (γ-PGA) is a polymer of L or D - glutamic acid units produced by microorganisms as a defense mechanism as an act of stress tolerance. Production of γ-PGA by microbial source has been gaining attention due to its low cost of production and varied application with high compatibility and high biodegradability. Its application ranges from food industry to waste water treatment. γ-PGA is a major constituent of Japanese food - natto. γ-PGA has vast applications in food, pharmaceuticals, healthcare, water treatment and other fields. The high research interest that currently is developing for γ-PGA is due to its potential as a biomedical material with a high biocompatibility and a fair biodegradability. In fact, γ-PGA is extensively used as a food additive and it is known to be hydrolytically degradable by water with or without intervening of proteases. An incessant number of publications dealing with the use of γ-PGA as drug delivery system are appearing in these last few years and several processes have been developed at large scale which are able to afford great amounts of this compound for industrial uses.

Keywords: γ-PGA; Nutraceuticals; Microorganisms; Foods; Delivery

1. INTRODUCTION

Poly γ-glutamic acid is a biodegradable poly amino acid which is soluble in water. γ-PGA has three stereochemical structures i.e., D-glutamic acid, L-glutamic acid, and copolymer of both D- and L-glutamic acid. γ-PGA was first discovered as a capsule of Bacillus anthracis, and later was found majorly in natto. γ-PGA was first discovered by Ivonovics and Bruckner[1]. γ-PGA produced by bacterial fermentation has peptide bonds between glutamic acid's amino group and the glutamic acid's carboxyl group. With contrast to proteins which has amide bonds between α-amine group and the α-carboxyl group, γ-PGA has it between α-amine group and the γ-carboxyl group. This improves the resistance against breakdown by proteases recognising the α-amide bond. Varies with proteins also by the fact that γ-PGA is formed by membrane bound process rather than sequential process of transcription and translation. Polymerisation of L-glutamic acid to γ-PGA is catalysed out by γ-PGA synthase complex in a ribosome independent manner. Lavorotary helix structure stabilises the γ-PGA in aqueous solution by forming hydrogen bonds. γ-PGA when applied as a food additive is known to be degradable by water hydrolytically.

2. STRUCTURAL AND MOLECULAR CHARACTERISTICS OF γ-PGA

γ-PGA can be of either of these conformations; an α-helix, a β-sheet or a helix-to-random coil transition or an enveloped aggregate. The conformational changes occur due to environmental conditions such as variations in pH, polymer concentration, and ionic strength[2,3]. As in the case of pH-7, γ-PGA adopts a largely α-helical conformation, but at a higher pH changes to β-sheet based conformation[2,4]. γ-PGA has molecular weight in the range of 100,000 to over 1,000,00032. The molecular weight depends on factors such as rate of fermentation, medium composition, alkaline hydrolysis, ultrasonic degradation, microbial or enzymatic degradation and the catalytic reaction rate in breaking up of hydrolytic bonds in poly γ-glutamic acid[5]. γ-PGA of higher molecular weight limits the industrial application due to high viscosity, unmanageable rheology, and difficult modification[6]. Therefore, the application of γ-PGA varies corresponding to its molecular weight and mode. The most common method for determination of molecular mass of γ-PGA is gel permeation chromatography (GPC). The techniques involve a range of mobile phases and calibrates against standards of γ-PGA with varied molecular masses[7]. The molecular mass determines the characteristics of the compound and γ-PGA produced by *Bacillus* species ranges from 105 Da to 106 Da[8]. The application of γ-PGA depends on its molecular mass. Drug delivery systems requires γ-PGA of low molecular masses, where methods such as ultrasonic degradation, alkaline hydrolysis and microbial or enzymatic degradation. The ultrasonic degradation proved to be effective technique to reduce the molecular mass.

Amino acid analysis is primarily carried out by Thin

Layer Chromatography (TLC), where the detection of γ-PGA is carried by hydrolysing it to glutamic acid and analysing it against a standard[9]. The process is carried out as follows: the purified γ-PGA is hydrolysed with 6 M Hydrochloric acid at 100 °C for specific hours depending on the γ-PGA concentration, then followed by HCl removal by evaporation and the glutamic acid presence is analysed using TLC. Generally solvent systems of butanol/acetic acid/water (3:1:1, w/w/w) and ninhydrin spray is employed in the detection of amino acid[10]. are usually performed to determine. The homogeneity and esterification characteristics of γ-PGA can be determined by [1]H- and [13]C- Nuclear Magnetic Resonance (NMR) spectroscopy. The chemical shifts are determined by comparing with the standards[11]. The Fourier Transform Infra-Red Spectroscopy (FTIR) is employed for the determination of carbonyl, hydroxyl, C-N, aliphatic N-H stretching and other characteristics of amide groups[10]. The IR spectra of γ-PGA indicated strong amide absorption at ~1620 cm^{-1} – 1655 cm^{-1}, a weaker carbonyl C=O absorption at ~1394 cm^{-1} – 1454 cm^{-1}, a strong hydroxyl OH absorption at ~3400 cm^{-1} – 3450 cm^{-1} and a characteristic strong C–N groups absorption in the range from 1085 cm^{-1} to 1165 cm^{-1}. All these characterisation gives a broader applicability of the compound in various fields.

3. FUNCTIONS AND ENANTIOMERIC CONFORMATION OF γ-PGA

The functions of the γ-PGA depend on the organism secreting and the presence of peptidoglycan bound. Peptidoglycan bound γ-PGA's assist in development of virulence and can act as a source of glutamate during the initial stages of starvation[12,13]. When it is being released into the environment, γ-PGA helps the survival of organism during adverse conditions[14,15]. The bacterial cells are protected against phage infections by γ-PGA by preventing the antibodies from gaining access to the bacterium[16]. *Staphylococcus epidermidis* also secretes surface-associated γ-PGA protecting it from peptides of antimicrobial origin[18]. In certain cases, γ-PGA can also act as a glutamate source for bacteria during starvation in the late stationary phase[17,18]. *Natrialba aegyptiaca, Sporosarcina halophili* and *Planococcus halophilus* survives in hostile environments by using γ-PGA to decrease the local salt concentrations[19]. *Bacillus amyloliquefaciens* C06 improves its characteristics of forming biofilm and motility by using γ-PGA where the cells are made to stick together in a confined pattern which leads to the essential nutrients absorption needed for motility from the environment[20].

γ-PGA possess a varied enantiomeric composition, determining the extraction of γ-PGA after the process of fermentation. If γ-PGA consist of only either L or D enantiomers, then it dissolves in ethanol. If L and D are equally present, then γ-PGA precipitates in ethanol. Research found that the antifreeze property of γ-PGA is not affected by its enantiomeric characteristics, but is affected by the molecular mass. The culture condition and the micro-organism were found to have an impact on the enantiomeric properties of the compound[19]. Further study on the enantiomeric properties and the effect of production media is necessary for the medium optimisation. These characteristics can be modulated for the γ-PGA production with desired properties and molecular mass.

4. SYNTHESIS AND MICROBIAL PRODUCTION OF γ-PGA

The biosynthesis of γ-PGA occurs with L-glutamic acid units as the source, where L-glutamic acid is produced in two different ways either exogenous or endogenous. By endogenous production of L-glutamic acid, a carbon source is converted to acetyl-CoA and TCA cycle intermediates. The ketoglutaric acid from the TCA cycle serves as a direct precursor of glutamic acid synthesis[21]. In exogeneous production of L-glutamic acid, a carbon source is converted to L-glutamine by the action of the enzyme glutamine synthase. Glutamine synthase is the precursor for γ-PGA. The γ-PGA synthesis occurs in various stages- γ-PGA racemisation, γ-PGA polymerisation, γ-PGA regulation and γ-PGA degradation[18]. The produced γ-PGA can be of different enantiomeric characteristics or the similar ones, depending on the production medium. The synthetically produced γ-PGA has a major drawback in terms of its molecular mass of at least 10 kDa, which limits its application. But bacterial production of γ-PGA results in polymer in the range of molecular mass 100 kDa to 1000 kDa[22,23]. But the microbial production too has a major drawback in commercialisation due to its cost of production. Therefore, the studies on the production of γ-PGA from microbe is concentrated highly on the optimisation of growth media and conditions with the scope of producing a high yield.

The medium involved for the production of γ-PGA by different bacteria is important in determining the γ-PGA yield, since it directly influences the characteristics of γ-PGA. In various cases it has been proved that the concentrations of NaCl in the medium affects the yield of γ-PGA and secreting γ-PGA of varied molecular masses[24]. To analyze the effect of ionic strength on the production of γ-PGA by *B. licheniformis* ATCC 9945a, the NaCl in the medium was distributed in the range of 0 per cent to 4 per cent the results showed that increase in NaCl concentration increased the molecular mass of the γ-PGA produced which thereby improved its production, properties and applications by a factor of 1.8 (from 1.26106 kDa to 2.26106 kDa). γ-PGA of higher molecular mass is very promising for various industrial applications.

5. INFLUENCE OF PRODUCTION MEDIUM ON γ-PGA PRODUCTION

The production of γ-PGA is correlated to the key nutrients in the medium, production conditions such as temperature, time period, pH and the microbial strain. *B. licheniformis* ATCC 9945 found to utilize glutamic acid, glycerol, citric acid, NH4Cl and citrate from the medium for production of 23.00 g/L of γ-PGA in a batch culture, where citrate utilisation was rapid at pH 6.5. With fed batch reaction Mn^{+2} in medium was found to be critical for maintaining cell viability, and in utilisation of carbon sources and the γ-PGA production was 17 g/L in this case 6.35.00 g/L of γ-PGA production was achieved with pulsed-feeding of citric acid (1.44 g/L) and L-glutamic acid (2.4 g/L)[25,26]. NaCl in the fermentation medium was found to significantly affect the yield and molecular weight of γ-PGA

in a batch culture[27]. *B. subtilis* IFO 3335 consumed glutamic acid and citric acid from the production medium to produce 20.00 g/L of γ-PGA. When the citric acid was replaced with other carbon sources, polysaccharide formed either had little or no γ-PGA. Addition of sufficient ammonium ions was found to be necessary for efficient conversion of citric acid to glutamic acid[28]. Addition of glutamic acid elongated the production of 15.60 g/L of γ-PGA with *B. subtilis* strain involving sucrose, $(NH4)_2SO_4$ and glutamic acid in the production medium. NaCl concentration in the fermentation medium significantly affects the yield of γ-PGA1. *B. lichenifomis* CCRC 12826 yielded 19.80 g/L of γ-PGA where the production medium consisted of glycerol, glutamic acid, NH_4Cl and citric acid. The L-glutamic acid proved to have greater level of interaction with glycerol[29].

Glycerol was found to influence the yield as well as molecular weight of γ-PGA. Mn^{+2} and Mg^{+2} ions showed an effect on volumetric yield and D-/L glutamate ratio of γ-PGA[30]. *B. subtilis* (natto) MR-141 strain was cultivated in the medium consisting of maltose, soy sauce and sodium glutamate yielding 35.00 g/L of γ-PGA. On transporting L-glutamic acid into the cells it was polymerised into the glutamic acid units of γ-PGA. NaCl in the medium was found to affect both the yield as well as foaming[31,32]. *B. licheniformis* NCIM 2324 strain used up glycerol, citric acid, $(NH4)_2SO_4$, glutamic acid, glutamine and ketoglutaric acid for producing 35.75 g/L of γ-PGA. Addition of ketoglutaric acid and a- L-glutamine to the medium increased the yield as well as molecular weight of γ-PGA[33].

6. APPLICATIONS

In food industry as shown in Fig. 1, γ-PGA is widely used as a food supplement and osteoporosis preventing agent[34]. Natto mucilages containing γ-PGA had greatly improved the calcium solubility and enhanced texture[35]. In the case of wheat bread, the addition of γ-PGA reduces its hardness including the storage period and enhanced the thermal and rheological properties of wheat dough. γ-PGA was proved to enhance the sponge cake's texture and decreased the uptake of oil during deep-fat frying. γ-PGA of molecular mass of 20 kDa showed higher antifreeze activities than the other common antifreeze agents like glucose. Since it has no significant interference with the foods taste, they are being used as a cryoprotectant[36]. Similarly, γ-PGA of molecular mass - 27 kDa was used as a cryoprotectant for preserving probiotic bacteria, to improve their chances of survival during production. γ-PGA is extensively applied in food industry to relieve bitterness from vegetables[37,38]. Medically, γ-PGA-coated with super paramagnetic iron oxide is used as a metal chelator[14,15], which demonstrated high heavy metal removal efficiency from stimulated gastrointestinal fluid and a metal solution[39]. Cisplatin when covalently attached to γ-PGA shows reduction in the toxicity of cisplatin, while effectively decreasing the tumour size of xenografted human breast tumours in nude mice. This resulted in the lengthened survival of nude mice grafted with Bcap-37 tumour cells. Paclitaxel poliglumex, a macromolecule which is the conjugate of paclitaxel and γ-L-PGA, showed extra ordinary benefits over conventional paclitaxel. The active agent paclitaxel was discharged in the tumour tissue by accumulation of paclitaxel

poliglumex. Chitosan and γ-PGA demonstrated potential application in wound dressing and in the field of tissue engineering. The resulted molecule consists of effective level of moisture content and showed good mechanical properties allowing the easy removal of dressing from the wound surface without destroying renewed tissues[40]. An hydrogel was formed from a mixture of gelatin and γ-PGA aqueous solution in the presence of water-soluble carbodiimide. The gel demonstrated better air-leak sealing and lung adhesion than conventional fibrin glue[41,42].

Absorption of calcium in the intestine was increased by the administration of γ-PGA in post-menopausal status women by inhibiting the formation of an insoluble calcium complex with phosphate and γ-PGA can be potentially used for disorders of borne treatment[43]. γ-PGA is applied for the waste water treatment where it is covalently incorporated into microfiltration membranes via attachment with pore surfaces which resulted in super-high heavy metal sorption ability[44]. γ-PGA is used as a bio-sorbent for the removal of Ni^{2+}, Cu^{2+}, Mn^{2+} and Al^{3+}. γ-PGA produced by *B. subtilis* R 23 showed a high flocculating activity that could be further enhanced by the addition of cations and hence γ-PGA been used as a flocculant[45]. The effective removal of basic dyes by γ-PGA from aqueous solution and was found that 98 per cent of the dye adsorbed on γ-PGA recovered at pH-13[46,47]. B. subtilis B6-1 using soybean Lipopeptides and γ-PGA produced by sweet potato residues was found to adequately repress cucumber wilts, increased nutrient and consumption[48].
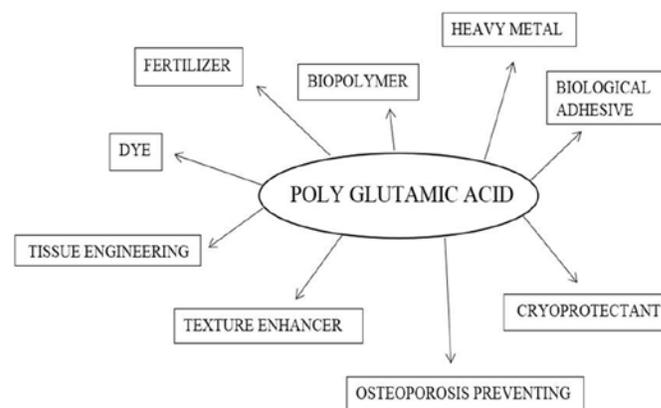
Figure 1. Applications of gamma poly glutamic acid.

7. CONCLUSIONS

Owing to the biodegradability and stability rate γ-PGA has a wider scope of application from the field of food industry to medicinal use. But it is highly necessary to control the molecular weight and derivatives of γ-PGA for its better application in medical and food industries. New derivatives can lead to revolution in the drug delivery systems. The studies on microbial production will result in efficient and economical way of production, but the issue lies with the understanding of biosynthetic pathway, molecular weight and stability of γ-PGA. These factors determine its applications capabilities. The γ-PGA has potential applications in fields such as medicine, cosmetics and food processing. Further research and optimisation of production will assist in the complete usage of the compound in near future.

REFERENCES

1. Shih, L. & Van, Y.T. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. *Bioresource Technology*, 2001, **79**(3), 207-225.
    doi: 10.1016/S0960-8524(01)00074-8.

2. Urushibata, Y.; Tokuyama, S. & Tahara, Y. Characterization of the *Bacillus subtilis* ywsC gene, involved in γ-polyglutamic acid production. *Journal of Bacteriology*, , 2002, **184**(2), 337-343.
    doi: 10.1128/JB.184.2.337-343.2002.

3. Yang, X. Preparation and characterization of γ-poly glutamic acid copolymer with glycol diglycidyl ether. *Procedia Environmental Sciences*, 2011, **8**, 11-15.
    doi: 10.1016/j.proenv.2011.10.004.

4. Zeng, W.; Lin, Y.; Qi, Z.; He, Y.; Wang, D.; Chen, G. & Liang, Z. An integrated high-throughput strategy for rapid screening of poly γ-glutamic acid producing bacteria. *Appl. Microbiol. Biotechnol.*, 2013, **97**(5), 2163-2172.
    doi: 10.1007/s00253-013-4717-0

5. Kocianova, S.; Vuong, C.; Yao, Y.; Voyich, J.M.; Fischer, E.R.; DeLeo, F.R. & Otto, M. Key role of poly-γ-DL-glutamic acid in immune evasion and virulence of staphylococcus epidermidis. *J. Clin. Invest.*, 2005, **115**(3), 688-694.
    doi: 10.1172/JCI23523

6. Shih, L. & Wu, J.Y. Biosynthesis and application of poly γ-glutamic acid: *microbial production of biopolymers and polymer precursors: applications and perspectives.* Horizon Scientific Press, Norfolk, 2009, 101-141.

7. Bhat, A.R.; Irorere, V.U.; Bartlett, T.; Hill, D.; Kedia, G.; Morris, M.R.; Charalampopoulos, D. & Radecka, I. Bacillus subtilis natto: a non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *Amb Express*, 2013, **3**(1), 36.
    doi: 10.1186/2191-0855-3-36.

8. Shih, L. & Van, Y.T. The production of poly-γ-glutamic acid from microorganisms and its various applications. *Bioresource Technology*, 2001, **79**(3), 207-225.
    doi: 10.1016/S0960-8524(01)00074-8.

9. Shih, L. & Van, Y.T. The production of poly-γ-glutamic acid from microorganisms and its various applications. *Bioresource Technology*, 2001, **79**(3), 207-225.
    doi: 10.1016/S0960-8524(01)00074-8.

10. 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. *Biotechnology Letters*, 2000, **22**(7), 585-588.

11. Bhat, A.R.; Irorere, V.U.; Bartlett, T.; Hill, D.; Kedia, G.; Morris, M.R.; Charalampopoulos, D. & Radecka, I. Bacillus subtilis natto: a non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *Amb Express*, 2013, **3**(1), 36.
    doi: 10.1186/2191-0855-3-36.

12. Soliman, N.A.; Berekaa, M.M. & Abdel-Fattah, Y.R. Polyglutamic acid (PGA) production by *Bacillus Sp.* SAB-26: application of Plackett–Burman experimental design to evaluate culture requirements. *Appl. Microbiol. Biotechnol.*, 2005, **69**(3), 259.

13. Tamang, J.P. Naturally fermented ethnic soybean foods of India. *J. Ethnic Foods*, 2015, **2**(1), 8-17.

14. Kimura, T.; Takahashi, S.; Akiyama, S.; Uzawa, T.; Ishimori, K. & Morishima, I. Direct observation of the multistep helix formation of poly-L-glutamic acids. *J. Am. Chem. Soc.*, 2002, **124**(39), 11596-11597.
    doi: 10.1021%2Fja026639f.

15. Ogunleye, A.; Bhat, A.; Irorere, V.U.; Hill, D.; Williams, C. & Radecka, I. Poly-γ-glutamic acid: production, properties and applications. *Microbiology*, 2015, **161**(1), 1-17.
    doi: 10.1099/mic.0.081448-0

16. Meerak, J.; Iida, H.; Watanabe, Y.; Miyashita, M.; Sato, H.; Nakagawa, Y. & Tahara, Y. Phylogeny of γ-polyglutamic acid-producing *Bacillus* strains isolated from fermented soybean foods manufactured in Asian countries. *J. Gen. Appl. Microbiol.*, 2007, **53**(6), pp.315-323. https://doi.org/10.2323/jgam.54.159

17. King, E.C.; Blacker, A.J. & Bugg, T.D. Enzymatic breakdown of poly-γ-D-glutamic acid in Bacillus licheniformis: identification of a polyglutamyl γ-hydrolase enzyme. *Biomacromolecules*, 2000, **1**(1), 75-83.
    doi: 10.1021/bm990001n

18. Buescher, J.M. & Margaritis, A. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. *Crit. Rev. Biotechnol.*, 2007, **27**(1), 1-19.
    doi: 10.1080/07388550601166458.

19. Ho, G.H.; Ho, T.I.; Hsieh, K.H.; Su, Y.C.; Lin, P.Y.; Yang, J.; Yang, K.H. & Yang, S.C. γ-Polyglutamic Acid Produced by *Bacillus Subtilis* (Natto): structural characteristics, chemical properties and biological functionalities. *J. Chin. Chem. Soc.*, 2006, **53**(6), 1363-1384.
    doi: 10.1002/jccs.200600182

20. 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. *Biotechnology for Biofuels*, 2016, **9**(1), 134.
    doi: 10.1186/s13068-016-0537-7

21. Lin, W.C.; Yu, D.G. & Yang, M.C. Blood compatibility of novel poly γ-glutamic acid polyvinyl alcohol hydrogels. *Colloids Surf., B*, 2006, **47**(1), 43-49.
    doi: 10.1016/j.colsurfb.2005.11.013.

22. Satter, M.A.; Ara, H.; Jabin, S.A.; Abedin, N.; Azad, A.K.; Hossain, A. & Ara, U. Nutritional composition and stabilization of local variety rice bran BRRI-28. *Int. J. Sci. Technol.*, 2014, **3**(5), 2049-7318.

23. Shih, L. & Van, Y.T. The production of poly-γ-glutamic acid from microorganisms and its various applications. *Bioresource Technology*, 2001, **79**(3), 207-225.
    doi: 10.1016/S0960-8524(01)00074-8.

24. Ho, G.H.; Ho, T.I.; Hsieh, K.H.; Su, Y.C.; Lin, P.Y.; Yang, J.; Yang, K.H. & Yang, S.C. γ-Polyglutamic acid produced

by *Bacillus Subtilis* (Natto): Structural characteristics, chemical properties and biological functionalities. *J. Chin. Chem. Soc.*, 2006, **53**(6), 1363-1384.
doi: 10.1002/jccs.200600182

25. 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*. *Biotechnology Letters*, 2000, **22**(7), 585-588.

26. Singer, J.W. Paclitaxel poliglumex XYOTAX™, CT-2103: a macromolecular taxane. *J. Controlled Release*, 2005, **109**(1-3), 120-126.
doi: 10.1016/j.jconrel.2005.09.033.

27. Bhat, A.R.; Irorere, V.U.; Bartlett, T.; Hill, D.; Kedia, G.; Morris, M.R.; Charalampopoulos, D. & Radecka, I. *Bacillus subtilis* natto: a non-toxic source of poly-γ-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *Amb Express*, 2013, **3**(1), 36.
doi: 10.1186/2191-0855-3-36.

28. Bhunia, B.; Mukhopadhy, D.; Goswami, S.; Mandal, T. & Dey, A. Improved production, characterization and flocculation properties of poly (γ)-glutamic acid produced from *Bacillus subtilis*. *J. Biochem. Technol.*, 2012, **3**(4), 389-394.

29. Shih, L. & Van, Y.T. The production of poly-(γ-glutamic acid) from microorganisms and its various applications. *Bioresource Technology*, 2001, **79**(3), 207-225.
doi: 10.1016/S0960-8524(01)00074-8.

30. Jian, X.; Shouwen, C. & Ziniu, Y., Optimization of process parameters for poly γ-glutamate production under solid state fermentation from *Bacillus subtilis* CCTCC202048. *Process Biochemistry*, 2005, **40**(9), 3075-3081.
doi: 10.1016/j.procbio.2005.03.011.

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

32. Xu, H.; Jiang, M.; Li, H.; Lu, D. & Ouyang, P. Efficient production of poly γ-glutamic acid by newly isolated *Bacillus subtilis* NX-2. *Process Biochemistry*, 2005, **40**(2), 519-523.
doi: 10.1016/j.procbio.2003.09.025.

33. Pandey, A.; Soccol, C.R. & Mitchell, D. New developments in solid state fermentation: I-bioprocesses and products. *Process Biochemistry*, 2000, **35**(10), 1153-1169.
doi: 10.1016/S0032-9592(00)00152-7.

34. Lee, N.R.; Lee, S.M.; Cho, K.S.; Jeong, S.Y.; Hwang, D.Y.; Kim, D.S.; Hong, C.O. & Son, H.J. Improved production of poly-γ-glutamic acid by *Bacillus subtilis* D7 isolated from Doenjang: a korean traditional fermented food, and its antioxidant activity. *Applied Biochem. and Biotechnol.*, 2014, **173**(4), 918-932.
doi: 10.1007/s1201

35. Shyu, Y.S. & Sung, W.C. Improving the emulsion stability of sponge cake by the addition of γ-polyglutamic acid. *J.*

Mar. Sci. Technol., 2010, **18**(6), 895-900.
doi: 10.6119/JMST.

36. Mitsuiki, M.; Mizuno, A.; Tanimoto, H. & Motoki, M. Relationship between the antifreeze activities and the chemical structures of oligo-and poly glutamic acids. *J. Agric. Food Chem.*, 1998, **46**(3), 891-895.
doi: 10.1021/jf970797m.

37. Sakai, K.; Sonoda, C. & Murase, K. Bitterness relieving agent. *JP Patent* WO0021390, 2000.

38. Tork, S.E.; Aly, M.M.; Alakilli, S.Y. & Al-Seeni, M.N. Purification and characterization of gamma poly glutamic acid from newly *Bacillus licheniformis* NRC20. *Int. J. Biol. Macromol.*, 2015, **74**, 382-391.
doi: 10.1016/j.ijbiomac.2014.12.017.

39. Ye, H.; Jin, L.; Hu, R.; Yi, Z.; Li, J.; Wu, Y.; Xi, X. & Wu, Z. Poly γ, l-glutamic acid cisplatin conjugate effectively inhibits human breast tumor xenografted in nude mice. *Biomaterials*, 2006, **27**(35), 5958-5965.
doi: 10.1016/j.biomaterials.2006.08.016.

40. Hsieh, C.Y.; Tsai, S.P.; Wang, D.M.; Chang, Y.N. & Hsieh, H.J. Preparation of γ-PGA/chitosan composite tissue engineering matrices. *Biomaterials*, 2005, **26**(28), 5617-5623.
doi: 10.1016/j.biomaterials.2005.02.012.

41. Park, Y.J.; Liang, J.; Yang, Z. & Yang, V.C. Controlled release of clot dissolving tissue type plasminogen activator from a poly L-glutamic acid semi interpenetrating polymer network hydrogel. *J. Controlled Release*, 2001, **75**(1-2), 37-44.
doi: 10.1016/S0168-3659(01)00360-1.

42. Khalil, I.R.; Irorere, V.U.; Radecka, I.; Burns, A.T.; Kowalczuk, M.; Mason, J.L. & Khechara, M.P. Poly-γ-glutamic acid: biodegradable polymer for potential protection of beneficial viruses. *Materials*, 2016, **9**(1), 28.
doi:10.3390/ma9010028.

43. Tanimoto, H.; Mori, M.; Motoki, M.; Torii, K.; Kadowaki, M. & Noguchi, T. Natto mucilage containing poly-γ-glutamic acid increases soluble calcium in the rat small intestine. *Biosci., Biotechnol., Biochem.*, 2001, **65**(3), 516-521.
doi: 10.1271/bbb.65.516.

44. Bajaj, I.B. & Singhal, R.S. Sequential optimization approach for enhanced production of Poly γ-glutamic acid from newly isolated *Bacillus subtilis*. *Food Technol. Biotechnol.*, 2009, **47**(3).
doi: 10.1016/S0032-9592(00)00152-7.

45. Ho, G.H.; Ho, T.I.; Hsieh, K.H.; Su, Y.C.; Lin, P.Y.; Yang, J.; Yang, K.H. & Yang, S.C. γ-polyglutamic acid produced by *Bacillus subtilis* (Natto): Structural characteristics, chemical properties and biological functionalities. *J. Chin. Chem. Soc.*, 2006, **53**(6), 1363-1384.
doi: 10.1002/jccs.200600182

46. Ho, G.H.; Ho, T.I.; Hsieh, K.H.; Su, Y.C.; Lin, P.Y.; Yang, J.; Yang, K.H. & Yang, S.C. γ-polyglutamic acid produced by *Bacillus Subtilis* (Natto): Structural characteristics, chemical properties and biological functionalities. *J. Chin. Chem. Soc.*, 2006, **53**(6), 1363-1384.

doi: 10.1002/jccs.200600182

47. 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*, 2007, **2**(9), 664-670.
    doi: 10.3923/jm.2007.664.670.

48. Wang, Q.; Chen, S.; Zhang, J.; Sun, M.; Liu, Z. & Yu, Z. Co-producing lipopeptides and poly-γ-glutamic acid by solid-state fermentation of *Bacillus subtilis* using soybean and sweet potato residues and its biocontrol and fertilizer synergistic effects. *Bioresource technology*, 2008, **99**(8), 3318-3323.
    doi: 10.1016/j.biortech.2007.05.052.

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