Isolation and Characterization of Poly-γ-glutamic Acid Producing Bacteria from Plant Rhizoplane

Sirirat Siripornvisal* and Teerayuth Suddee

Department of Microbiology, Faculty of Science and Technology
Phranakhon Si Ayutthaya Rajabhat University, Ayutthaya, Thailand

Received: 28 September 2017, Revised: 21 May 2019, Accepted: 4 June 2019

Abstract

The purpose of this study was to isolate and evaluate the diversity of poly-γ-glutamic acid (γ-PGA) producing bacteria from rhizoplane of three Poaceae plants viz. rice (*Oryza sativa* Linn.), maize (*Zea mays* Linn.) and sugarcane (*Saccharum officinarum* Linn.), which are considered the most important agronomic crops of Thailand. A total of 368 isolates of rhizoplane bacteria were obtained from the root samples: 200 isolates from rice roots, 112 isolates from maize roots and 56 isolates from sugarcane roots. All isolates were screened for γ-PGA production consecutively by plate and tube culture assay. There were 186 isolates which exhibited γ-PGA producing capability. The γ-PGA concentrations obtained ranged from 12.62 - 18.46 g/L. Of those 186 isolates, 16 isolates were capable of producing γ-PGA higher than 15 g/l and these isolates were selected as the most efficient γ-PGA producers for further molecular characterization. The molecular genetic study based on 16S rRNA genes analysis revealed that the selected γ-PGA producers were closely related to 9 *Bacillus* species, namely *B. amyloliquefaciens* subsp. *amyloliquefaciens*, *B. atrophaeus*, *B. methylotrophicus*, *B. siamensis*, *B. subtilis* subsp. *inaquosorum*, *B. subtilis* subsp. *subtilis*, *B. tequilensis*, *B. vallismortis* and *B. velesensis*. All of them are belonging to *B. subtilis* and *B. amyloliquefaciens* groups. These results indicate that the rhizoplane of Poaceae plants are an important reservoir of natural isolates of γ-PGA producing bacteria.

Keywords: poly-γ-glutamic acid, γ-PGA, rhizoplane, Poaceae plants, *Bacillus*
DOI 10.14456/cast.2019.17

*Corresponding author: E-mail: sirirat2@yahoo.com

1. Introduction

Biopolymer, γ-polyglutamic acid (γ-PGA), is a natural and multifunctional poly-amino acid which can be produced by a range of bacteria. It is constituted of D- and/or L-glutamic acid units which are polymerized by gamma amide linkages [1]. As a biopolymer material, γ-PGA has several attractive physicochemical and biological properties such as high water solubility, excellent absorb ability, metal-binding capacity, good thickening capacity and biodegradability [2]. This polymer is also edible and non-toxic towards humans and environment [3]. These properties of γ-PGA are attractive for applications in many fields. This polymer can be used as a novel drug delivery material [4, 5], an osteoporosis-preventing factor in medicine [6, 7], an antifreeze agent, a food thickener [8-10] and a flocculent or absorption agent for biotechnological and environmental applications [11, 12].

Bacteria of the genus *Bacillus* are the best known γ-PGA producer [13, 14]. It was widely known that glutamic acid is the stringent precursor for γ-PGA biosynthesis and the γ-PGA-producing bacteria can obtain from two different sources; exogenously acquired from environment or endogenously synthesized by itself. This leads to the classification of the γ-PGA producing bacteria into two groups, i.e. glutamic acid dependence- and independence strains, according to the requirement of exogenous glutamic acid [15]. From the economic viewpoint, industrial production of γ-PGA may be limited by the high cost of glutamic acid and it would be attractive to shift to the utilization of glutamic acid by independent strains instead.

In the past recent years, short lists of glutamic acid-independent strains have been published such as *Bacillus methylotrophicus* [2], *B. subtilis* C10 [15] and *B. amyloliquefaciens* LL3 [16]. These bacteria can self-synthesize a considerable yield of γ-PGA without the addition of glutamic acid. In some glutamic acid-independent strains such as *B. licheniformis* ATCC 9945a, an addition of a small amount of glutamic acid in the media can significantly enhance γ-PGA synthesis [17]. It is clear that the glutamic acid-independent strains have potential to be improved by further comprehensive researches in which metabolic pathways and the enzymes can be related to γ-PGA synthesis under the optimal culture conditions. However, a few number of the glutamic acid-independent strains have been studied. In addition, the native glutamic acid-independent strains that have been reported, were found to synthesize a little amount of γ-PGA, generally lower than 15g/l [2]. Therefore, it remains meaningful to search for glutamic acid-independent strains with high-levels of γ-PGA production, as these strains involve lower costs and simplified processes for industry. The objective of this study was to isolate a number of γ-PGA producing bacteria from rhizoplane of three Poaceae plants viz. rice (*Oryza sativa* Linn.), maize (*Zea mays* Linn.) and sugarcane (*Saccharum officinarum* Linn.) and to reveal the co-occurrence of both glutamic acid-dependence and glutamic acid-independent strains in these habitats.

2. Materials and Methods

2.1 Isolation and screening of γ-PGA producing bacteria

Samples of root of three Poaceae plants *viz*. rice (*Oryza sativa* Linn.), maize (*Zea mays* Linn.) and sugarcane (*Saccharum officinarum* Linn.) were collected from cultivation area in central provinces of Thailand, including Ang Thong, Phra Nakhon Si Ayutthaya and Saraburi.

To isolate rhizoplane bacteria, the root samples were gently washed with sterilized water with the aids of sonication to remove adhered soils. Then the final rinsing water was spread on a minimal medium containing glutamic acid. After incubation period for 24 h at 37°C, single mucoid colonies were selected for secondary screening [18].

Secondary screening was done by streaking each mucoid colony on a differential medium (1 % glucose, 0.5 % yeast extract, 0.5 % L-glutamic acid, 0.05 % KH_2PO_4, 0.05 % K_2HPO_4, 0.01 % $MgSO_4$ $7H_2O$, and 1.5 % agar, pH 7.2±0.1) supplemented with 0.006% (w/v) neutral red as differential indicator. Any colony which interacted with the dye and formed a specific concentric zone after incubation at 37 °C was selected for further analysis as a potent γ-PGA-producing strain [19].

To differentiate whether the selected isolates are glutamic acid-dependent or -independent strains, the isolates were inoculated on a modified Basal medium without the supplement of L-glutamic acid (1% tryptone, 1% beef extract, 1% yeast extract, 1% glucose, 0.5% NaCl and 1.5% agar). Isolates which form observable mucoid colonies on the medium were considered as glutamic acid-independent strains [15].

2.2 Tube culture assay for γ-PGA production

Rapid growing colonies which produced clear concentric zone were selected and inoculated into 50 ml sterile tubes containing 5 ml of fermentation medium (3% glucose, 0.25% yeast extract, 2% L-glutamate, 0.05% KH_2PO_4, 0.05 % K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, pH7.2±0.1) and incubated in a shaking incubator at 37 °C with shaking at 200 rpm for 48 h.

After incubation, the culture broth was diluted with an appropriate volume of deionized water to reduce the viscosity of the medium. Cells were separated from the medium by centrifugation at 12,000 rpm for 20 min and the supernatant was precipitated with four volumes of ice-cold ethanol. After centrifugation, the sediment was collected and dissolved in an appropriate volume of deionized water. Any insoluble contaminants was removed by centrifugation and the solution was then used for further quantification of γ-PGA. The yield of γ-PGA was determined by measurement absorbance of the sample solution at 216 nm using a UV/VIS spectrophotometer (Lambda 25; PerkinElmer, USA). A standard curve was plotted between the average blank corrected absorbance of each standard at 216 nm and its concentration (20-200 µg/ml). Bacterial isolates which produce considerable quantity of γ-PGA were selected and subjected to the identification and phylogeny study [19].

2.3 Bacterial identification and phylogeny study

Selected bacteria were grown in LB medium with shaking at 37°C overnight. Cells were collected by centrifugation. Genomic DNA was extracted using the Qiagen DNA extraction kit per manufacturer's protocol (Qiagen Inc., Valencia, USA). Almost complete 16S rDNA fragment was amplified by polymerase chain reaction (PCR) in a thermocycler (GeneAmp PCR system 9700, PerkinElmer-Applied Biosystems, USA) with the specific primers, 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) [20]. Purification of the PCR products was performed using a commercial kit per manufacturer's protocol (QIAGEN PCR Purification Kit, Qiagen Inc., Valencia, USA). The purified 16S rDNA fragments were sent to Macrogen Sequencing Service (Macrogen Inc., Seoul, Korea). The obtained sequences of 16S rRNA gene were assembled and manually corrected using the BioEdit program version 7.0 [21]. The primary comparison of the obtained sequences with the sequences available in GenBank was performed using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned with the corresponding sequences of the closest bacterial species using the CLUSTAL_W program incorporated in the MEGA-X software package. Pair-wise evolutionary distances were calculated and a neighbour-joining phylogenetic tree was constructed using the MEGA-X. The stability of phylogenetic tree was assessed by bootstrap analysis with 1000 replicates.

3. Results and Discussion

3.1 Isolation and screening of γ-PGA producing bacteria

The polymer γ-PGA has attracted much attention as an environment-friendly material and many researchers have put efforts on searching for novel bacterial strains with more efficient production of γ-PGA. From previous literatures, there are a growing number of reports related to the isolation and screening for new bacteria with γ-PGA producing capability and almost entirely focused on the bacteria from food sources, particularly from fermented soybean products. Additionally, some of the recent knowledge has suggested that γ-PGA is a polymeric substance found in *Bacillus* biofilm and plays a role in the bacterial colonization on plant surfaces including fruit and root surface [22, 23]. In this study, we hypothesized that γ-PGA producing bacteria may be the common inhabitant of root rhizoplane of higher plants because of its capability to produce adhesive biofilm with γ-PGA as the main component.

The isolation of rhizoplane bacteria with γ-PGA producing capability was conducted with the combination of conventional and newly proposed methods. First, rhizoplane bacteria were isolated from the root samples using a minimal medium containing 0.5 % L-glutamic acid. According to the conventional criteria, single mucoid colonies emerged on the medium were presumed to be γ-PGA producing bacteria and were chosen for further screening using the differential medium proposed by Zeng *et al*. [19]. From the isolation and screening, a total of 368 isolates of probable producer of γ-PGA were obtained, 200 isolates from rice rhizoplane, 112 isolates from maize rhizoplane and the remaining 56 isolates from sugarcane rhizoplane.

In addition, a result of the discrimination of the γ-PGA producing isolates on glutamic acid-free medium revealed 86 isolates as glutamic acid-independent stains, representing about 23 percent of the total isolates obtained, implied that the population of glutamic acid-independent strain was in much less abundance.

3.2 γ-PGA production capability

The tube culture assay was conducted to quantitatively evaluate the capability of the isolated bacteria producing γ-PGA in a formulated glutamate containing medium. The results revealed 186 isolates (approximately 50 % of the total isolates) as potentially efficient γ-PGA producer with γ-PGA yields ranging from 12.62 - 18.46 g/l of culture broth. Among them, there were 16 isolates which produced higher than 15 g/l γ-PGA (Table 1). Thus these isolates were considered as the most efficient strains and were selected for further identification and phylogeny study.

Table 1. γ-PGA content produced by 16 most efficient strains after 48 h cultivation and their type of γ-PGA production.

Isolate	Sources	γ-PGA production (g/l)	Type of γ-PGA production
PGA 005	Rice	15.64	GD*
PGA 006	Rice	16.88	GD
PGA 009	Rice	18.36	GID**
PGA 21	Rice	15.06	GD
PGA 29	Rice	16.56	GD
PGA 52	Rice	17.36	GD
PGA 54	Rice	15.88	GID
PGA 62	Rice	16.92	GD
PGA 69	Rice	18.46	GD
PGA 75	Maize	18.01	GID
PGA 77	Maize	17.42	GD
PGA 84	Maize	17.33	GD
PGA 100	Sugarcane	17.81	GD
PGA 102	Sugarcane	18.01	GD
PGA 103	Sugarcane	15.48	GD
PGA 109	Sugarcane	16.92	GID

*Glutamic acid-dependence
**Glutamic acid-independence

3.3 Bacterial identification and phylogeny study

Based on the preliminary observation on morphology and some physiological properties, all the selected isolates were found to be Gram positive, rod shaped, spore forming and catalase positive bacteria, which are the common features of bacteria of the genus *Bacillus*.

Following the phenotypic characterization, the result from 16S rRNA gene analysis has confirmed that all the 16 isolates are phylogenetically close to *Bacillus* species as shown in Table 2 and in Figure 1. The results are in alignment with the most of previous studies which reported that bacteria of the genus *Bacillus* were the main producer of γ-PGA, including *B. amyloliquefaciens* subsp. *amyloliquefaciens, B. atrophaeus, B. methylotrophicus, B. siamensis, B. subtilis* subsp. *inaquosorum, B. subtilis* subsp. *subtilis, B. tequilensis, B. vallismortis* and *B. velesensis*.

Table 2. Sixteen most efficient strains producing γ-PGA after 48 h cultivation and their related taxa.

Isolate	Sources	Closely related taxa	Strain	Similarity (%)
PGA 005	Rice	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens*	DSM7$^{(T)}$	99
PGA 006	Rice	*Bacillus vallismortis*	DV1-F-3$^{(T)}$	100
PGA 009	Rice	*Bacillus siamensis*	KCTC 13613$^{(T)}$	98.9
PGA 21	Rice	*Bacillus atrophaeus*	JCM 9070$^{(T)}$	99.9
PGA 29	Rice	*Bacillus siamensis*	KCTC 13613$^{(T)}$	99.9
PGA 52	Rice	*Bacillus siamensis*	KCTC 13613$^{(T)}$	99.9
PGA 54	Rice	*Bacillus methylotrophicus*	XY18$^{(T)}$	99.9
PGA 62	Rice	*Bacillus tequilensis*	KCTC 13622$^{(T)}$	99.9
PGA 69	Rice	*Bacillus tequilensis*	KCTC 13622$^{(T)}$	99.9
PGA 75	Maize	*Bacillus subtilis* subsp. *subtilis*	NCIB 3610$^{(T)}$	99.9
PGA 77	Maize	*Bacillus siamensis*	KCTC 13613$^{(T)}$	99.9
PGA 84	Maize	*Bacillus siamensis*	KCTC 13613$^{(T)}$	99.9
PGA 100	Sugarcane	*Bacillus tequilensis*	KCTC 13622$^{(T)}$	100.0
PGA 102	Sugarcane	*Bacillus tequilensis*	KCTC 13622$^{(T)}$	99.9
PGA 103	Sugarcane	*Bacillus subtilis* subsp. *inaquosorum*	KCTC 13429$^{(T)}$	100.0
PGA 109	Sugarcane	*Bacillus velesensis*	CR-502 $^{(T)}$	99.9

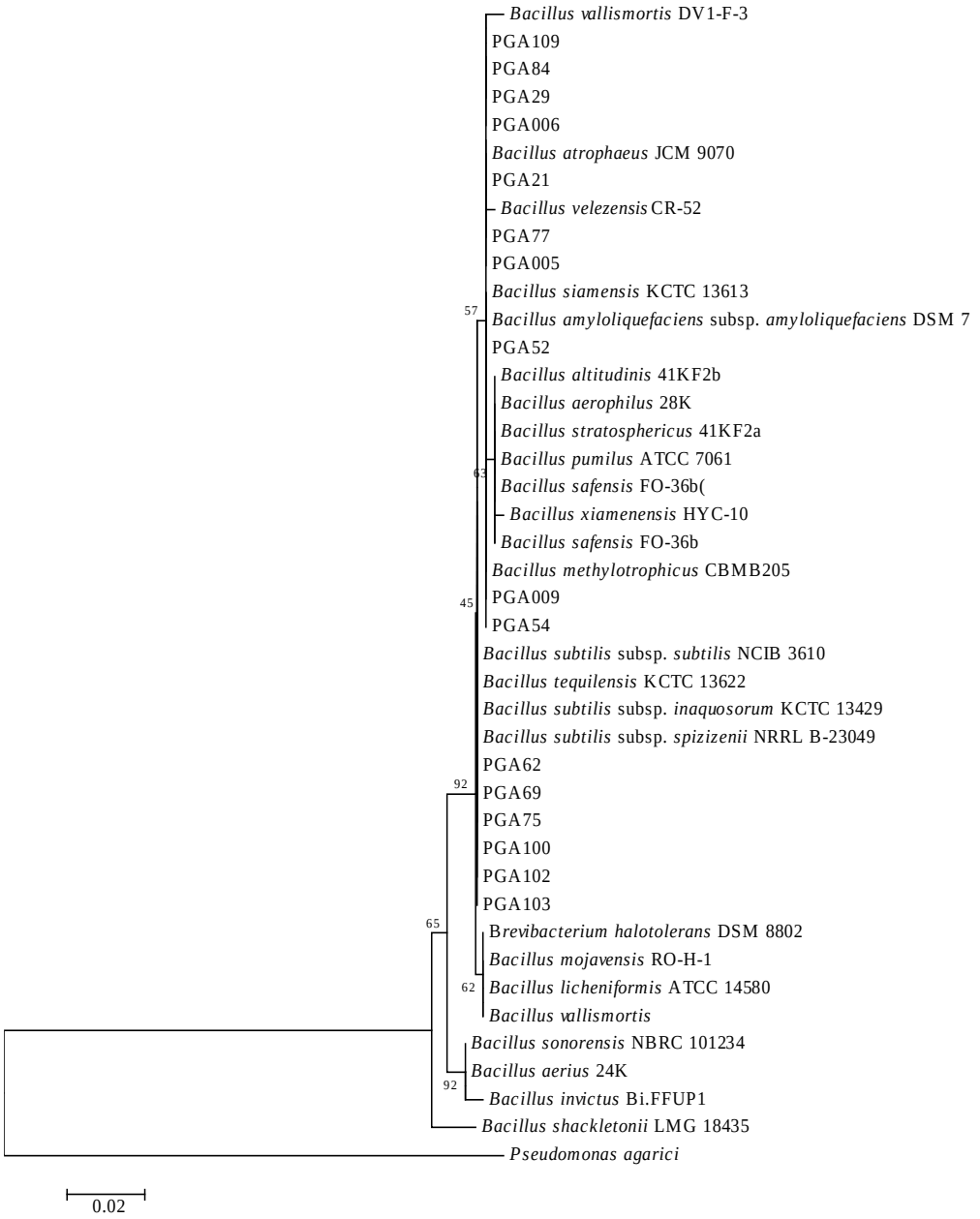

Figure 1. Phylogenetic tree of 16S rDNA sequences of the γ-PGA producing strains isolated from rhizoplane of rice, maize, sugarcane showing the relationship with members of the genus *Bacillus.* The phylogenetic tree was constructed by the neighbor-joining method. The type strain of *Pseudomonas agarici* was used as an outgroup organism. Numerals at nodes indicate bootstrap values (%) derived from 1,000 replications.

4. Conclusions

The results of this study have suggested that rhizoplane of three Poaceae plants, i.e. rice, maize and sugarcane are the natural habitat of γ-PGA producing bacteria, particularly of the genus *Bacillus*. Out of 200 isolates, 16 most efficient strains of the genus *Bacillus* produced γ-PGA between 15.06-18.46 g/l. Furthermore, it might be presumed that natural isolates of γ-PGA producing bacteria may also be prevalent on rhizoplane of other plant families.

5. Acknowledgement

This research work was supported by the National Research Council of Thailand.

References

[1] Ashiuchi, M. and Misono, H., 2002. Biochemistry and molecular genetics of poly-γ-glutamate synthesis. *Applied Microbiology and Biotechnology*, 59, 9-14.

[2] Peng, Y., Jiang, B., Zhang, T., Mu, W., Miao, M. and Hua, Y., 2015. High-level production of poly (γ-glutamic acid) by a newly isolated glutamate-independent strain, *Bacillus methylotrophicus*. *Process Biochemistry*, 50, 329-335.

[3] Tang, D.W., Yu, S.H., Ho, Y.C., Huang, B.Q., Tsai, G.J., Hsieh H.Y., Sung, H.W. and Mi, F.L., 2013. Characterization of tea catechins-loaded nanoparticles prepared from chitosan and an edible polypeptide. *Food Hydrocolloids*, 30(1), 33-41.

[4] Liang, H.F., Chen, C.T, Chen, S.C., Kulkarni, A.R., Chiu, Y.L., Chen, M.C. and Sung, H.W., 2006. Paclitaxel-loaded poly (γ-glutamic acid)-poly(lactide) nanoparticles as a targeted drug delivery system for the treatment of liver cancer. *Biomaterials*, 27, 2051-2059.

[5] Li, C., 2002. Poly (l-glutamic acid)-anticancer drug conjugates. *Advanced Drug Delivery Reviews*, 54, 695-713.

[6] Tanimoto, H., Fox, T., Eagles, J., Satoh, H., Nozawa, H., Okiyama, A., Morinaga, Y. and Fairweather-Tait, S., 2007. Acute effect of poly-γ-glutamic acid on calcium absorption in post-menopausal women. *Journal of the American College of Nutrition*, 26, 645-649.

[7] Yang, L.C., Wu, J.B., Ho, G.H., Yang, S.C., Huang, Y.P. and Lin, W.C., 2008. Effects of poly-γ-glutamic acid on calcium absorption in rats. *Bioscience, Biotechnology and Biochemistry*, 72, 3084-3090.

[8] Shih, I.L., Van, Y.T. and Sau, Y.Y., 2003. Antifreeze activities of poly (gamma-glutamic acid) produced by *Bacillus licheniformis*. *Biotechnology Letters*, 25, 1709-1712.

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

[10] Lee, C.Y. and Kuo, M.I., 2011. Effect of γ-polyglutamate on the rheological properties and microstructure of tofu. *Food Hydrocolloids*, 25, 1034-1040.

[11] Wang, F., Zhao J., Wei, X., Huo, F., Li, W., Hu, Q. and Liu, H., 2014. Adsorption of rare earths (III) by calcium alginate-poly glutamic acid hybrid gels. *Journal of Chemical Technology and Biotechnology*, 89, 969-977.

[12] Zheng. H., Gao, Z., Yin, J., Tang, X., Ji, X. and Huang, H., 2012. Harvesting of microalgae by flocculation with poly (γ-glutamic acid). *Bioresource Technology*, 112, 212-220.

[13] Shih, I.L. and Van Y.T., 2001. The production of poly-γ-glutamic acid from microorganisms and its various applications. *Bioresource Technology*, 79, 207-225.
[14] Buescher, J.M. and Margaritas, A., 2007. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. *Critical Review in Biotechnology*, 27, 1-19.
[15] Zhang, H., Zhu, J., Zhu, X., Cai, J., Zhang, A., Hong, Y., Huang, J., Huang, L. and Xu, Z., 2012. High-level exogenous glutamic acid-independent production of poly-(gamma-glutamic acid) with organic acid addition in a new isolated *Bacillus subtilis* C10. *Bioresource Technology*, 116, 241-246.
[16] Cao, M., Geng, W., Liu, L., Song, C., Xie, H., Guo, W., Jin, Y. and Wang, S., 2011. Glutamic acid independent production of poly-γ-glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of *pgs* BCA genes. *Bioresource Technology*, 102, 4251-4257.
[17] Ko, Y.H. and Gross, R.A., 1997. Effects of glucose and glycerol on γ-poly (glutamic acid) formation by *Bacillus licheniformis* ATCC 9945. *Biotechnology and Bioengineering*, 57, 430-437.
[18] Ueda, Y., Frindte, K., Knief, C., Ashrafuzzaman, M. and Frei, M., 2016. Effects of elevated tropospheric ozone concentration on the bacterial community in the phyllosphere and rhizoplane of rice. *PLoS One* 11, e0163178. https://doi.org/10.1371/journal.pone.0163178
[19] Zeng, W., Lin, Y., Qi, Z., He, Y., Wang, D., Chen, G. and Liang, Z., 2013. An integrated high-throughput strategy for rapid screening of poly (gamma-glutamic acid) -producing bacteria. *Applied Microbiology and Biotechnology*, 97, 2163-2172.
[20] Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Dymock, D. and Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology*, 64, 795-799.
[21] Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.
[22] Liu, J., He, D., Li, X-Z., Gao, S., Wu, H., Liu, W., Gao, X. and Zhou, T., 2010. γ-Polyglutamic acid (γ-PGA) produced by *Bacillus amyloliquefaciens* C06 promoting its colonization on fruit surface. *International Journal of Food Microbiology*, 142, 190-197.
[23] Yu, Y., Yan, F., Chen, Y., Jin, C., Guo, J.-H. and Chai, Y., 2016. Poly-γ-Glutamic acids contribute to biofilm formation and plant root colonization in selected environmental isolates of *Bacillus subtilis*. *Frontiers in Microbiology*, 7, 1811. doi: 10.3389/fmicb.2016.01811