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Full Length Article

Studies of a γ-Polyglutamic Acid-producing *Bacillus* Strain Isolated from Chinese Fermented Soybean Foods

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Abstract

In this study, we isolated a bacterium Dg5041 which can synthesis γ-polyglutamic acid (γ-PGA) from the Chinese natto-like fermented soybean food. This γ-PGA-producing strain was studied by phenotypic, phylogenetic, spectrophotometric and HPLC analyses. Based on 16S rRNA gene sequences, a phylogenetic tree was constructed and showed that isolate Dg5041 and *Bacillus subtilis* were in the same cluster. According to the phenotypic and phylogenetic outcomes of this report, the strain Dg5041 can be identified as *B. subtilis*. The supernatant of the fermentation broth after centrifugation was determined by CTAB method, and the yield of γ-PGA reached 18.04 g L^{-1}, reaching the higher level than reported in the literature. The γ-PGA fermentation product has a maximum absorption peak at 192 nm, indicating that the purified product is almost a single product, consistent with γ-PGA standard products. The HPLC chromatogram of hydrolyzed γ-PGA spectrum studies verified that the hydrolysate of γ-PGA fermentation product was glutamate. © 2019 Friends Science Publishers

Keywords: Bacillus; Fermented soybean foods; γ-polyglutamic acid; HPLC; Phylogeny; 16S rRNA gene sequences

Introduction

Poly-γ-glutamic acid (γ-PGA) is a biopolymer made from D-/L-glutamate units. These repeating units linked by amide bonds between α-amino and γ-carboxylic acid groups. This anionic polymer is biodegradable, water-soluble, non-immunogenic and non-toxic for humans and environment (Poo *et al*., 2010). A variety of functions of γ-PGA have been applied in medicine (Otani *et al*., 1996), foods, cosmetics, agriculture, water treatments, and other applications (Poo *et al*., 2010, Luo *et al*., 2016, Cao *et al*., 2018).

To date, most industrial production of γ-PGA relies exclusively on microbial fermentation from biomass (Lee *et al*., 2018). A number of *Bacillus* species and some other microorganisms are γ-PGA producer. The biosynthesized γ-PGA turns out to be a capsule component of the bacterial cell, acts as an energy source for bacteria, enables immune evasion, and lessens the salt concentration of the surrounding environment (Hanby and Rydon, 1946). Most industrial γ-PGA producers are gram-positive bacteria belonging to the genus *Bacillus*, including *B. subtilis*, *B. licheniformis*, *B. thermotolerant* and *B. anthracis* (Lee *et al*., 2018). Therefore, main studies of large-scale γ-PGA production aimed these bacterial growth conditions at higher-yield, manipulate enantiomeric composition, and adjust the molecular mass (Ashiuchi *et al*., 2001). However, while much laborious manipulations have been attempted on industrial γ-PGA production, only a few has been achieved. The developments of γ-PGA production are restricted by their low yield and high cost of production due to the mechanism of γ-PGA biosynthesis is still need further investigations, the metabolic pathway of γ-PGA is complicated and the diverse regulation mode in the cell (Scoffone *et al*., 2013, Hsueh *et al*., 2017, Lee *et al*., 2018). Different main strategies are being improved to increase the production efficiency of bacterial γ-PGA (Luo *et al*., 2016). These studies include screening for novel γ-PGA-producing strains and optimizing growth conditions to increase yield.

In the present work, a bacterium Dg5041 was isolated and found producing γ-PGA from natto-like fermented soybean food in China. Phenotypic classification and 16S rRNA gene sequencing were used to identify the isolate Dg5041. To investigate the phylogeny of this γ-PGA-producer, a phylogenetic tree was constructed according to partial 16S rRNA gene sequences of isolate Dg5041 and *Bacillus* species. Identification of fermentation products of the strain Dg5041 was also studied.

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Materials and Methods

Experimental Material and Cultivation of Strain

The γ-PGA standard sample was obtained from Shandong Freda Pharmaceutical Group Co., Ltd (Shandong, China). The analytical grade of chemical reagents were purchased from Kelong Co., Ltd. (China). The strain Dg5041 was isolated from natto-like fermented soybean food obtained at local markets in China. The strain Dg5041 was cultured on the agar medium at 30°C for 2 days. Then, a loop of bacterial cells was inoculated into the seed medium (L^{-1}): 10 g peptone, 5 g beef extract and 10 g NaCl at pH 7. The seed culture was added (2% inoculum ratio) into a 250 mL Erlenmeyer flask containing 50 mL of fermentation medium: 10 g peptone, 25 g glucose, 30 g sodium L-glutamate, 1 g Na_2HPO_4, 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ at pH 7 per L. The seed culture and fermentation culture were incubated at 37°C for 24 and 48 h on a shaker (DHP-9162, China) with shaking at 140 rpm.

Extraction of γ-PGA

The fermentation culture was centrifuged at 20,000 rpm for 30 min. The collected supernatant was then adjusted to pH 3 with 1 M HCl and mixed with three volumes of ethanol. The ethanol precipitate was obtained by centrifugation and then rinsed with ethanol. After drying in vacuum, the resuspended precipitate was dialyzed against deionized water. The dialyzed solution sample was manipulated to quantify γ-PGA (Ito *et al*., 1996).

Phenotypic Characterization

The morphology, size, movement and spore production of isolate Dg5041 were examined with optical microscope. Based on Bergey's Manual of Systematic Bacteriology [Bergey's Manual of Systematic Bacteriology (1986). Baltimore: William and Wilkins], the biochemical tests of isolate Dg5041 was used for taxonomic identification. Strain Dg5041 was Gram-stained and was subjected to a series of test including: production of catalase; anaerobic growth; Voges–Proskauer reaction; nitrate reduction; fermentation of sugars; gas production from glucose; growth at different NaCl concentrations, pH and temperature ranges; citrate utilization; degradation of starch, gelatin, casein, tyrosine, and urea.

Partial 16S rRNA Gene Sequencing

The partial 16S rRNA gene sequence of strain Dg5041 was amplified by PCR using universal primers;

forward 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGTGATCCAGCCGCA-3' (Weisburg *et al*., 1991). The expected 1,429 bp product was purified with a PCR purification kit (Qiagen, Germany) and sequenced. The sequencing of this partial 16S rRNA fragment was performed via ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in both directions with universal primers used for amplification.

Phylogenetic Analysis

Partial 16S rDNA sequence of strain Dg5041 and homologous sequences from *Bacillus* related strains retrieved from GenBank were aligned using Clustal W (Thompson *et al*., 1994). The MEGA 7.0 was used to analysis the resulting alignment and construct the phylogenetic tree with neighbor-joining (NJ) method (Saitou and Nei, 1987). Sequence divergences among the strains were quantified using Kimura-2-paramater distance model. A total of 1,000 bootstrap replication were computed for assessment of the tree topology (Kumar *et al*., 2016).

CTAB Assay of Fermentation Product Samples

The concentration of fermentation product was determined by the cetyltrimethylammonium bromide (CTAB) spectrophotometric method (de Cesaro *et al.*, 2014). The 2 mL of γ-PGA fermentation broth or γ-PGA standard sample were added to equal volume of CTAB solution at room temperature for 3 min. The turbidity of micelle-like complex measured with the spectrophotometer at 400 nm and the absorbance of the mixture was determined.

Spectrophotometric Analytical Procedure

UV absorbance value of γ-PGA standard sample or fermentation product sample were read at 216 nm using UV/Vis spectrophotometer (Zeng *et al*., 2012). Ultraviolet scanning: The 0.5 g L^{-1} γ-PGA standard sample and fermentation product were scanned in a UV/Vis spectrophotometer at wavelength range of 190-400 nm.

HPLC Analysis of γ-PGA and Glutamate

The sample contained γ-PGA was hydrolyzed in 6 M HCl at 90°C for 24 h, and then the hydrolysate was neutralized with 6 M NaOH. The resulting hydrolysate and 0.2 g L^{-1} glutamate standard sample are subjected to Shimadzu HPLC using an eluted with acetonitrile: H_2O = 1:1 at a flow rate of 1.0 mL min^{-1}. The γ-PGA samples are subjected to Shimadzu HPLC using an ODSHypersil C18 column (250 mm × 4.6 mm) and eluted with 0.015 M NaH_2PO_4-Na_2HPO_4 (pH 6.98): methanol = 95:5 at a flow rate of 1.0 mL min^{-1}. The absorbance of γ-PGA or glutamate samples were monitored at 210 or 200 nm with SPD-10A UV/Vis detector, respectively.

Results

Identification of Strain Dg5041

Morphologically Dg5041 bacterium cells were arranged in rod-shaped singles or chains that was gram-positive and

0.7~0.8 μm × 2.0~3.0 μm in size (Fig. 1A). Spore-forming Dg5041 strain could grow under anaerobic condition and used their flagella for a swarming motility. The colonies of this strain appeared cream color, rough texture, and circular with undulate margins on nutrient agar (Fig. 1B). Dg5041 bacterium produce flocculent growth in liquid media (Fig. 1C). Based on these phenotypic characterizations, strain Dg5041 was considered as *Bacillus* species.

According the test results of Table 1, strain Dg5041 was able to hydrolyze starch, gelatin, casein and tyrosine and reduced nitrate. In addition, this strain had positive reactions for catalase, Voges-Proskauer test, and nitrate reduction and negative reactions for lecithinase, oxidase and urease. Strain Dg5041 utilized a glucose, arabinose, xylose, maltose, and mannitol. Gas production from glucose was found. The inhibition of NaCl concentration was 10%, and the cultivable pH values were 5.7 and 6.8. With these biochemical features mentioned above, strain Dg5041 was similar as *B. subtilis* (Yang *et al.,* 2015). In addition, when testing the enzyme stability was tested at various temperatures (30°C, 40°C, 50°C and 60°C); the proteases of strain Dg5041 were affected at 60°C. After close observation and multiple physiological and biochemical tests, it was suggested that strain Dg5041 could be identified as *B. subtilis*.

A 1,429-bp PCR fragment from the partial 16S rRNA gene of strain Dg5041 was evaluated by phylogenetic analysis. Based on the evolutionary distance analyzed from 1,000 replicates, the construction of the phylogenetic tree with neighbor-joining (NJ) was generated. The phylogenetic tree of strain Dg5041 and *Bacillus* related strains showed 3 distinct clusters (Fig. 2), which were divided on a scale of 0.02 nucleotide substitution. The results showed that strain Dg5041 and previously validated *Bacillus* species belong to different clusters, although they had high 16S rRNA gene sequence similarities. Based on partial 16S r rRNA gene sequences, the phylogenetic tree showed that isolate Dg5041 and *B. subtilis* were in the same cluster. These findings suggested that strain Dg5041 was classified as *B. subtilis*.

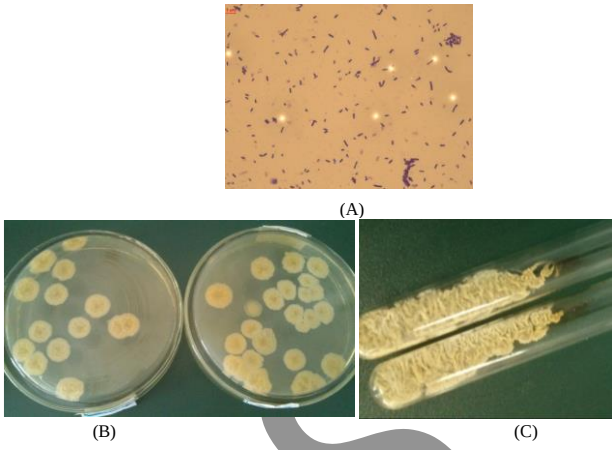

Fig. 1: The Gram-staining morphology (A), colonial morphology (B) and cell growth morphology (C) of strain Dg5041

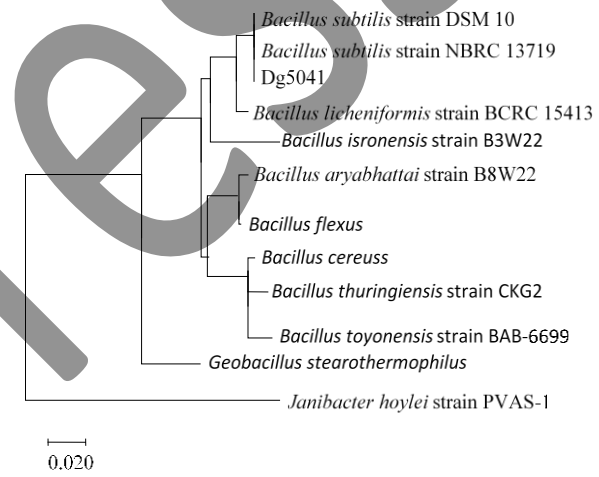

Fig. 2: Evolutionary relationships of the analyzed strain Dg5041 with its closest known taxa. The evolutionary history was inferred using the Neighbor-Joining method. The tree was constructed based on the evolutionary distance calculated from 16S rRNA gene partial sequences using Kimura 2-parameter method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances. Bar (0.002) indicates substitutions per nucleotide position

Identification of Fermentation Products of Strain Dg5041

The CTAB were convenient and highly specific to γ-PGA and form a micelle-like CTAB/γ-PGA complex. This assay was allowed to determine the concentration of γ-PGA from microbial broth directly. The turbidity-based calibration curve provided a useful linearity on the γ-PGA concentration range of 0.2–2 μg mL^{-1}, in spite of γ-PGA stereochemistry (Ashiuchi, 2011). Therefore, CTAB turbidimetric method was used to detect the concentration of γ-PGA in fermentation culture of strain Dg5041. The yield of γ-PGA reached 18.04 g L^{-1}, reaching the higher level reported in the literature.

The γ-PGA standard sample and fermentation product were placed in the UV-Vis spectrophotometer, the distilled water was used as the baseline. The wavelength 190~400 nm was scanned; the UV scan diagram of γ-PGA standard sample and the fermentation product were shown in Fig. 3A and 3B, respectively. The γ-PGA fermentation product had a maximum absorption peak at 192 nm, indicating that the purified product was almost a single product, consistent with γ-PGA standard products.

With acetonitrile as the flow phase, the HPLC chromatogram of the L-glutamate standard solution and the hydrolyzed sample of γ-PGA fermentation product were shown in Fig. 4A, 4B and 4C in the condition that the flow rate is 0.5 mL min^{-1}, the sample quantity is 10 μL and the wavelength was 250 nm. Comparing Fig. 4A and 4B,

Table 1: Biochemical test and identification of strain Dg5041

Properties	Strains	
	Bacillus subtilis (Yang *et al*., 2015)	Dg5401
Catalase	＋	＋
Oxidase	Variable	－
Anaerobic condition	facultative aerobe	＋
Voges-Proskauer reaction	+	＋
Nitrate Reduction	＋	＋
Hydrolysis of Starch	＋	＋
Hydrolysis of Gelatin	＋	＋
Fermentation test of sugar and alcohol	D-Glucose(+)	D-Glucose(＋)
	L-Arabinose(+)	L-Arabinose(＋)
	D-Xylose(+)	D-Xylose(＋)
	D-Mannitol (＋)	D-Mannitol (＋)
	Gas from Glucose(-)	Gas from Glucose(－)
L-Arginine dehydrolase L-Lysin decarboxylase L-Ornithine decarboxylase	L-Arginine dehydrolase (-)	L-Arginine dehydrolase (－)
	L-Lysin decarboxylase(-)	L-Lysin decarboxylase(－)
	L-Ornithine decarboxylase(-)	L-Ornithine decarboxylase(－)
Tolerance of NaCl	The minimal inhibitory concentration of NaCl was 7%	2%NaCl(＋), 5%NaCl(＋), 7%NaCl(＋), 10%NaCl(－)
Citrate utilization test	＋	＋
Tolerance of pH in nutrient broth	5-9	pH6.8(＋), pH 5.7(＋)
Lecithinase test	－	－
Indole production	－	－
the enzyme stability was tested at different temperatures	most affected at 60 °C	30°C (＋), 40°C (＋), 50°C (＋), 60°C (－)
Hydrolysis of casein	＋	＋
Hydrolysis of tyrosine	-	－
Urease	－	－

the peak time of the hydrolyzed γ-PGA fermentation product was 5.58 min, which was consistent with the peak time of the glutamate standard, indicating that this hydrolysis product was glutamate. Furthermore, HPLC analysis was used to determine hydrolysis of the γ-PGA standard sample under the same chromatographic conditions, and the chromatogram was obtained as shown in Fig. 4C.

Discussion

In nature, many functions of γ-PGA depended on the microorganism and their environment. One of the γ-PGA utilities of *Bacillus* sp. gave relative viscosity and stickiness in many Asian fermented soybean foods (Chettri *et al*., 2016; Tamang *et al.*, 2016). In the present work, the γ-PGA producer Dg5041 was obtained from Chinese natto-like soybean food. The morphological characterizations of strain Dg5041 were similar to *Bacillus* species. Biochemical properties of strain Dg5041 are the same as *B. subtilis* (Yang *et al*., 2015). Besides, the phenotypic characterization of strain Dg5041, it was also identified by partial 16S rRNA gene sequencing and phylogenetic evaluation. Strain Dg5041 was constructed a monophyletic clade with *B. subtilis* DSM 10 and NBRC 13719. The *B. subtilis* and *B. licheniformis* strain BCRC 15413 were relatively closed and belong to γ-PGA-producing *Bacillus* species. Based on the consistency between the results of the biochemical characterization and the 16S rRNA gene sequence analysis, strain Dg5041 was recognized as *B. subtilis*.

The UV scan diagram of γ-PGA standard sample or fermentation product had a maximum absorption peak at 192 nm. These consistent absorption peaks revealed that our purified fermentation product was almost γ-PGA product. This γ-PGA absorption peak was different from those of proteins at 260 nm and 280 nm. The UV scan diagram of this study confirmed that the fermentation product was γ-PGA which was not a typical protein and did not contain aromatic amino acids and nucleic acids. The previous studies indicated that UV absorption spectrum of γ-PGA usually displayed a maximum absorption peak at 216 nm. These spectral variations were probably caused by the conformation of γ-PGA in aqueous solution of the different experimental conditions (Zeng *et al*., 2012). We suggested that the absorbance was measured at 192 nm could provide another simple and accurate alternative method for γ-PGA assay with UV spectrophotometer. Besides, the HPLC chromatogram of hydrolyzed γ-PGA standard and fermentation samples had a maximum absorption peak, and the peak time of this absorption peak was consistent with that of glutamate standard sample, which indicated that the hydrolysis product of our fermentation sample was mainly glutamate.

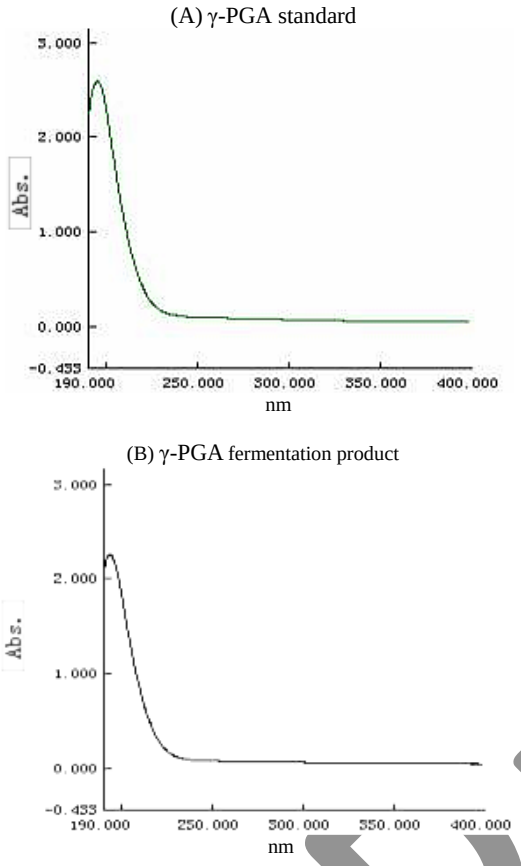

Fig. 3: The UV scan diagram of the γ-PGA standard (A) and fermentation product (B)

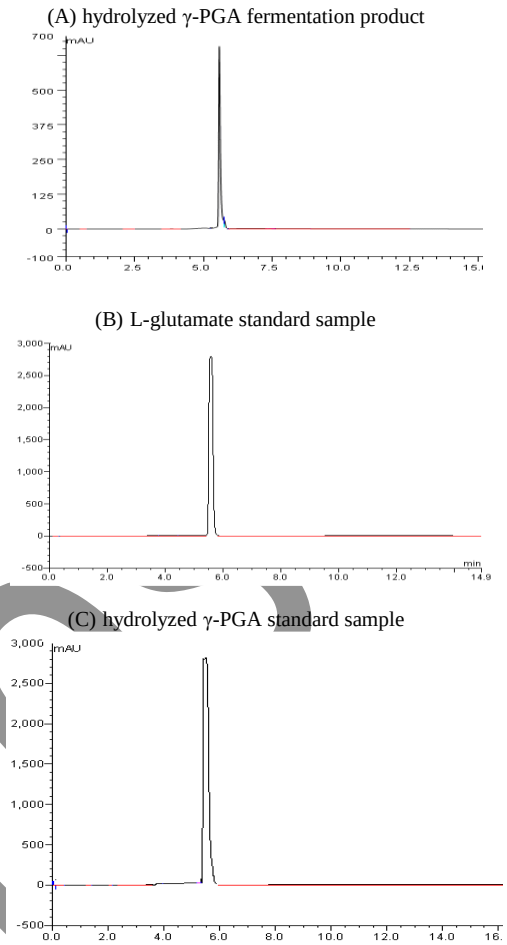

Fig. 4 (A-C): HPLC chromatogram of hydrolyzed γ-PGA fermentation product, L-glutamate standard sample, and hydrolyzed γ-PGA standard sample

Current studies reported that DL-γ-PGA, a mixed component, existed in extracellular mucilage for some strains of *B. subtilis*. About 50-80% D-glutamate and 20-50% L-glutamate was presented in mucilage of *B. subtilis* from for Natto fermented soybean food (Ashiuchi *et al*., 1998). However, about 60-70% D-glutamate and 30-40% L-glutamate presented in mucilage of *B. subtilis* from traditional Korean fermented soybean seasoning (Ashiuchi *et al*., 2001; Poo *et al*., 2010). Recent chiral HPLC study reveals these typical chromatograms, and also suggest that the majority of DL-γ-PGA from *B. subtilis* is D-glutamyl residues (Ashiuchi, 2011). Comparing with previous studies, the hydrolysate of γ-PGA fermentation product of strain Dg5041 was composed of glutamate. Besides, the HPLC chromatogram of hydrolyzed fermentation product showed the overlap of the main peak and the slightly shoulder peak. The main peak could be recognized as D-glutamate according to above-mentioned studies about DL-γ-PGA components in mucilage of *B. subtilis*. Besides, the UV scan diagram of hydrolyzed γ-PGA fermentation product was the same as that of glutamate standard sample. All these findings of this study suggested the fermentation product of *B. subtilis* Dg5041 is γ-PGA.

Conclusion

A bacterium strain Dg5041 was successfully isolated from Chinese fermented soybean foods. This Gram-positive, rod-shaped, and spore-forming bacterium was recognized as *B. subtilis* by using phenotypic and phylogenetic studies. The present findings revealed that *B. subtilis* Dg5041 produced DL-γ-PGA in the culture medium. the majority of γ-PGA from Dg5041 was D-glutamyl residues. This native γ-PGA producer can yield approximately 18.04 g L^{-1} of γ-PGA via fermentation processes. Comparing with other *Bacillus* species, strain Dg5041 has the potential to produce a high-level yield of γ-PGA. Further investigation is needed to optimize the culture condition for *B. subtilis* Dg5041 in order to enhance productivity of γ-PGA.

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