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Isolation of Halotolerant *Bacillus licheniformis* WX-02 and Regulatory Effects of Sodium Chloride on Yield and Molecular Sizes of Poly-γ-Glutamic Acid

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Abstract A poly-γ-glutamic acid (γ-PGA) productive strain, halotolerant bacterium WX-02 was isolated from the saline soil of China (Yingcheng). By physiological, biochemical, and 16S rDNA sequence analysis methods, the strain was identified as *Bacillus licheniformis*. The effect of NaCl concentration on γ-PGA production by WX-02 was investigated in modified E (ME) medium. It was found that the γ-PGA production was salt-inducible, and the highest volumetric yield of γ-PGA (13.86 g/l) was attained with 8% of NaCl. It was also observed that the molecular size of γ-PGA decreased when the NaCl concentration increased. This was the first report of isolation and identification of a γ-PGA productive strain, halotolerant *B. licheniformis*. This study provided a simple strategy for controlling the yield and molecular size of γ-PGA by WX-02.

Keywords *Bacillus licheniformis* · Halotolerant · Poly-γ-glutamic acid · Molecular size · Yield

Introduction

The poly-γ-glutamic acid (γ-PGA) is a viscous slime biopolymer containing D- and L-glutamate residues produced mainly by several species of *Bacillus*. It is water-soluble, biodegradable, edible, and nontoxic towards human and environment. Multifarious applications of γ-PGA have been developed in food, cosmetics, medicinal industries, and agriculture [1, 2].

Several researchers found that the culture broth became highly viscous along with the production of γ-PGA due to the inherent property of this biopolymer. The increased broth viscosity was likely to result in O_2 limitation by decreasing the volumetric O_2 transfer, thus would lead to a decrease in γ-PGA yields [3, 4]. The broth viscosity could be decreased by addition of NaCl to the medium [5, 6]. However, most of the *Bacillus subtilis* strains from

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Natto starters cannot grow sufficiently under high saline conditions. The yields of γ-PGA were obviously reduced in medium containing more than 3% of NaCl [6]. Therefore, halotolerant *B. subtilis* (*chungkookjang*) was isolated from a highly salty Korean soybean paste to overcome these problems. Interestingly, *B. subtilis* (*chungkookjang*) could grow in the medium containing 25% of NaCl, and the broth apparent viscosity decreased with the increase in salt concentration, but the γ-PGA volumetric yield decreased by 29.5% when the NaCl concentration increased from 0.5% to 5% [6]; thus, it is more attractive to understand the regulatory effects of NaCl on γ-PGA production. *B. subtilis* and *Bacillus licheniformis* are the most prominent bacteria producing γ-PGA [4–10]. To the best of our knowledge, the isolation of halotolerant *B. licheniformis* as a γ-PGA producer has not been reported.

In the present work, we isolated and identified halotolerant *B. licheniformis* WX-02 producing γ-PGA from the saline soil of China (Yingcheng). Furthermore, some advantageous characteristics of the strain such as salt-inducible γ-PGA production and the γ-PGA synthesized with lower molecular sizes under higher concentrations of NaCl were systematically illustrated.

Materials and Methods

Materials

KH_2PO_4 and methanol were of the high-performance liquid chromatography (HPLC) grade, and the rest of the chemicals were of the AR grade. All the chemicals and culture medium components were obtained from Sinopharm Chemical Reagent Co., LtdS. China. The saline soil samples were collected from Yingcheng saline of China.

Isolation of the Bacterium

The soil samples were suspended in distilled water and boiled for 10 min, then the suspension was spread out onto the modified Luria–Bertani (LB) solid medium (per liter: tryptone 10 g, yeast extract 5 g, NaCl 100 g, agar 15 g, pH 7.2). The plates were incubated at 37 °C for 72 h. Highly mucoid bacteria colonies were picked up and were inoculated into 50 ml 6% NaCl-containing modified E (ME) medium (per liter: L-glutamic acid 20 g, glucose 20 g, citric acid 12 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeCl_3{\cdot}6H_2O$ 0.04 g, $CaCl_2{\cdot}2H_2O$ 0.15 g, $MnSO_4{\cdot}H_2O$ 0.104 g, pH 6.5), its ingredients were the same as E medium's, except substitution glucose for glycerol [11], then cultured at 37 °C and 200 rpm for 4 days.

Identification of the Bacterium

The strain was identified by physiological, biochemical, and 16S rDNA sequence analysis methods. Physiological and biochemical identification was performed according to Bergey's Manual of Systematic Bacteriology [12]. The 16S rDNA sequence was determined as described by Li et al. [13]. Genomic DNA was prepared by the reported method [14]. The 16S rDNA was amplified using universal primers, 16sf95 (TGAC-GAGTGGCGGACGGGTG) and 16sr394 (CCATGGTGTGACGGGCGGTGTG). The amplified polymerase chain reaction (PCR) was performed at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1.5 min, and a final extension step of 72 °C for 10 min. The PCR product was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). The nucleotide sequences were determined by the chain

termination method on an ABI Prism 370 automatic sequencer (Applied Biosystems, Foster City, CA, USA) with 16sf95 and 16sr394 primers. The sequences of closely related strains were retrieved from the GenBank and Ribosomal Database Project libraries [15]. Retrieved sequences were aligned by CLUSTAL W program and manually edited. A phylogenetic tree was constructed with MEGA 4.0 [16]. Bootstrap confidence values for branching nodes were inferred by the generation of 1,000 resampling trees.

Culture Conditions

B. licheniformis WX-02 cells were inoculated into 10 ml LB medium (per liter: tryptone 10 g, yeast extract 5 g , NaCl 10 g, pH 7.2). After cultivation at 37 °C and 200 rpm for 10 h in a shaking incubator, it was used as a seed culture. For flask culture, the seed culture broth (150 μl) was transferred into 250-ml flask containing 50 ml ME medium and cultured at 37 °C and 200 rpm for 4 days.

Determination of Biomass

To monitor the cell growth, aliquots of samples were drawn at preset time intervals. Cells were separated from the culture broth by centrifugation for 10 min at 13,700×*g* and 4 °C using a Himac CR21G centrifuge (Hitachi, Tokyo, Japan) with R20V rotor. Dry cell weight (DCW) was determined by drying washed precipitation at 80 °C to a constant weight.

Purification of γ-PGA

The recovery and purification of γ-PGA was carried out by the method reported by Do et al. [17]. Cells were separated by centrifugation for 15 min at 9,540×*g* using a Himac CR21G centrifuge (Hitachi) with R20V rotor after lowering the pH value of culture broth to 3. The supernatant was neutralized with NaOH solution, poured into four volumes of ethanol, then mixed and kept for 12 h at 4 °C, and centrifuged at for 10 min at 9,540×*g*. The pellet was dissolved in distilled water and any insoluble contaminants were removed by centrifugation at 13,700×*g* for 10 min. The aqueous crude γ-PGA solution was purified by dialysis (molecular weight cutoff, 3,500) in distilled water for 24 h and then was lyophilized to prepare pure γ-PGA.

Determination of γ-PGA

γ-PGA was analyzed according to reported method [18, 19]. The pure γ-PGA was hydrolyzed in 6 mol/l HCl at 110 °C for 24 h. The hydrolysate was neutralized with 6 mol/l NaOH, and the quantity of glutamate in the hydrolysate was determined by Agilent 1100 HPLC system with a Lichrospher C_{18} column (25 cm×4.6 mm, Agilent Technologies Corporate, USA). Ten millimoles per liter KH_2PO_4 plus 5.0% methanol (*v*/*v*, pH 2.5) was used as mobile phase at a flow rate of 1.0 ml/min. Glutamate was confirmed as the sole product in the hydrolysate by retention time using the authentic standards, and the amount of γ-PGA was given as the total glutamate.

Molecular Size Estimation of γ-PGA

The molecular size of γ-PGA was estimated by agarose gel electrophoresis [20]. Portions (10 μl) of pure γ-PGA were resolved by electrophoresis at 6 V/cm for 30 min on 1.0%

agarose gels using TAE running buffer [40 mmol/l Tris-hydroxylaminomethane, 1 mmol/l EDTA, 0.14% (*v*/*v*) acetic acid]. The γ-PGA samples on the gel were visualized by staining with methylene blue [0.23% (*w*/*v*) methylene blue, 23% (*v*/*v*) ethanol, 0.008% (*w*/*v*) KOH] for 10 min, followed by destaining with water.

Determination of Broth Apparent Viscosity

The broth apparent viscosity was measured using the rotational viscometer NDJ-1 (Shanghai Hengping Science Instruments Co., China) when the maximum γ-PGA concentration was attained in the ME medium containing various concentrations of NaCl.

Statistical Analyses

Each experiment was carried out at least in triplicates, and three parallel experiments were performed for each experiment. One-way analysis of variance and *t* test were used to interpret the difference in means at the 95% confidence level. All statistical analyses were performed using Statistica 6.0 software package.

Results and Discussion

Isolation of the γ-PGA-Producing Strain with Halotolerance

Three salt-tolerant, γ-PGA high productive strains were isolated from saline soil collected from China (Yingcheng). They produced more than 10 g/l γ-PGA, which was as high as those of typical *B. subtilis* γ-PGA overproducers [18]. The best producer, strain WX-02, was selected and used for further investigation.

Identification of the Bacterium

Table 1 showed the taxonomical characteristics of the strain WX-02. The colony micrograph of WX-02 was changeable in the solid medium. The colony was rough, folding with the hollow center and irregular edge on the LB solid medium. In contrast, the colony was highly mucous, with smooth surface and regular edge on the LB solid medium containing 10% of NaCl.

About 1,263-bp sequence of the 16S rDNA determined in this study was submitted to the GeneBank database (EU564336). This sequence was compared to 16S rDNA sequences of related strains. It showed similarity of 99% to that of *B. licheniformis* DSM 13 (X68416). The subsequently constructed phylogenetic tree revealed the isolate clusters with representatives of the closely related organisms (Fig. 1). From these results, the strain was classified as *B. licheniformis*. The strain was deposited at China Center for Type Culture Collection (CCTCC M208065).

Effect of NaCl Concentration on the Production of γ-PGA

The effect of NaCl concentration on DCW, γ-PGA volumetric yield, and γ-PGA productivity per unit of biomass ($Y_{P/X}$) at its maximum γ-PGA concentration was investigated in the ME medium containing various concentrations of NaCl. As shown in Table 2, though the cell growth decreased, the γ-PGA yield was enhanced when the NaCl

Table 1 Taxonomical characteristics of the strain WX-02.

Property	Characterization	Property	Characterization
Gram stain	+	Acid from D-xylose	+
Shape	Rod,2–3×0.8–1 μm	Acid from mannitol	+
Sporulation	+	Gas from glucose	−
Motility	+	Degradation of tyrosine	−
Nitrate reduction	+	Deamination of phenylalanine	−
Anaerobic growth	+	Urease production	+
Starch hydrolysis	+	Indole formation	+
Casein hydrolysis	+	Catalase test	+
Gelatin hydrolysis	+	Growth in NaCl (12%)	+
VP test	+	Growth on pH 8.0	+
Citrate utilization	+	Growth on pH 5.7	+
Propionate utilization	+	Growth at 30 °C	+
Acid from D-glucose	+	Growth at 50 °C	+
Acid from L-arabinose	+	Growth at 55 °C	−

+: positive

−: negative

concentrations increased. The highest yield of 13.86 g/l was obtained when the NaCl concentration was 8%, increasing by 5.28 times as compared with that of the control without NaCl. To the best of our knowledge, there was no report on the halotolerant *B. licheniformis* for γ-PGA production, so WX-02 was the first halotolerant γ-PGA productive strain in *B. licheniformis*. Though the halotolerant *B. subtilis* (*chungkookjang*) could grow in the medium containing 25% of NaCl, γ-PGA volumetric yield decreased by 29.5% when the NaCl concentration increased from 0.5% to 5% [6], suggesting that NaCl had a negative effect on the γ-PGA production in halotolerant *B. subtilis* (*chungkookjang*).

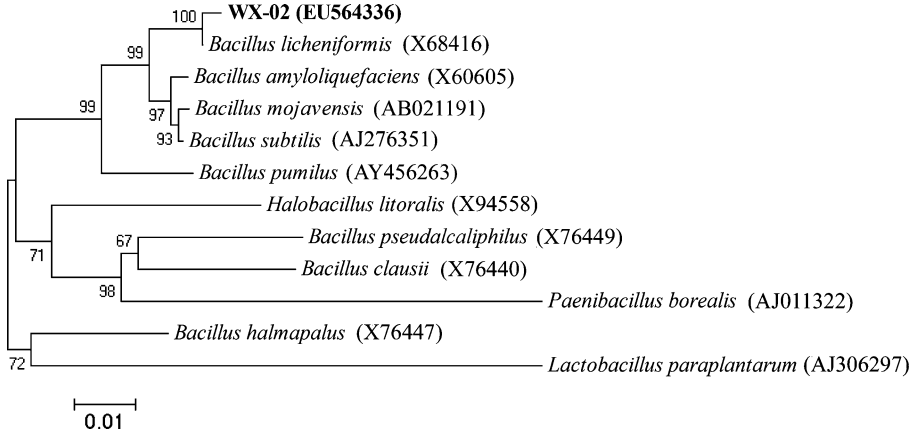

Fig. 1 Phylogenetic tree based on 16S rDNA sequences showing the position of strain WX-02 (accession number EU564336) among its closely related organisms. *Numbers in parentheses* are accession numbers of published sequences. Bootstrap values were based on 1,000 replicates. The tree was constructed by the neighbor-joining method. The *scale bar* represents 0.01 nucleotide substitution per position

Table 2 The effect of NaCl concentration on DCW, γ-PGA yield, and γ-PGA productivity per unit of biomass ($Y_{P/X}$) in batch cultures.

NaCl concentration (%)	DCW (g/l)	γ-PGA yield (g/l)	$Y_{P/X}$ (g/g)
0	3.42±0.22	2.16±0.09	0.61±0.03
2	3.3±0.15	2.93±0.23	0.83±0.08
4	3.21±0.13	5.20±0.14	1.64±0.04
6	3.04±0.16	11.20±0.27	3.85±0.02
8	2.82±0.15	13.56±0.24	4.81±0.01
10	1.79±0.05	12.49±0.22	6.69±0.11

By addition of different NaCl concentrations (0%, 2%, 4%, 6%, 8%, 10%) to the ME medium for flask cultures, the DCW and γ-PGA yield was measured, respectively, when the maximum γ-PGA concentration was attained. $Y_{P/X}$ = g γ-PGA produced per gram of cells. The results were the average of three separate fermentations.

Therefore, the NaCl effects on γ-PGA production were different between *B. licheniformis* WX-02 and *B. subtilis* (*chungkookjang*). We concluded that *B. licheniformis* WX-02 had the potential to address the limitations of high saline conditions and hence exhibited industrial applicability as a supplier of γ-PGA.

Figure 2 showed that the broth apparent viscosity at its maximum γ-PGA yield increased as NaCl concentration increased to 8%. This suggested that the NaCl effect, decreasing apparent viscosity of broth and increasing volumetric O_2 transfer, was not one key influencing factor for γ-PGA in our trial condition. These results showed that there were other key factors of the NaCl effect. According to the model of the inducement of γ-PGA production in *B. subtilis* strain RO-FF-1 reported by Stanley and Lazazzera [21], the two-component regulators *ComPA* and *DegSU* activated the transcription of *degQ*, which activated the transcription of the *ywsC* operon responsible for γ-PGA biosynthesis.

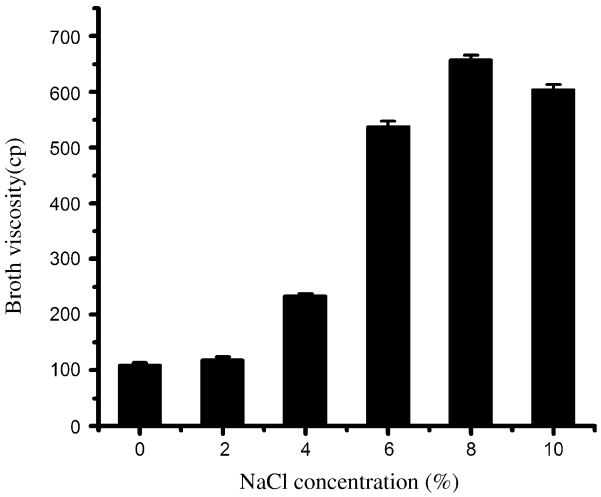

Fig. 2 Effect of NaCl concentration on the apparent viscosity of the broth. By addition of different NaCl concentrations (0%, 2%, 4%, 6%, 8%, and 10%) to the ME medium for flask cultures, the broth apparent viscosity was measured, respectively, when the maximum γ-PGA concentration was attained. The results were the average of three separate fermentations

Interestingly, high salinity could activate the transcription of *DegSU* in *B. subtilis* QB4256 [22]. Consequently, it was possible that high salinity activated transcription of the *ywsC* operon finally promoted the γ-PGA production. This might be the main reason of the NaCl effects. These results suggested that the γ-PGA production from *B. licheniformis* WX-02 was salt-inducible.

The γ-PGA productivity per unit of biomass ($Y_{P/X}$) was enhanced as the NaCl concentration increased, which suggested that the γ-PGA was synthesized to increase the survival of *B. licheniformis* WX-02 when exposed to high osmotic stress. γ-PGA might support the hydration status of *B. licheniformis* under strong dehydrating conditions based on its strong water-binding capacity [19]. It was suggested that the γ-PGA from *B. licheniformis* WX-02 might possess extremolyte-like functionality [23].

Effect of NaCl Concentration on the Molecular Sizes of γ-PGA

Figure 3 showed the effect of NaCl concentration on the molecular sizes of γ-PGA. The molecular sizes of γ-PGA decreased when the NaCl concentrations increased. Different

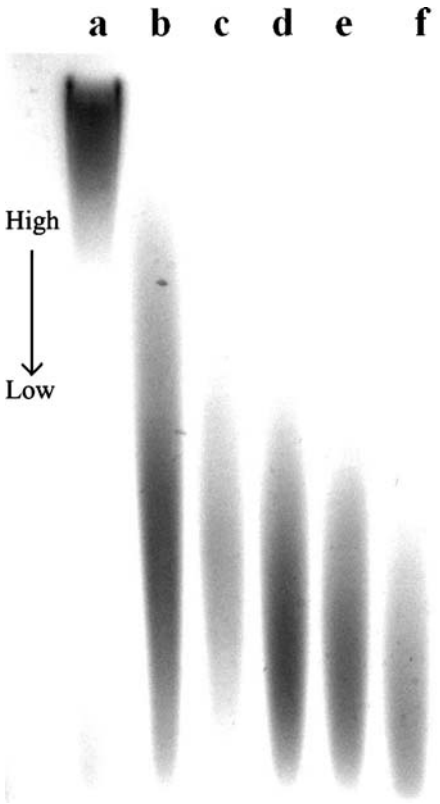

Fig. 3 Effect of NaCl concentration on the molecular sizes of γ-PGA. Using ME medium containing different NaCl concentrations (0%, 2%, 4%, 6%, 8%, and 10%), when the maximum γ-PGA concentration was attained, γ-PGA was purified, respectively. Then, γ-PGA was subjected to agarose gel electrophoresis and visualized by methylene blue staining. Each band corresponded to the γ-PGA accumulated in the medium in the absence (*lane a*) or presence of NaCl at 2% (*lane b*), 4% (*lane c*), 6% (*lane d*), 8% (*lane e*), and 10% (*lane f*). The *arrow* indicated the direction of electrophoresis

molecular sizes of γ-PGA were required for different purposes. It was significant to control synthesis of γ-PGA with different molecular sizes for its application [24]. The present work provided a simple method for producing γ-PGA with different molecular sizes. The effects of NaCl on γ-PGA molecular sizes were different among *Bacillus* species [3, 6, 18]. It was still unclear how the NaCl resulted in such a variation in γ-PGA molecular sizes among different *Bacillus* species. The present study emphasized the effect of NaCl on γ-PGA molecular sizes and provided a unique model organism for further study on the regulation of γ-PGA molecular sizes by NaCl.

Conclusion

The γ-PGA production from *B. licheniformis* WX-02 was salt-inducible. The maximum volumetric yield was attained when the NaCl concentration was 8%, and the γ-PGA molecular sizes decreased as NaCl concentration increased in the ME medium. These results indicated that WX-02 was the first halotolerant *B. licheniformis* for γ-PGA production, as well as a valuable strain for regulation of the yields and molecular sizes of γ-PGA by NaCl.

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