CELLULASE OF ENDOPHYTIC *BACILLUS* SP. FROM GLYCYRRHIZA URALENSIS F. AND ITS APPLICATION FOR EXTRACTION OF GLYCYRRHIZIC ACID

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Glycyrrhizic acid is the main component of the medicinal plant *Glycyrrhiza uralensis* Fisch. It is widely used as a sweetener and an effective active ingredient with multiple physiological functions. Endophytes are microorganisms that coexist with plants and can produce cellulase. This cellulase enzyme can be used to overcome dissolution barriers of plant active ingredients by degrading plant cell wall. In the present study, a cellulase-producing strain with high cellulase activity was isolated from fresh *Glycyrrhiza uralensis* Fisch, and identified using the Congo red staining method and the DNS method. Glycyrrhizic acid yield was determined by the HPLC method. A highly reactive cellulase-producing strain, with a high extraction capacity of glycyrrhizic acid, was obtained. The strain was named GG-3, and bioinformatic analysis showed that it was a *Bacillus* sp. Findings obtained after optimization of the enzyme production and glycyrrhizic acid extraction process showed that glycyrrhizic acid yield increased by 32.52% and 31.35% after extraction with GG-3 enzyme, compared with the use of the traditional extraction method and commercial cellulase extraction method, respectively.

Keywords: cellulase, endophytes, glycyrrhizic acid

INTRODUCTION

Licorice refers to the dried roots of the legume *Glycyrrhiza uralensis* Fisch.[1] Licorice is used for medicinal purposes in China, as reported in the "Shen Nong's Materia Medica". In addition, licorice has been used in Europe and Asia for more than four thousand years.[2-5] *Glycyrrhiza uralensis* is widely grown throughout Europe and East Asia,[6] and commonly used as a food condiment. Licorice is included in pharmacopoeias of East Asia and some European countries,[7] being the oldest widely used herbal medicine worldwide.[8] Licorice is frequently used in clinical medications in China, and nine out of ten prescriptions contain licorice. It is used to treat skin infections, bronchial diseases, sore throat, flatulence and pain, muscle convulsions and physical weakness.[9] Moreover, licorice is widely used in food, beverage, cosmetics, tobacco processing, brewing and other manufacturing industries, owing to its sweetness and other unique properties.[10-12] For example, condiments, such as soy sauce and sweet chili sauce, exhibit a unique flavor after addition of licorice. Licorice is also used for flavoring sweets, such as Red Vines and London Drops.[11] The US FDA has approved the safety of licorice (GRAS, Report No. 28), implying that it has no harmful effects on humans.[11] The addition of licorice extract to tobacco provides a rich smoke flavor and improves the moisturizing properties and shelf life of tobacco.[13] A licorice extract gel is used for effective treatment of skin

pigmentation and skin inflammation.[14]

Studies report that licorice is rich in natural active compounds. Notably, more than 20 kinds of triterpenes, more than 300 flavonoids, polysaccharides, coumarins and alkaloids have been isolated from licorice.[1] Glycyrrhizic acid (Fig. 1) is the main active ingredient in licorice.[2] Glycyrrhizic acid is widely used as a sweetener in the food industry, owing to its high sweetness.[4] Some studies report that glycyrrhizic acid has anti-inflammatory, anti-viral, anti-cancer, hepatoprotective, immune-enhancement and significant anti-diuretic effects.[15,16] It is widely used clinically to prevent acute and chronic hepatitis, bronchitis, AIDS and cancer.[3-5] Moreover, findings from recent studies indicate that glycyrrhizin can inhibit the replication of SARS-CoV-2 virus, thus exhibiting anti-coronavirus (COVID-19) effects.[17]

Glycyrrhizic acid is the most active and widely used active substance in licorice, thus studies have widely explored methods for its effective extraction. Methods such as solvent extraction,[4] ultrasonic and microwave-assisted extraction[18] are used for isolation of natural active ingredients, including glycyrrhizic acid. However, the solvent extraction method is characterized by a long period of time required for extraction of natural products, significant loss of organic solvents and high energy consumption. Furthermore, the solvent does not penetrate into the cellulose cell wall and the final product is easily contaminated by organic solvents, thus compromising its quality.[19] In addition, methods such as ultrasonic and microwave-assisted extraction require investment of specific extraction equipment, and fragmentation of wooden cell walls by these methods is relatively limited. Therefore, it is imperative to develop an extraction method for effective extraction of active ingredients from plants. The enzyme-assisted extraction method has several advantages, including fast extraction speed, low solvent loss, low energy consumption, easy recovery and high capability of recycling.[20,21] The enzyme-assisted extraction method is an effective approach for replacing conventional solvent extraction methods.[22]

Lignocellulose is the most abundant organic polymer occurring naturally and is one of the main components of plant cell walls. Lignocellulose forms a complex with flavonoids and polyphenols through covalent bonds to further improve its stability. High stability of lignocellulose limits the extraction of biologically active compounds from plant tissues.[20-23] Cellulase can effectively destroy the structure of plant cell walls, help the dissolution of effective components in tissues, and improve extraction efficiency.[21,24,25] Plant endophytes are microorganisms that ubiquitously exist in plant tissues, plant organelles or cell spaces, and do not cause diseases in plants. Endophytes promote growth and resistance of individual plants and obtain nutrients from the host, thus presenting a mutually beneficial symbiotic relationship.[22] Previous studies report that highly active cellulase-producing strains coexist with plants.[22] This implies that endogenous cellulase has higher specificity in acting on plant cell walls. The application of endogenous cellulase enzyme-assisted extraction can thus increase the yield of active ingredients, significantly reduce the cost of extraction, and improve efficacy of enzyme-assisted plant extraction.[19,25,26]

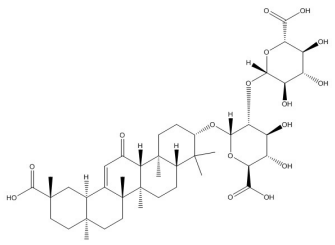

Figure 1: Glycyrrhizic acid (3beta)-30-hydroxy-11,30-dioxoolean-12-en-3-yl2-O-beta-D-glucopyranuronosyl-alpha-D-glucopyranosiduronic acid

The aim of the present study was to optimize and improve the traditional extraction method through the use of endophytes to produce cellulase enzyme, which was subsequently used for extraction of glycyrrhizic acid from licorice (*G. uralensis* Fisch). The findings of this study provide a basis for developing new methods for extraction of medically important compounds from plants by using endogenous cellulase enzymes.

EXPERIMENTAL

Plant materials, chemicals and reagents

The fresh complete plant (*G. uralensis* Fisch) was collected from Qingcheng County, Gansu Province in the middle of April 2017, China. The dried plant licorice was purchased from the Yellow River medicinal material market in Lanzhou, Gansu. All plants had been identified as licorice by Professor Yang Lin, School of Life Science and Engineering, Lanzhou University of Technology. Glycyrrhizic acid standard (98%) was purchased from Aladdin Co., Ltd. (Shanghai, China). Commercially available cellulase (from *Aspergillus niger*, 400 units/mg) was purchased from Biotopped (Beijing, China). Other chemical reagents are of analytical grade, purchased from Guangfu Reagent Company (Tianjin, China).

Isolation and screening of cellulase-producing endophytes

Roots, stems and leaves of fresh licorice plants were harvested and used for isolation of endophytes. Endophytes were isolated as follows: the fresh whole licorice plant was washed under running water, and then the tissue surface was disinfected with ethanol at a concentration of 75% and sodium hypochlorite at a concentration of 3%. Sterilized plant tissues cut by a scalpel were aseptically ground to obtain homogenates in a mortar or all-glass tissue homogenizer. Homogenates were inoculated into separation medium I-IV (Table 1) to obtain single colonies. The endophytes were then inoculated into the screening medium V (Table 1). Further, cellulase-producing strains were screened using the Congo red staining method. The ratio of the diameter of the transparent circle to the diameter of the colony, and enzyme activity were determined for subsequent analysis.

Determination of endophytic cellulase activity

Selected cellulase-producing strains were inoculated into a liquid medium and incubated to obtain fermentation broth. The enzyme solution was obtained by centrifugation for subsequent determination of enzyme activity. The same amount of inactivated enzyme solution as that of the control was used to eliminate the effect of reducing sugar in the enzyme solution. Cellulase activity was determined according to the DNS method previously described by Miller *et al.*[27] The amount of enzyme that hydrolyzed the substrate to produce 1 μmol of reducing sugar (calculated as glucose) per minute was defined as one unit of enzyme activity (U).

Table 1
Different media for isolation, screening, and fermentation of cellulase-producing endophytes

Medium	No	Ingredients
Separation medium	I	10 g licorice powder, 5 g ammonium sulfate, 15 g agar, 5 g sodium chloride, add distilled water to make the volume of 1000 mL, pH 7.0
	II	Licorice powder 5 g, ammonium sulfate 5 g, agar 15 g, sodium chloride 5 g, add distilled water to make the volume of 1000 mL, pH 7.0
	III	10 g of licorice powder, 5 g of ammonium sulfate, 15 g of agar, 5 g of sodium chloride, 20 mL of potato stock solution and distilled water to make the volume of 1000 mL, pH 7.0
	IV	After alcohol extraction, 10 g of licorice powder, 5 g of ammonium sulfate, 15 g of agar, 5 g of sodium chloride, 20 mL of potato stock solution and distilled water to make the volume of 1000 mL, pH 7.0
Screening medium	V	10 g sodium carboxymethyl cellulose, 10 g peptone, 15 g agar, 5 g sodium chloride, add distilled water to make the volume of 1000 mL, pH 7.0
Fermentation medium	VI	Medium I-V without agar
Beef extract peptone medium	VII	Beef extract 3.0 g, peptone 10.0 g, NaCl 5.0 g, agar 15.0 g, add distilled water to make the volume of 1000 mL, pH 7.0

Identification of endophyte strains

Genomic DNA was extracted from GG-3 strain, using an Ezup Column Bacterial Genome DNA Extracting Kit (Sangon Biotech, Shanghai, China). Further, 16S rDNA from the extracted DNA was amplified by PCR using primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and primer 1520R (5'-AAGGAGCTGATCCAGCCGCA-3'). The reaction system comprised 10×PCR buffer, 2.5 mmol/L dNTPs, 5 μmol/L forward primer, 5 μmol/L reverse primer, Taq enzyme, template DNA and ddH_2O. PCR analysis was performed according to previously reported methods.[28,29] The 16S rDNA gene sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST tool. The CLUSTAL X 1.81 program was used for sequence alignment. MEGA version 7.0 software was used for construction of a phylogenetic tree

Extraction glycyrrhizic acid from *G. uralensis* Fisch

Reflux extraction

The reflux extraction method was used for extraction of glycyrrhizic acid from licorice, as described previously.[30] In brief, 5 g of dry sieved licorice powder was added into a round bottom flask (particle size of licorice powder between 400 to 500 μm), then 30% ammonia ethanol was added at a ratio of 1:10 for reflux extraction. Reflux extraction was performed in a water bath at 90 °C for 1.5 h.[31,32] The extracts were filtered and combined, and the pH was adjusted to pH 2.0 with 5% H_2SO_4. Further, extracts were centrifuged (4000 rpm for 15 min) and the precipitate was obtained. The precipitate was dried in an oven at a constant temperature of 60 °C, then it was weighed and the weight was recorded. The content of glycyrrhizic acid was determined by the HPLC method, as described below, and the extraction rate of the glycyrrhizic acid was determined. All experiments were conducted in triplicates

Enzyme-assisted extraction

Crude cellulase solution obtained from GG-3 strain was added to 5 g of licorice powder. The enzyme activity of cellulase in the crude solution was 30 U/g. The enzyme reaction was conducted for 22 h (37 °C, 180 rpm/min). Subsequently, 30% ammonia ethanol was added to the enzyme hydrolysate, and the mixture refluxed at 90 °C for 1.5 h in a ratio of 1:10. The pH of the mixture was adjusted to 2.0 using 5% H_2SO_4, then centrifuged to obtain a precipitate. The precipitate was dried in a constant temperature oven at 60 °C, and the weight was recorded. The content of glycyrrhizic acid was determined by the HPLC method, as described below. The extraction rate of the glycyrrhizic acid was determined. A commercially available cellulase solution dissolved in phosphate buffer at pH 7.0 was used as a control. The procedure was the same as that used for the experimental group. All experiments were conducted in triplicate.

Determination of glycyrrhizic acid level

The content of glycyrrhizic acid was determined by high performance liquid chromatography (HPLC), as described by Lindsay *et al.*[33] The HPLC system comprised a Waters 515 HPLC pump, a 6-valve sample injection port with a sample loop, a sample volume of 10 μL, and a Waters UV-2487 detector was used. Analyses were carried out using a reversed-phase C18 column (Agilent Zorbax ODS3-C18, 250×4.6 mm, D, S-5.0 μm, 12 nm) at room temperature. The mobile phase was methanol-water (40:60 v/v) at a flow rate of 0.4 mL/min.

Effects of different factors on enzyme activity and growth of cellulase-producing strains

The effects of different factors on the fermentation growth and activity of cellulase enzyme extracted from strain GG-3 were evaluated. GG-3 strain was inoculated into variable medium V (Table 1) with 1% inoculum, and supplemented with different carbon sources (including sucrose, glucose, $NaHCO_3$, starch and CMC-Na), different nitrogen sources (such as $(NH_4)_2SO_4$, urea, beef extract, yeast extract and peptone), different pH values (3.5, 5.5, 7.5, 9.5, 11.5), and different carbon to nitrogen ratios (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5). The fermentation broth biomass was determined at OD600 and cellulase activity was evaluated. All experiments were conducted in triplicate.

Effect of different factors on enzymatic properties of cellulase

The effect of different factors, including temperature, pH, and metal ion concentration, on the enzymatic properties of cellulase isolated from GG-3 strain were evaluated. Cellulase was subjected to different culture temperatures (20 °, 30 °, 40 °, 50 ° and 60 °C), different culture pH (6.0, 7.0, 8.0, 9.0 and 10.0), and different metal ions (K^+, Mg^{2+} and Zn^{2+}) at different concentrations (0.01%, 0.03%, 0.05%, 0.07%, 0.1%, 0.3%, 0.75% and 0.75%), then the cellulase activity was determined. All experiments were performed in triplicate.

Process parameter optimization

A single factor experiment was designed to evaluate the effects of different ammonia ethanol content, extraction time and different extraction

temperatures on the extraction rate of glycyrrhizic acid. Furthermore, a 3-factor 3-level orthogonal test was designed according to the single factor test results to determine the final process parameters. Cellulase solution produced by the GG-3 strain was added to 50 g of licorice powder for extraction of glycyrrhizic acid, then the effect of different parameters was evaluated. The HPLC method was used to determine the content of glycyrrhizic acid, as described above and the extraction rate of glycyrrhizic acid was determined. Commercially available cellulase and water were used as controls to verify the feasibility of the optimal extraction process.

Statistical analysis

The average of all data was obtained from three repeated experiments, and analyzed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Screening and isolation of cellulase-producing endophyte strains

A total of 20 endophytic strains with different morphologies were isolated from the roots, stems and leaves of licorice. Further, 13 cellulase-producing strains were selected using the Congo red staining method (Fig. 2a). PDA medium is the most widely used medium for isolation of endophytes.[34] However, strains isolated using PDA medium are generally readily available conventional strains. Studies on plant endophytic bacteria population based on metagenomics technology show that endophytic bacteria in plants are more diverse compared with those obtained from pure culture.[35] Therefore, different media were used for isolation of specific enzyme-producing strains in the present study. Morphology, A-value, high enzyme activity and enzyme-assisted glycyrrhizic acid extraction results of the different strains are summarized in Table 2. The results showed that the enzyme produced by GG-3 strain had the highest activity and was the most effective in the extraction of glycyrrhizic acid. The 16S rDNA sequence of strain GG-3 was solved, and homologous sequence alignment was performed. A phylogenetic tree was constructed (Fig. 2b). The findings showed that the GG-3 strain had a high sequence similarity at 100% with *Bacillus pumilus* strain. Therefore, the selected GG-3 strain was preliminarily identified as *Bacillus* sp. Meanwhile, a study by Ariffin *et al*.[36] once reported that *Bacillus pumilus* EB3 isolated from decaying lignocellulose of oil palm empty fruit bunch was similarly cellulase competent.

Effects of different factors on enzyme activity and growth of cellulase-producing endophyte

The effects of different factors on enzyme activity and growth of endophyte producing cellulase are presented in Figure 3. The organic carbon source had a significantly higher effect on GG-3 strain growth and enzyme activity optimization, compared with the inorganic carbon source (Fig. 3a and b). The use of sodium carboxymethyl cellulose as the carbon source improved the growth of the strain. However, the enzyme activity was the highest with sucrose as the carbon source, compared with other carbon sources. These results are consistent with previous results, confirming that the carbon source improves the yield of cellulase produced by *Trichoderma viride*,[37] and that carboxymethyl cellulose is the best carbon source for the production of cellulase by several *Bacillus* spp.[38] The nitrogen source is also an important factor in bacterial growth and enzyme production. Moreover, the organic nitrogen source significantly improved the growth and enzyme production of the GG-3 strain (Fig. 3c and d). The strain showed the highest growth rate with beef extract as the nitrogen source. Cellulase enzyme exhibited the highest activity with the yeast extract at the nitrogen source. Previous studies report that peptone and yeast extract can significantly promote growth and enzyme production of strain GG-3.[39] Deepmoni Deka and Abou-Taleb reported that the yeast extract significantly affects enzyme yield.[38] The growth of the strain reached the maximum level when the carbon-to-nitrogen ratio of the fermentation medium of strain GG-3 was 1:2 (Fig. 3e and f). The enzyme production rate of the strains decreased when different ratios were used. The results showed that a carbon to nitrogen ratio of 1:1.5 was optimal for enzyme production by the strain. Maximum biomass and enzyme activity of the strain was achieved when the pH of the fermentation medium of strain GG-3 was 7.5 (Fig. 3g and h).

Table 2
Characteristics of cellulase-producing endophytes and their application for extraction of glycyrrhizic acid

Strain	Morphological characteristics	A-value	Enzyme activity (U/mL)	Extraction yield (%)
GG-3	White colonies, smooth, projections, neat edge	1.70	42.689	4.32±0.10
G-B	Milky white colonies, smooth, opaque, irregularly jagged on the edges	1.00	31.444	3.12±0.14
GF-5	Gray colonies, smooth, projections, neat edge	0.75	32.703	3.04±0.13
G-0.5-1	White colonies, surface folds, jagged edges	1.70	34.085	4.24±0.11
G-1-1	Light green colony, wrinkled surface, neat edges	0.60	33.017	4.18±0.10
G-1-2	White colonies, frosted, prominent, neat edges	1.20	30.343	3.10±0.13
Commercial cellulase		-	94.05	3.08±0.14

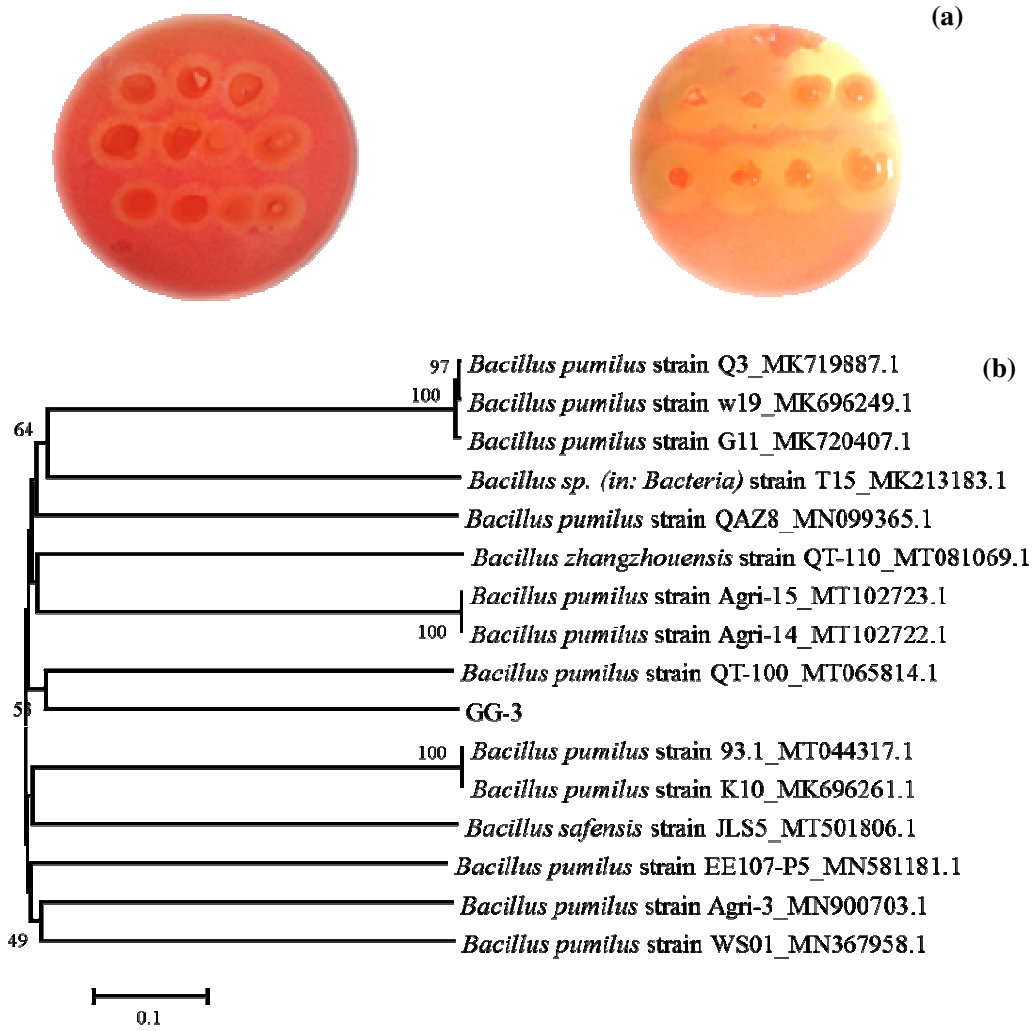

Figure 2: Endophyte producing cellulase isolated from *G. uralensis* Fisch; (a) Cellulase-producing stains screened by Congo red staining method; (b) Congo Red Staining Screening of GG-3 Strain

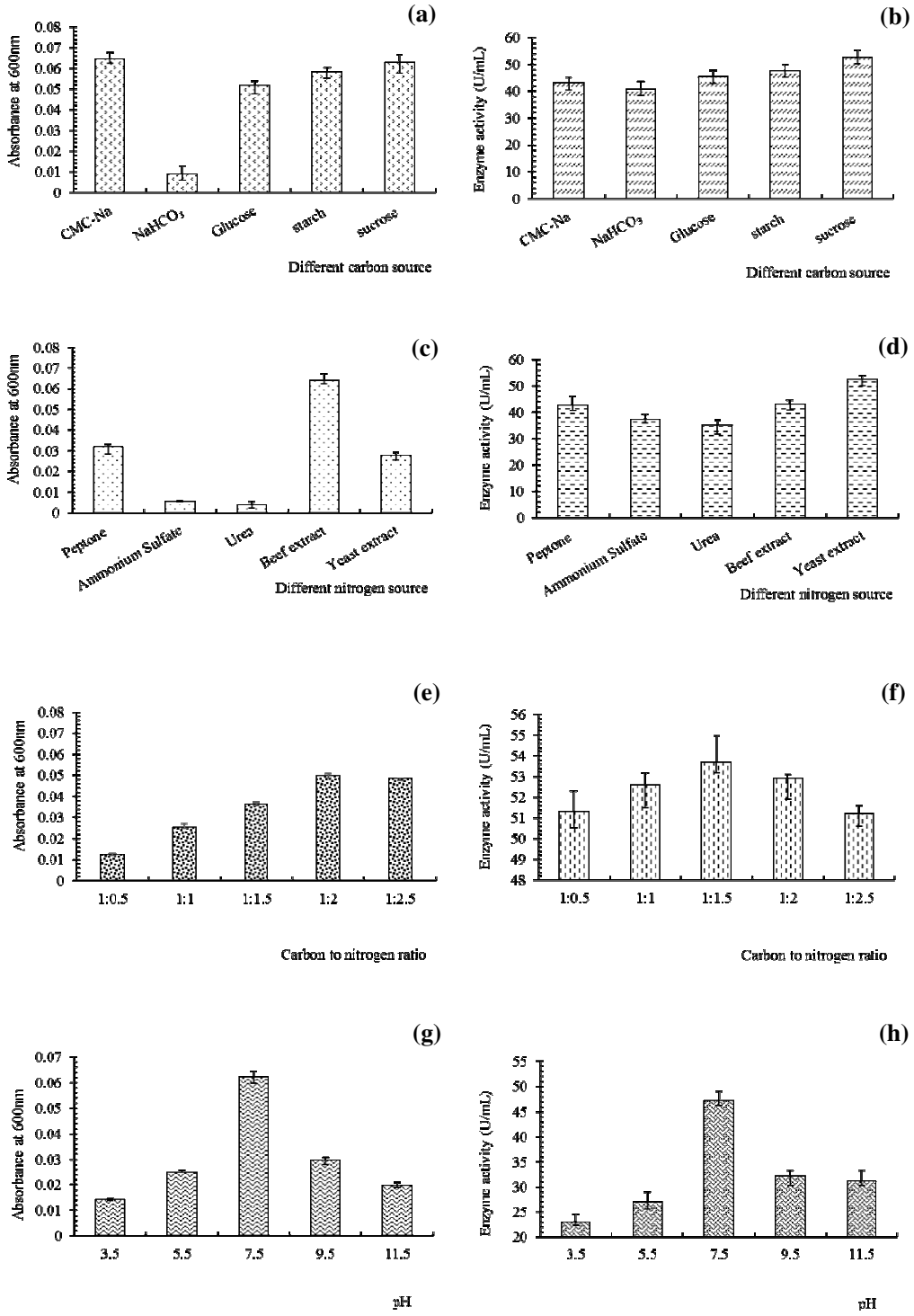

Figure 3: Effects of different (a, b) carbon sources (sodium carboxymethyl cellulose, glucose, sodium bicarbonate, starch and sucrose), (c, d) nitrogen sources (ammonium sulfate, peptone, urea, beef extract and yeast extract), (e, f) carbon-nitrogen ratios, and (g, h) pH, on the growth and enzyme production of endophytic bacteria GG-3

Effect of different factors on the enzymatic properties of cellulase produced by strain GG-3

The effects of different factors on the enzymatic properties of cellulase produced by the GG-3 strain are presented in Figure 4. The enzyme activity of the GG-3 strain exhibited a bell-shaped distribution with the change in temperature. Enzyme activity was lost at temperatures above 40 °C (Fig. 4a). Exposure of the enzyme to different pH values exhibited a bell-shaped distribution. Enzyme activity reached its peak at pH 8 (Fig. 4b). This result is consistent with the optimum pH of extracellular cellulase of leopard moth symbiotic *Bacillus subtilis* Bc1.[40] Enzyme activity reached the peak value when the K^+ concentration was 0.07%. The enzyme was inhibited by an increase in K^+ concentration continued above 0.07%, thus decreasing the enzyme activity (Fig. 4c). A maximum enzyme activity was observed with Mg^{2+} concentration at 0.10%. The increase in Mg^{2+} concentration above 0.10% inhibited the enzyme, and enzyme activity decreased (Fig. 4d). Maximum enzyme activity of the GG-3 strain was achieved with Zn^{2+} concentration at 0.03%. The increase in Zn^{2+} concentration above 0.05% caused inhibition of the enzyme, thus decreasing enzyme activity (Fig. 4e).

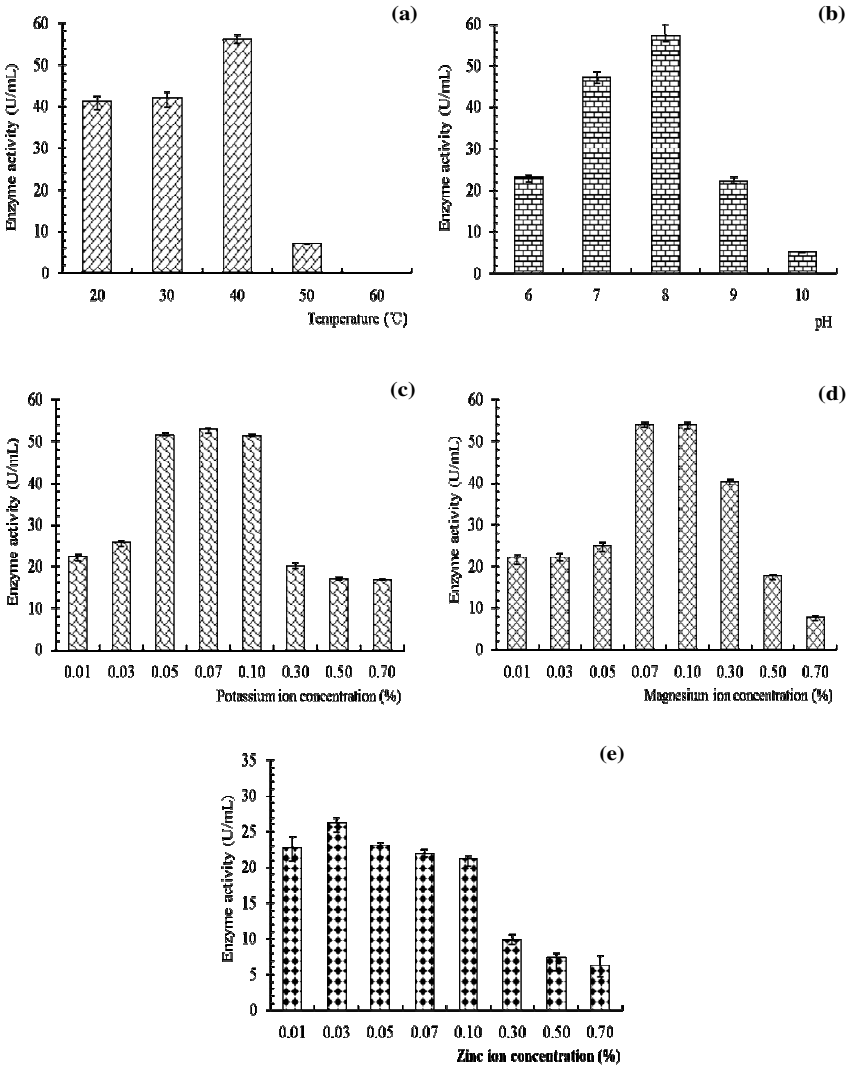

Figure 4: Effects of different (a) temperatures, (b) pH, (c) potassium ion concentrations, (d) magnesium ion concentrations, and (e) zinc ion concentrations, on the enzymatic properties of cellulase from GG-3 strain

Effect of different factors on extraction of glycyrrhizic acid

The effects of different factors on the extraction rate of glycyrrhizic acid are presented in Figure 5. A maximum extraction rate of glycyrrhizic acid was observed when the concentration of ammonia ethanol was increased to 30%. The extraction rate of glycyrrhizic acid did not show significant change with an increase in ammonia ethanol concentration above 30% (Fig. 5a). The increase in the extraction rate of glycyrrhizic acid was not significant when the single extraction time was increased to 1.5 h (Fig. 5b). The extraction rate of glycyrrhizic acid reached a plateau when the extraction temperature was 70 °C, indicating that further increase in extraction temperature had no significant effect on the extraction rate (Fig. 5c).

Optimization of glycyrrhizic acid extraction process through orthogonal experiments

Orthogonal experiments were performed to determine the optimum conditions for extraction of glycyrrhizic acid. A three-factor three-level orthogonal test based on the extraction rate of glycyrrhizic acid was conducted based on the above single-factor test results (Table 3). The effects of the three evaluation factors were determined according to the R values obtained by the method of range analysis (Table 3), and the order was as follows: extraction temperature > extraction time > ammonia ethanol concentration. The results showed that extraction temperature had the highest effect on the extraction process and was the main factor affecting the extraction rate of glycyrrhizic acid. Orthogonal experiment results showed that the optimal level of glycyrrhizic acid extraction rate was $A_1B_3C_3$. The extraction rate of glycyrrhizic acid using 5 g of licorice was the highest at 3.36% under the parameters: GG-3 enzyme treatment, reflux extraction with 30% ammonia ethanol, a single extraction time of 2 h and an extraction temperature of 90 °C.

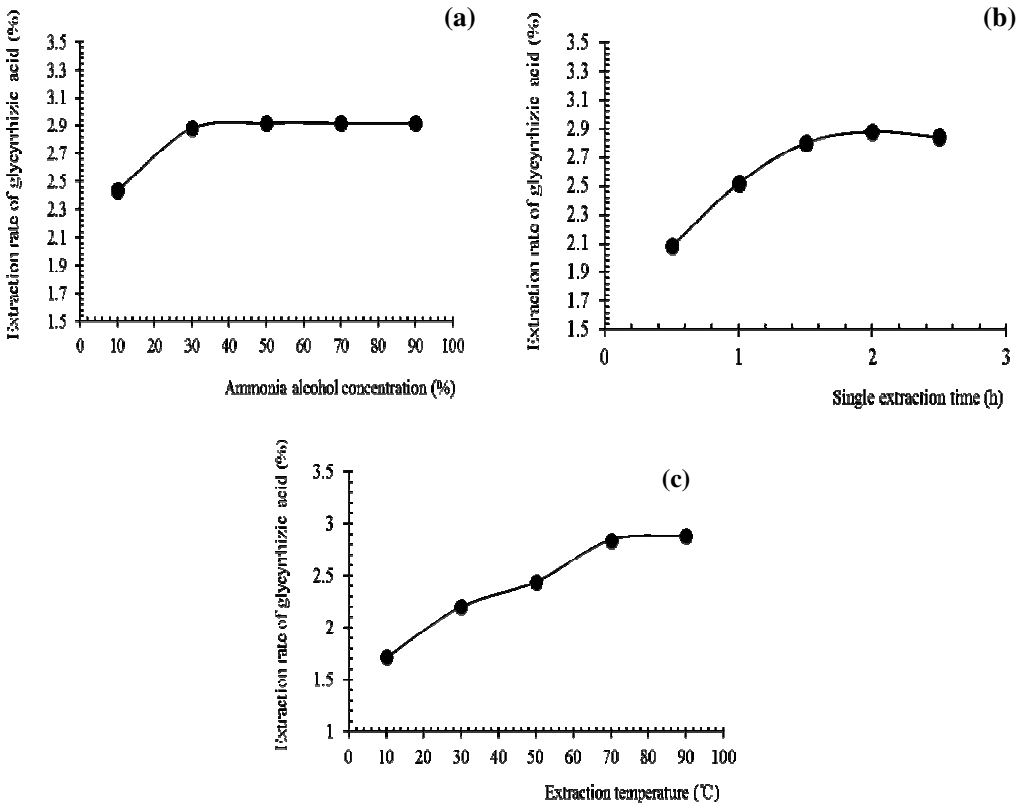

Figure 5: Optimization of enzyme-assisted extraction process. Effects of different (a) concentrations of ammonia ethanol, (b) single extraction times, and (c) extraction temperatures, on the extraction rate of glycyrrhizic acid from licorice

Table 3
Orthogonal experimental design table for extraction of glycyrrhizic acid

Experimental number	Experimental conditions and levels			Experimental results (%)
	A	B	C	
	Ammonia ethanol concentration (%)	Extraction time (h)	Extraction temperature (°C)	
1	30	1	50	2.44
2	30	1.5	70	2.88
3	30	2	90	3.36
4	50	1	70	2.56
5	50	1.5	90	3.28
6	50	2	50	2.52
7	70	1	90	2.92
8	70	1.5	50	2.68
9	70	2	70	3.24
I	0.434	0.396	0.372	
II	0.418	0.442	0.434	
III	0.442	0.456	0.478	
K_1	0.145	0.132	0.124	
K_2	0.139	0.147	0.145	
K_3	0.147	0.152	0.159	
R	0.008	0.02	0.035	

Table 4
Results of extraction of glycyrrhizic acid from licorice by different methods

Sample serial number	Glycyrrhizic acid quality (g)	Extraction rate (%)	Increase rate (%)
Solvent extraction group	1.230	2.460	0
Commercial cellulase group	1.241	2.482	0.8943
G-G-3	1.630	3.26	32.52

The results for the extraction of glycyrrhizic acid with 50 g licorice using this new process are presented in Table 4. The commercial cellulase group had no significant difference in the extraction rate of glycyrrhizic acid, compared with the traditional water extraction (non-enzyme) group. However, the cellulase-assisted extraction method using endophyte GG-3 exhibited significantly higher extraction rate of glycyrrhizic acid, compared with the enzyme-free group and the commercial cellulase group. Moreover, thin-layer chromatography and HPLC analysis of GG-3 fermentation broth showed that the strain did not produce glycyrrhizic acid. These results indicated that cellulase enzyme produced by endophytes promoted the extraction of glycyrrhizic acid, while commercial cellulase failed to play its due role. Plant cell walls are the main physical barriers to the dissolution of effective active ingredients in plants.[20,23] The main structural components of primary and secondary cell walls of plants include cellulose, hemicelluloses, lignin and pectin. Intact hemicelluloses form grid-like crosslinks in the cell wall by connecting cellulose fibers, lignin and pectin, thus improving structural integrity, ductility and stability. Cellulase can degrade the intercellular layer and cellulose structure in plants and contribute to the dissolution of plant active ingredients. Therefore, it is widely used as a biological enzyme method to extract natural compounds.[19,21]

Although the biological enzyme method is the most economical and effective method, most studies have explored the extraction of natural compounds using commercial enzymes, and only a few have explored the extraction of natural compounds using extracellular enzymes isolated from plant endophytic bacteria. A study by Bell *et al*.[41] reported that active ingredients in plants, such as polysaccharides and phenols, have inhibitory effects on commercial cellulase and

pectinase. Therefore, as shown in our results, the yield of glycyrrhizic acid is not significantly improved when these commercial cellulases are used for extraction of glycyrrhizic acid from licorice. Our results also supported this hypothesis, and enzymes derived from endophytes have better specificity with high extraction yields of 32.52% and 31.35%, compared with the enzyme-free group and the commercial cellulase group. Moreover, a possible explanation for this result includes that plant endophytes are microorganisms that infect plants, but do not exhibit any symptoms.[29] This infection may occur in the early stage of plant growth, then the microorganism gains mutual benefit and co-exists with plants for a long time.[42] Plant endophytes thus degrade plant aging cell walls to obtain nutrients by producing cellulase and other enzymes. Endophytes promote renewal of plant cell walls and block external influences to keep the internal environment stable. Moreover, the growth and reproduction of the endophyte are promoted. As showed by Bell *et al*.,[41] commercial cellulase is inhibited by certain components of licorice, while the plant endophyte GG-3 of licorice is not affected by these components produced by plants due to its symbiosis with plants.

In addition, another possible explanation for this result is that cellulase from endophytes has a special cellulose binding site and high efficiency, compared with commercial cellulase enzyme. The findings of the present study showed that commercial cellulase and several strains with enzyme activity above 30U have high enzyme activity. However, the strains isolated from licorice have differences in the extraction rate of glycyrrhizic acid. Not all strains isolated from licorice exhibit high efficacy in the extraction of glycyrrhizic acid from licorice, which may depend on the binding situation of enzymes produced by strains and targets. This explains to some extent that cellulase enzyme from GG-3 strain had higher efficacy in the extraction of glycyrrhizic acid, partially because it can be effectively embedded on the cellulose binding site in licorice, increasing the catalytic degradation of the cell wall structure. Therefore, the cellulase produced by endophytes coexisting with plants can be used as a specific enzyme to aid in the extraction of glycyrrhizic acid. However, further studies should be conducted to explore whether the process can be used for larger-scale applications.

CONCLUSION

Endophytes coexist with plants, and cellulases derived from plant endophytes are an important source for obtaining specialized cellulases. Our results showed that cellulases from endophytes of *Bacillus* sp. GG-3 had higher specificity for plant cell wall degradation, when compared to commercial cellulases. After optimizing the enzyme production and extraction processes, the extraction rate of glycyrrhizic acid using the cellulase-assisted extraction method was significantly increased by 32.52% and 31.35%, compared with the enzyme-free group and the commercial cellulase group. The results showed that endogenous cellulase significantly improves the isolation of active ingredients from plants. The findings of this study would provide new methods and ideas for extraction of active ingredients from plants.

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