# Co-culture for improving the biosynthesis ability of Huperzine A

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# Abstract

Huperzine A (HupA) is for treating Alzheimer's disease(AD) which was mainly extracted from the *Huperzia serrata(HS)*. Especially, (-)-HupA extracted from *HS* with high inhibitory activity of acetylcholinesterase are scarce and the chemical synthesis of (+)- HupA is high toxicity with low acetylcholinesterase inhibitory activity. In this work, *Serratia marcescens* HL1 was firstly found and isolated from *HS*, that was identified according to their morphological characteristics and nuclear 16SDNA sequences. It could biosynthesize HupA(BHA) of 32.976 ± 0.21 mg/L which was co-cultivated after with endophytic fungi *Trichoderma harzianum* NSW-V. Moreover, this bacteria with shorter fermentation time could form better purity and crystal structure with the same physicochemical properties compared to (-)-HupA isolated from *HS*(PHA) according to the results of NMR and molecular docking. Furthermore, this study explored new indications for HupA which indicated it could protect β-cells of pancreatic islets.


# Introduction

Alzheimer's disease (AD) is one disease marked by gradual dementia and the cognitive function deterioration(Venkata Ratnam, K. et al. 2022; Kumar, A. & Yadav, A.K. 2023; Dadlani, V.G. et al. 2023; Yadav, M.R. et al. 2023). The drugs for treating AD include acetylcholinesterase(AChE) inhibitors (AChEIs) containing rivastigmine, donepezil, galantamine, and NMDA receptor antagonist memantine (Yadav, M.R. et al. 2023).

Fortunately, Huperzine A (HupA) is one of the AChEIs with good potency and selectivity, especially with the reversible inhibition of AChE (Kunal 2018; Xiaoqiang et al. 2008; Ratia et al. 2013; Xiao-Tian et al. 2014; Dadlani, V.G. et al.; 2023). It is a powerful and reversible AChE inhibitor with multi-target effect. Its inhibition of AChE is 3 times that of physostigmine and 30 times that of galantamine, and its peripheral adverse reactions are the lowest. Thus, HupA is safe (Xu, ZQ. et al. 2012; Wen-Xia, H. et al. 2020). Notably, plant extraction is the easiest way to obtain HupA.

Unfortunately, the content of HupA in *HS* is very low. Plant extraction has faced the bottleneck of lack of plant resources. In addition, chemical synthesis to produce HupA is one of the important ways of research and development, but the synthesis steps are complicated and expensive, and it is difficult to obtain pure optically active synthesis with (-)-HupA not with (+)-HupA which has some insurmountable limitations with low activity, high pollution(Wen-Xia, H. et al. 2020). (-)-HupA with high activity and high security was derived from the natural plants *HS* or *LS*. However, the products of chemical synthesis are mostly (+)-HupA. Notably, the activity of the (-)-HupA product is 30 times higher than that of the (+)-HupA product(Yang, H.L. et, al. 2020).

Previous studies have identified five fungi isolated from *HS* expressing HupA for treating AD (Wen-Xia, H. et al. 2020), the aim of the present study was to optimize the culture conditions by cocultivation that used to activate metabolic activity of endophytic fungi to overcome the problem of attenuation of metabolite synthesis and promote HupA further yields.

## Materials and methods

# Materials

The healthy whole plants of *HS* were collected in October, 2011 from Guangyuan of Sichuan Province, China. Solvents used for chromatography were of high-performance liquid chromatography (HPLC) grade. Standard HupA (99% purity) (SHA) was from Shanghai Siyu Bio-technology CO., LTD., Shanghai, China. Mouse islet beta cell Min6 (BFN608006398, purchased from Qingzi (Shanghai) Biotechnology Development Co., LTD.), DMEM medium (HYCLONE SH30022.01), fetal bovine serum (GIBCO 10099-141), 0.25% containing EDTA pancreatic enzyme (SC107-01, Cyman Innovation (Beijing) Biotechnology Co., LTD.), Three antibodies (C0224, Biyuntian Biotechnology Co., LTD.), PBS buffer (SC106-01, Cyman Innovation (Beijing) Biotechnology Co., LTD.), Bovine Serum albumin (AR1006, Bode Biotechnology Co., LTD.), palmitic acid (H8780, BHD, BHD) Beijing Solaibao Technology Co., LTD.), dimethyl sulfoxide (AR, commercially available).

### Bacteria from HS and Preparation of fermentation solution

The part of the experiments were carried out according to the endophytic fungal isolation method(Wen-Xia, H. et al. 2020).SHA and HupA from endophytic bacteria (BHA) were dissolved to 0.01 mol/L hydrochloric acid solution and prepared into 2, 0.2, 0.02, 0.002, 0.0002 mg/mL mass concentrations.The sample size was 20 μL. Repeat 5 times each time to determine good precision.

## Identification of Biological

# Morphological identification of strains

All of the strains were inoculated on PDA media at 28℃ for 2 d which were observed and Identificated by LM( light microscope ,eclipse 55i, Nikon, Japan) and STEM(scanning transmission electron microscopy, Talos F200iS/TEM, FEI, Netherlands).

## Biochemical identification of strains

The fresh bacterial solution of isolated strain was coated with PDA media and the bacterial morphology were observed under microscope. The relevant tests were performed according to the instructions of biochemical identification tests containing Ornithine, Glucose, Lactose, Galactose, Mannose, Urea, Nitrate reduction, Sucrose, Sorbose, Natrium citricum, V-P, MR, Indole test.

## Molecular identification of strains

16SrDNA was extracted and amplified. The primer sequence was 27F: 5 '-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5 '-TACGGCTACCTTGTTACGACTT-3'. The PCR reaction system consisted of adding Super Mix

15μL, Primer F (10p) 1μL, Primer R (10p) 1μL, template (ng/μL) 1μL, dd$H_2O$ 12μL, and total volume 30μL. PCR cycle condition predegeneration at 96℃ for 5min; 35 cycles were repeated at 96 ° C (20 sec) 62 ° C (20 sec) 72 ° C (30 sec). Repair extended at 72℃ for 10 min; The reaction was terminated at 16℃. PCR products were identified and sequenced by Beijing Liuhe Bada Gene Technology Co., LTD.

# NCBI-BLAST comparison

The analyses were conducted in MEGA 7.

## HPLC and NMR

This part refered to the conventional determination method (Wang Bo et al. 2024).

## Molecular docking

Download the AchE protein structure (PDB ID: IE66) from the RCSB PDB database (RCSB PDB: Homepage) and prepare the IE66 protein structure using the Glide module in Schrödinger software. By this module, the loop missing chain of protein was compensated with hydrogen atom added, water atom in protein removed, energy of protein minimized under OPLS4 force field. Ligprep module was used to optimize the structure of HupA compounds.

The Receptor Grid Generation module in Schrödinger software was used to construct the centered of 20 Å grid file on the co-crystalline compound on the basis of the processed protein, and the co-crystalline compound and HupA compound were interconnected with the AchE protein structure in a super standard precision mode.

# The protective effect of (-)-HupA on β-cells of pancreatic islets

The Cell type was mouse islet beta cell Min6 and the control model was mouse islet beta cell Min6 stimulated by palmic acid. The drug type was biosynthetic drug (-)-HupA and other screened drugs.

# Cell passage and culture

The cells were cultured at 37℃, 5% $CO_2$ incubator till to the cell fusion degree reached 80%, and were passed. The complete medium was sucked out and washed gently with PBS buffer twice. 1 mL trypsin containing EDTA with the concentration of 0.25% was added. After digestion for 1.5 min, the cells were washed off with complete medium (DMEM medium + 1% triantibody + 10% fetal bovine serum). Centrifuge at 1000 r/min for 5 min, discard the supernatant to collect the cells, spread them into T25 culture bottles for culture with 5 parallels, add 5 mL complete medium for each, disperse the cells evenly, and then place them in the cell incubator for culture.

# Solution configuration

Bovine serum albumin (BSA) powder with 1.0g was accurately weighed which was dissolved fully with PBS buffer and fixed volume to 10 mL, filtered and sterilized, and stored at -20℃after subpacking. Palmitate (PA) powder with 359.002 mg was weighed accurately and ultrasonic dissolved with 1.0 mL anhydrous ethanol. After dilution with anhydrous ethanol, it was diluted with 10% sterile BSA at the ratio of 1:19 to prepare 30 mM working liquid, which was used on the go. Different concentrations of PA working liquid were prepared to observe the effect of different concentrations of PA on cell activity and determine the optimal stimulation concentration. Weigh different compounds accurately, add dimethyl sulfoxide and dissolve to 25 mM. Dilute with PBS buffer into 10 mM working liquid and store at -20℃ for later use.

# The protective effect of HupA isolated from endophytic bacteria on cells

When the fusion degree of Min6 cells reached 80%, the cells were inoculated uniformly in 96-well culture plates and cultured overnight in a 5% $CO_2$ incubator at 37℃. The final concentration of PA in the culture medium was 300 μM by adding different concentrations of PA working fluid. After PA stimulating with 24 hours, CCK8 reagent was used to measure cell viability.

When the fusion degree of Min6 cells reached 80%, the cells were inoculated uniformly in 96-well culture plates and cultured overnight in a 5% $CO_2$ incubator at 37℃. Compound solution with final concentration of 100 μM was added for pre-protection with 30 min, and PA with final concentration of 300 μM was added for incubation with 24 hours. Then CCK8 reagent was used to measure cell viability.

## Results

### HupA-producing symbiotic strain isolated from HS

Six endophytic bacteria were isolated from the wild *HS* and four with high activity of HupA producing were selected (Fig. 1A). Among them, one strain named No.3 (Fig. 1B)have the advantage of high yield HupA when cocultured with endophytic fungus *Trichoderma harzianum* NSW-V obtained from *HS*(Fig. 1C, Fig. 2F) producing HupA *(*Wen-Xia, H. et al. 2020). As shown in Fig. 1, endophytic fungus *Trichoderma harzianum* NSW-V was cultured alone(Fig. 1D), which cultured with strain named No.3 (Fig. 1E).

# Yielding of HupA

The No.3 strain was coded CGMCC No.21397 with the yielding of HupA to be 32.175 ± 0.13mg/L by HPLC when it was cultured alone((Fig. 2C). And more, when it was cultured with endophytic fungus *Trichoderma harzianum* NSW-V, the yielding of HupA was 32.597 ± 0.19 mg/L by HPLC((Fig. 2F), when it was isolated from the co-culture environment with *Trichoderma harzianum* NSW-V, the yielding of HupA

was 32.976 ± 0.21 mg/L by HPLC(Fig. 2G). These results indicate that co-culture environment can promote the production of HupA. In particular, when the capacity of producing HupA decreased, co-culture could activate the strain's capacity to produce HupA .

# Crystallization of HupA

The product of HupA obtained from endophytic bacteria (BHA) had a high initial degree and the crystal precipitation was shown in Fig. 3.

# Morphological characteristics

No.3 strain as above mentioned was cultivated in PDA medium and grew well after 2 d at 25℃(Fig. 4A). The morphological characteristics was observed through light microscope and STEM. The morphological characteristics of the strain colonies were round, smooth, moist, protruding, milky white without pigment production, large and sticky on solid medium(Fig. 4A-1,Fig. 4A-2 ). The strain was gram negative, with various forms, nearly spherical and short rod-shaped(Fig. 4B, Fig. 4C-1). It can be clearly seen capsules (Fig. 4D, E-2, F-1)and spores (Fig. 4E-3, F-2) on some of the bacteria through STEM. The STEM images revealed that the strain was multiplied by direct division (Fig. 4G, H) and DNA was clearly observed(Fig. 4E-1).

# Physiological and biochemical identification

The biochemical identification results were shown in Table 1, which were consistent with the biological characteristics of *Serratia marcescens*. The isolated strain No. 3 was preliminary identified as *Serratia marcescens* by morphological observation and physiological and biochemical characteristics which named as *Serratia marcescens* HL1.

Table 1
Biochemical test results

| Item | Results |
|---|---|
| Ornithine | + |
| Glucose | + |
| Lactose | - |
| Galactose | - |
| Mannose | + |
| Urea | - |
| Nitrate reduction | - |
| Sucrose | + |
| Sorbose | + |
| Natrium citricum | - |
| V-P test | + |
| MR test | - |
| Indole test | - |
| Notes: “+”positive, “-”negative. | |

# The result of PCR and Phylogenetic analysis

The amplification band sizes were about 1000–2000 bp for the *Serratia marcescens* HL1, and there were no heterozygosis (Fig. 5). The full uncropped image of PCR amplification and agarose gel electrophoresis was shown(Fig.S1).

The strains of HL1, which was submitted to GenBank (accession numbers: MW632158, Seq.S1) was further compared by Blast on NCBI. The result was shown in Fig. 6. The HL1 16S-rDNA was 99.93% similar with *Serratia marcescens* FY (accession number CP053378.1) and *Serratia marcescens* AR_0131 (accession number CP029715.1), so the strain HL1 and *Serratia marcescens* had close evolutionary distance in the phylogenetic tree (Fig. 6). As a result, the strain HL1 was identified as *Serratia marcescens* HL1.

# Structure determination by NMR

By analyzing the data of carbon and hydrogen spectra, the structure of the product of HupA obtained by biosynthesis was consistent with that obtained by plant extraction(Fig. 7), which lays a solid foundation for the further implementation of industrialization.

# Molecular docking

It can be seen from the mode of action diagram that the binding cavity was a hydrophobic pocket composed of aromatic amino acids and fat-soluble amino acids (Fig. 8). The cavity diagram of the activity of (-)-HupA was obtained from HS (SHA) in acetylcholinesterase( Fig. 8A). AS result the HupA mainly relies on the hydrophobic force and Pi-Pi interaction when it bound to the pocket. For example, the positively charged ammonium group and the aromatic ring structure formed strong Pi-cation interaction and T-shaped Pi-Pi interaction with Trp279, respectively. It was noteworthy that the binding force of the (-)-HupA was stronger than that of the (+)-HupA. The binding energy affinity for docking of (-)-HupA was − 10.3 kcal/mol(Fig. 8B), while the binding energy affinity for docking of (+)-HupA was − 4.2 kcal/mol(Fig. 8C). The docking score was based on the size of binding energy affinity, and the smaller the value, the stronger the binding force. The rules of thumb was as following: affinity> -4 kcal/mol with very weak binding or considered no binding; -7 kcal/mol < affinity≦-4 kcal/mol with the medium binding force; affinity≦ -7 kcal/mol with the strong binding force.

# The protective effect of HupA isolated from endophytic bacteria on cells

The results of CCK8 showed that when PA concentration was 300μM, the cell viability was only 58.2%, so this concentration was selected as the best stimulation concentration. In the model group, it was shown that the cell survival rate was 58.2% after cell injury, and the cell activity was enhanced to 71.1% by adding HupA isolated from endophytic bacteria *Serratia marcescens* HL1(Table 2).

Table 2
Protective activity of the compound on Min6 cells stimulated by PA

| Compound | The rate of cell survival |
|---|---|
| BHA | 71.1% |
| Model | 58.2% |
| Notes: HupA obtained from endophytic bacteria (BHA) . | |

# Discussion

HupA, which was approved as a Class II new drug in 1994 in China, is a highly reversible and selective AChEI as a first-line drug for AD. Compared with similar drugs approved by the FDA, plant-derived HupA has the advantages of easy penetration of the blood-brain barrier, good safety, high oral bioavailability, long action time and minimal adverse reactions. Due to the shortage of plant resources, biosynthesis provides an important way to obtain highly active HupA.

*Serratia marcescens* is widely found in the natural environment, such as soil, water, plants and animal intestines. It can also be found in the gut of humans and animals as part of the normal flora, but can sometimes cause infections, especially in individuals with compromised immunity. However, this does not affect its application as a source of strains for the production of important secondary metabolites. In terms of industrial applications, *Serratia marcescens* has several uses in biotechnology. For example, exopolysaccharides are produced, which may be used as drug carriers or food additives(Chen, X et al. 2025). In addition, it is also used in biodegradation research, such as breaking down certain plastics or pollutants. *Serratia marcescens* with ability of good adsorption capability (Shen, J et al. 2021), improving soil biological properties (Li, L. et al. 2019), producing (2R, 3R)-butanediol (Sun, T. et al. 2023), an excellent benzo (a) pyrene degrader (Kotoky, R. & Pandey, P. et al. 2020) has been investigated. In addition, it is used as a model organism to study bacterial adhesion, biofilm formation or antibiotic resistance mechanisms(de Oliveira, R.S. et al. 2024). *Serratia marcescens* can colonize endophytes and promotes the growth of rice plants(de Oliveira, R.S. et al. 2024). Recent research advances may involve genomics, metabolomics analysis, or the development of novel therapeutic approaches, such as phage therapy or antimicrobial peptides(Esteves, N.C. & Scharf, B.E. et al. 2024). In addition, *Serratia marcescens* applications in synthetic biology, such as the engineered production of specific metabolites, are also areas of interest.

In summary, *Serratia marcescens* is a double-edged sword. As an inherent strain in soil, it plays an important role in ensuring the normal growth of plants and maintaining normal plant flora. And more, this study have also proved that co-culture of this strain with another endophytic strain can greatly improve the yield of secondary metabolites of HupA. The production of HupA by *Serratia marcescens* has not been reported and HupA as new drug to protect to β-cells of pancreatic islets is of great significance for the treatment of diabetic disease .

# Abbreviations

List of abbreviations were shown in Tab. S1.

# Declarations


### Funding

The work was sponsored by the Science and Technology Innovation Base-
Open and Sharing Platform of Science and Technology Resources Project of Shaanxi Province (2019PT-26), Biological breeding and green synthesis of endangered precious medicinal materials future Industrial Innovation Research Institute(Shaanxi Education No. 30 [2022]), State General Administration of Sport, based on multi-omics to explore the effects of aerobic exercise on the regulatory mechanism of organ and pancreas microenvironment(24KJCX062), Shaanxi Provincial Department of Education service local special(24JC080).


### Conflicts of interest/Competing interests

The work with no conflicts of interest or Competing interests.

### Availability of data and material

Data can be accessed in NCBI.

### Code availability

Not applicable.

### Authors' contributions

Han Wen-Xia was the head of experimental research. Han Zhong-Wen was responsible for the clinical drug research.Mi Yu was the corresponding author. Zhang-Han was responsible for HPLC. Li Wei-Ze carried out the active pharmaceutical ingredients. Guan Li carried out the active pharmaceutical ingredients and the protective effect of HupA isolated from endophytic bacteria on cells. Zhang Ning carried out the data analysis. Wang Lin carried out the strain activation. Jia Min was responsible for NMR. Mei Shan-Shan participated in the coordination.

### Ethics approval and consent to participate

The study protocol was approved by the ethics review board of Northwest University.

### Clinical trial number

Not applicable.

### Consent for publication

All authors approved to submit to the journal we have choose.

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# Figures

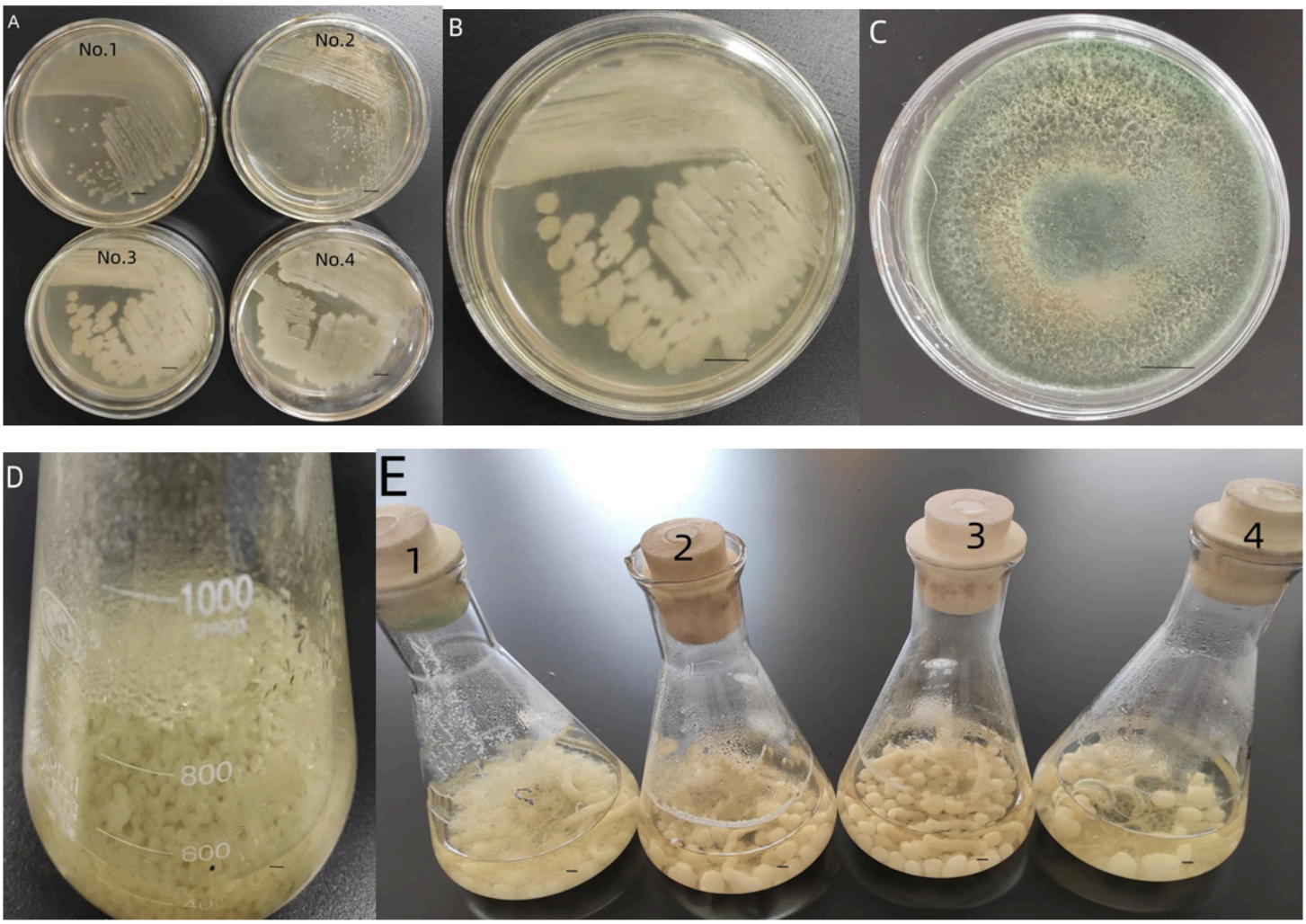
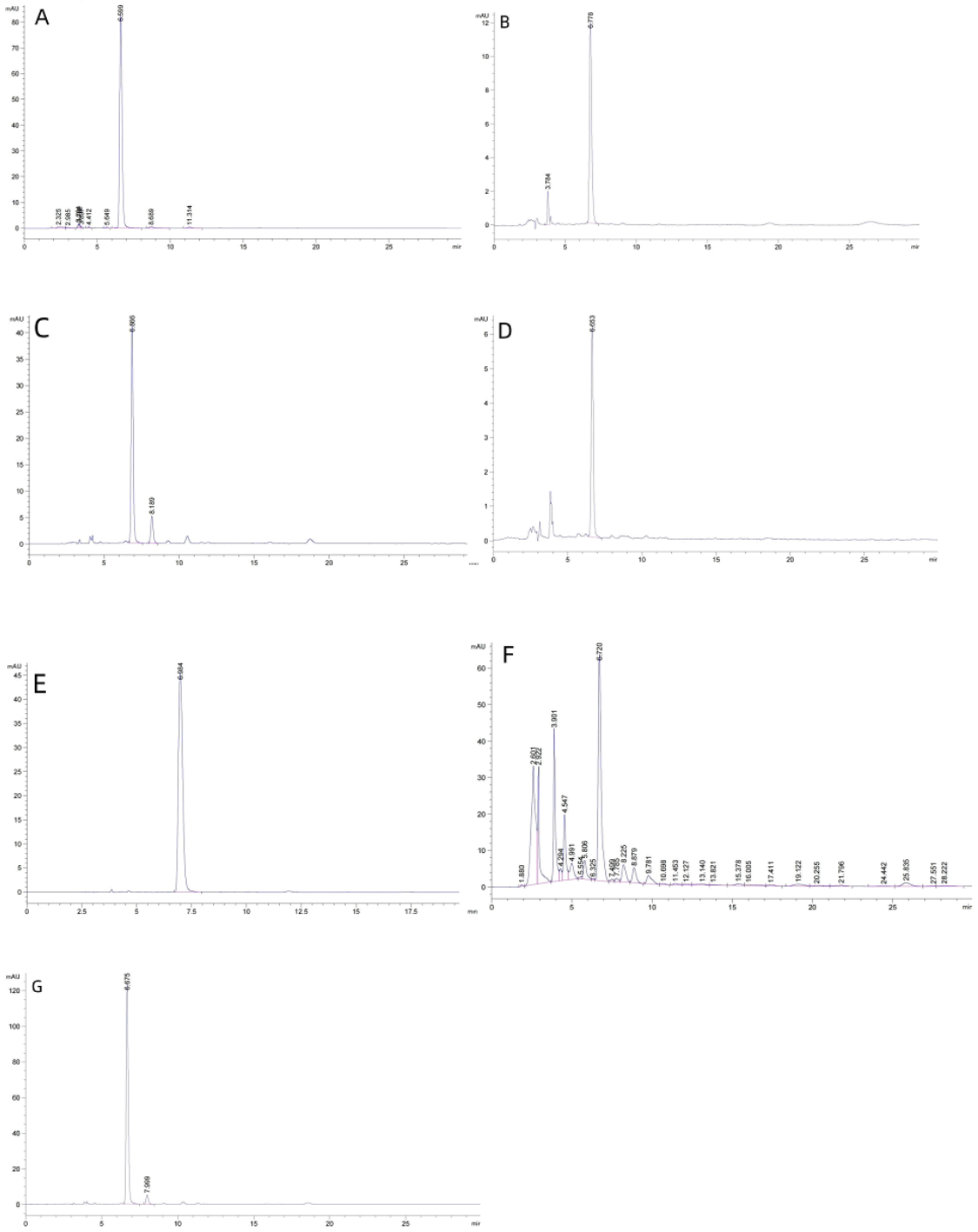



**Figure 1**

Morphological characteristics of bacteria in solid and liquid cultures **A**: colonial morphology for four endophytic bacteria with high activity of HupA producing on the upside of PDA medium after 2 d; **B**: colonial morphology of the upside of PDA medium for strain No.4 after 2 d; **C**: colonial morphology of the upside of PDA medium for endophytic fungus *Trichoderma harzianum* NSW-V after 5 d; **D**: mycelial pellets of *Trichoderma harzianum* NSW-V cultured in PDB alone after 5 d; **E**: mycelial pellets of

*Trichoderma harzianum* NSW-V cocultured with strain No.4 in PDB after 5 d. A,D,E =10 mm; B=5mm, C=15mm.

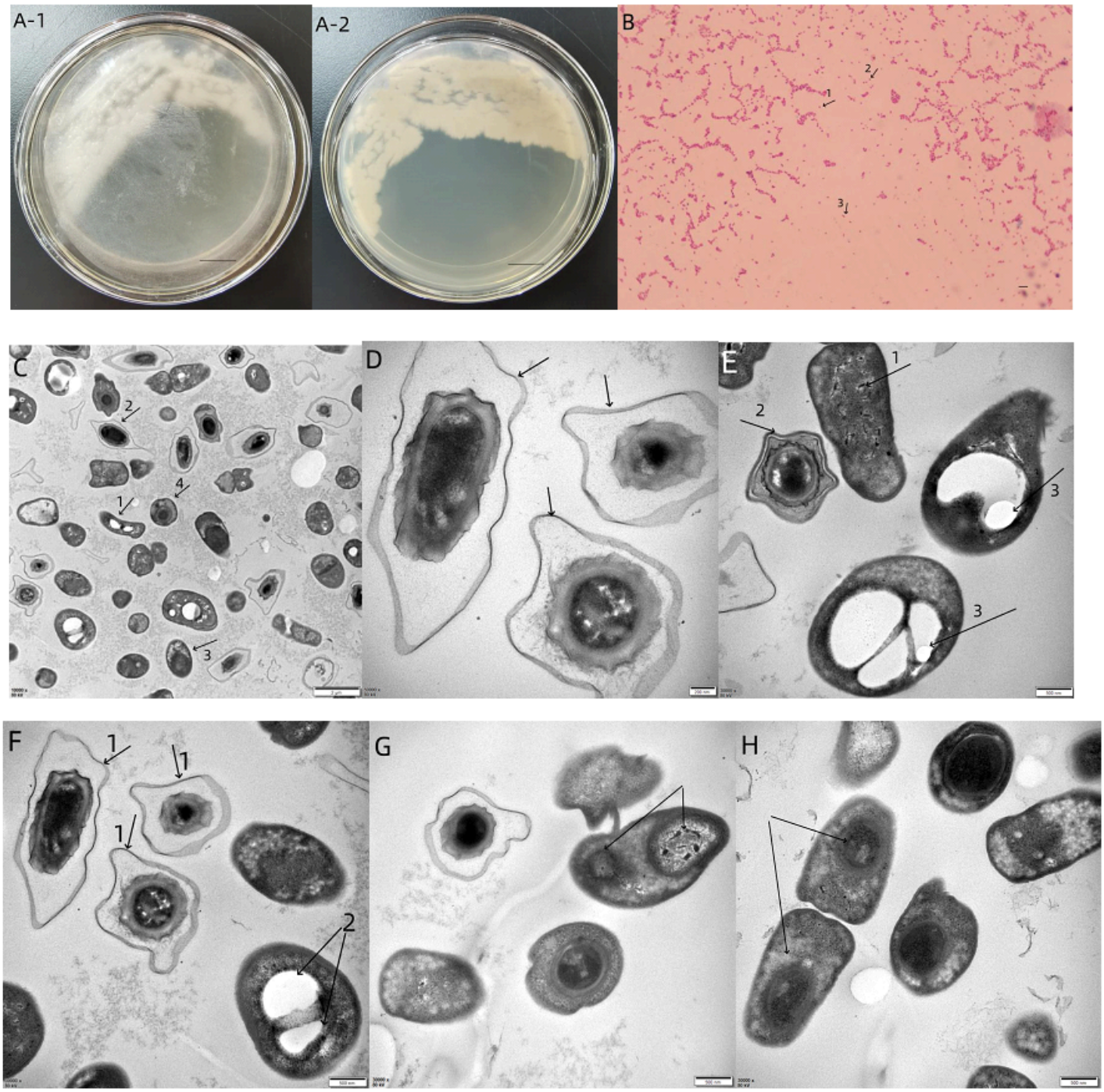



## Figure 2

The strains of 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**). The Standard HupA (SHA) (**E**) as controls; the NO. 3 strain was cultured with *Trichoderma harzianum* NSW-V(**F**), the NO. 3 strain was isolated from the co-culture environment with *Trichoderma harzianum* NSW-V(**G**).

## Figure 3

Crystallization of HupA (BHA)




## Figure 4

Morphological of strain No.3; **A**: colonial morphology of the backside and upside for strain No.3 after 2 d; **B**: Morphological characteristics of bacteria with different forms, 1: subcircular, 2: oval to short rod-shaped, 3: rod-shaped; **C**: Morphological characteristics of bacteria with different forms, 1: rod-shaped; 2,3: oval to short rod-shaped, 4: subcircular; **D**: capsule around the bacterial, arrow indicates the capsule; **E**: 1: DNA, 2: capsule, 3: spore; **F**: 1:capsule, 2: spore; **G-H**: bacterial division. A=12 mm; B=50μmm; C=2μm; D=200 nm; E-H=500 nm.

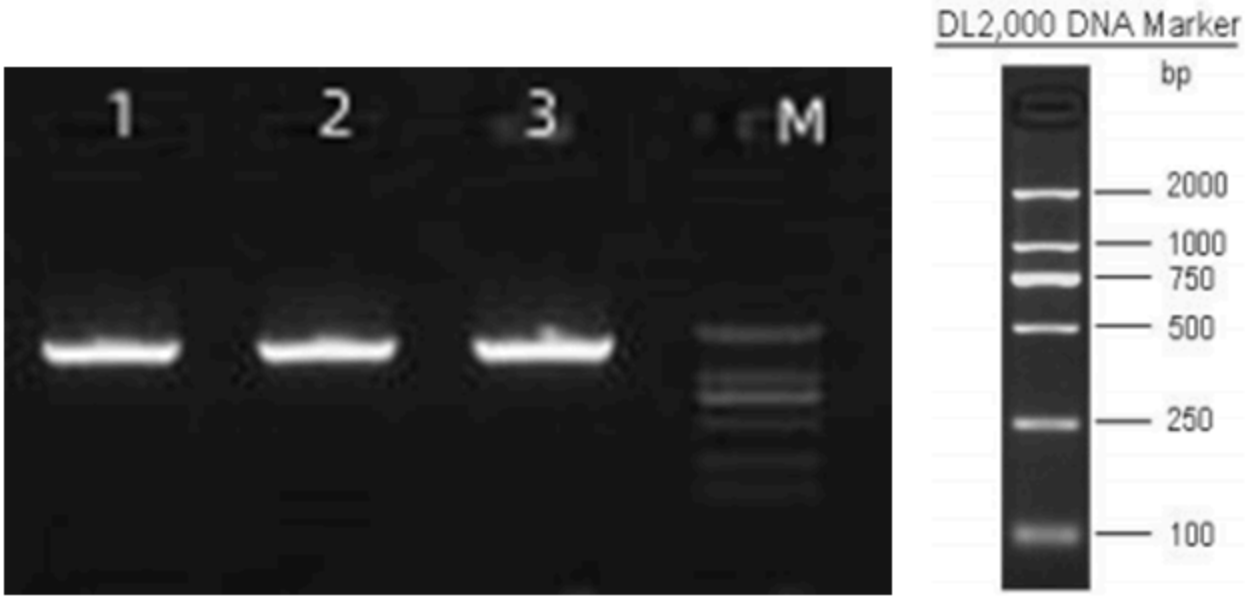
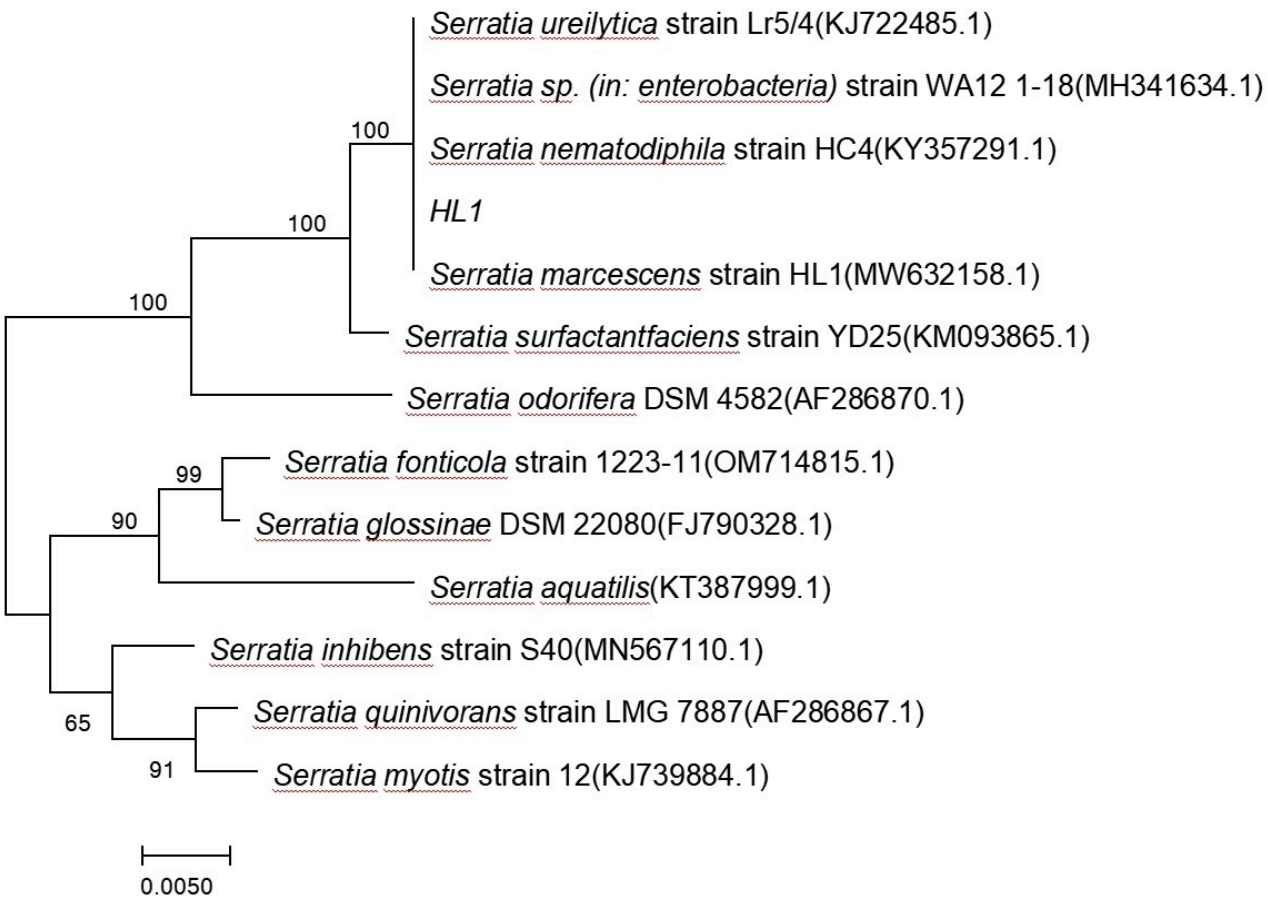


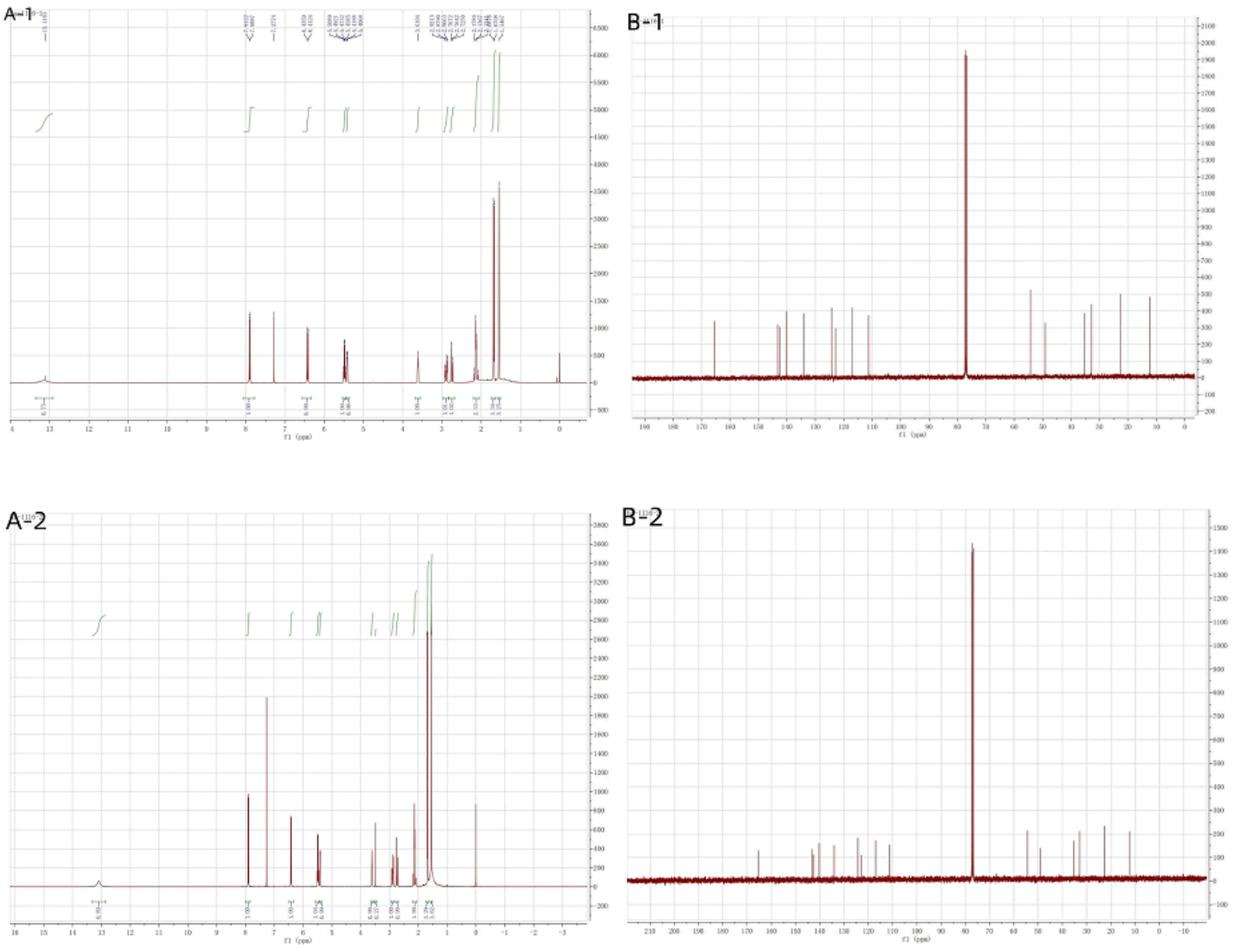

**Figure 5**

PCR amplification and the agarose gel electrophoresis. lane 1: the strain HL1, lane 2:the strain HL1, lane 3: the strain HL1, M: marker (100-2000 bp).



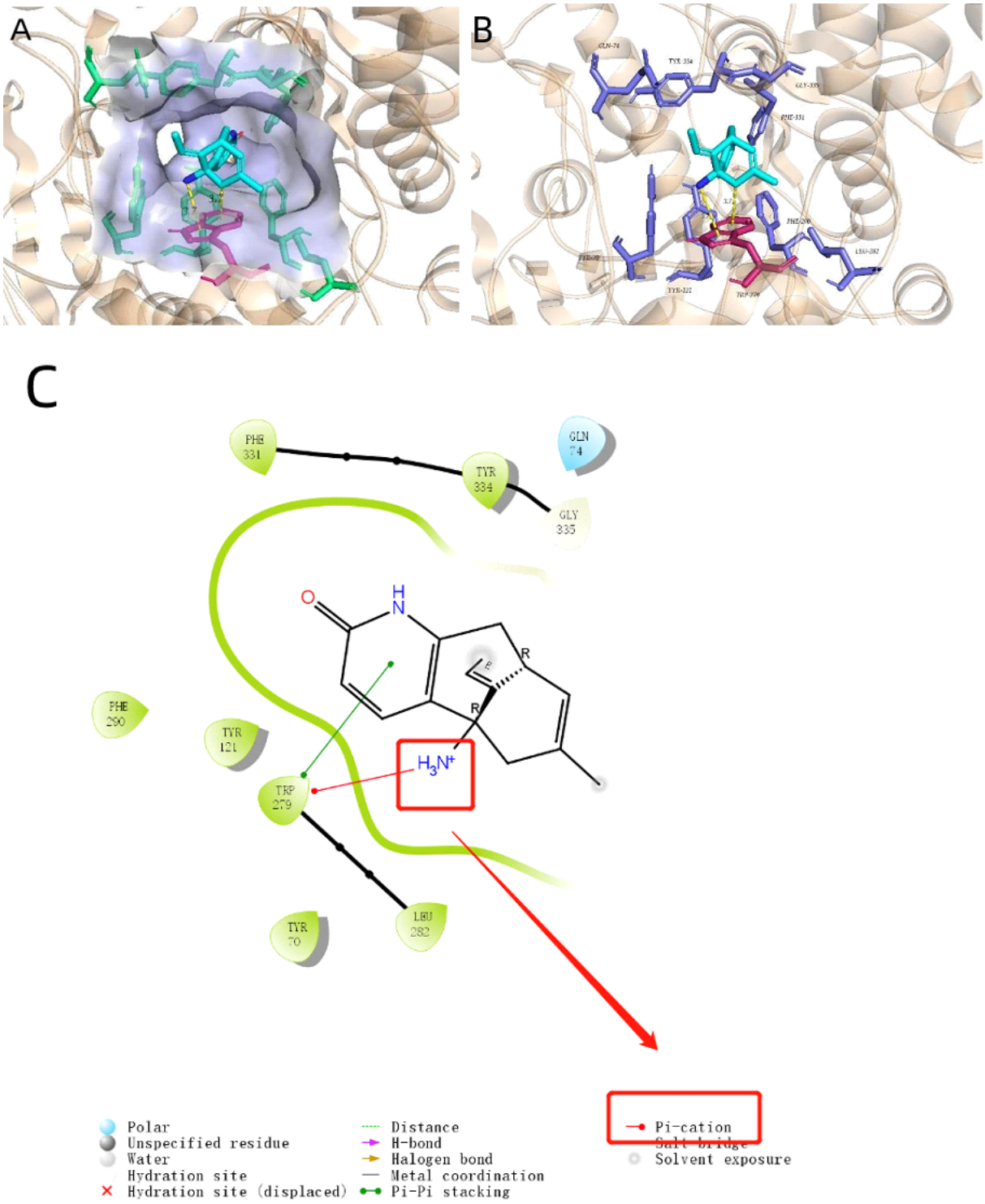
## Figure 6

Evolutionary history The phylogenetic tree constructed from 16S DNA for strain of HL1 was collected from GenBank.




## Figure 7

Determination of chemical structure of products of HupA by carbon and hydrogen spectra **A-1**: hydrogen spectra of HupA from plant extracts ; **B-1**: carbon spectra of HupA from plant extracts; **A-2**: hydrogen spectra of HupA from biosynthetic HupA; **B-2**: carbon spectra of HupA from biosynthetic HupA




## Figure 8

Molecular docking **A**: cavity diagram of the activity of (-)-HupA obtained from *HS* (SHA) in acetylcholinesterase;**B**: diagram of interaction mode between (-)-HupA obtained from endophytic bacteria (EHA) and key amino acid Trp279 (Pi-cation interaction and T-shaped Pi-Pi interaction); **C**: diagram of interaction mode between (+)-HupA obtained from chemical synthesis (CHA) and key amino acid Trp279 (Pi-cation interaction and T-shaped Pi-Pi interaction)

# Supplementary Files

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