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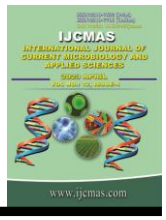

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Molecular Identification of Azithromycin Antibiotic Degradation Bacteria Isolated from Pharmaceutical Waste

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A B S T R A C T

The biological removal of antibiotic residue in the environment has earned great interest. This study presented the biodegradation of azithromycin using *B. cereus* isolated from pharmaceutical waste. This azithromycin-degrading bacterial strain grew well in the range of temperatures between 25°C and 40°C under aerobic condition. On the basis of the observation of the morphology, Gram staining, the determination of biochemistry as well as the subsequent analyse of 16S rDNA, the No. 2 bacteria was identified as *B. cereus* strain. *B. cereus* also indicated the possibility to use for treating azithromycin, the degradation ability was higher. Thus, *B. cereus* could be utilized as an effective biological tool for removing azithromycin residue in the environment.

Introduction

Vitamins The main source of the antibiotic residue in the environment is from the fragmentary metabolization of antibiotics by human and animal. Others come from hospital and veterinary waste, industrial antibiotic producing company, waste from ordinary animal feeds, and agricultural farm where plant biomass is increased by using antibiotics (Rajilic-Stojanovic *et al.*, 2012). Of the antibiotics waste, azithromycin is one of the most typical antibiotics found in the environmental pollutants. It belongs to the β-lactam group of antibiotic drugs. This kind of antibiotics discomposes the cell walls of the bacteria in their growth process. The present of azithromycin in the environment may accompany to the increase of the antibiotic resistant bacteria (Yang *et al.*, 2020).

An alternative for those expensive techniques is the microbial degradation technologies. They are inexpensive and more ecofriendly than chemical and physical methods. In the metabolic processes of microorganisms, numerous enzymes with high catalytic activity are generated that can modify the structure of antibiotic directly or indirectly, which can lower or inactivate the antibiotic activity (Li and Zhang, 2010). Moreover, the products of these biological processes are very simple such as water, CO_2, nitrogen, and simple organic compound. There

are many reports on biological degradation of antibiotics in the environment. Many studies on β-lactam antibiotic resistance of *Bacillus cereus* have also been reported (Fiedler *et al.,* 2019; Kim *et al.,* 2015). This paper evaluated the azithromycin degradation ability by morphological and molecular finger printing method for their physiological and biochemical characteristics were preliminarily studied in this paper.

Materials and Methods

Isolation source

The Pharmaceutical waste site with antibiotic strain were the source for bacterial isolation. The soil samples for microbiological analysis were collected in clean polythene bags. The soil samples were collected from pharmaceutical waste site of March to July, 2020. Soil sample was gathered from top area and 10 to 20 cm in deep.

Enrichment culture of strain

The samples were taken for 10 ml and filtered with gauze respectively. The samples were diluted and diluted to 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 respectively. In the sterile operating table, samples with different dilution concentrations were evenly coated in LB solid medium for 0.2 ml, and cultured in 32 C incubator for 5–7 d (Su *et al.,* 2017).

Isolation and purification of strains

The colonies were randomly picked enrichment lines separation, then were isolated single colonies obtained further separated to obtain single colonies of pure purification line, then pick a single colony is connected to the LB liquid medium, then add 4°C refrigerator spare (Su *et al.,* 2017).

Molecular identification of azithromycin degrading bacteria

The antibiotic degrading isolates were cultivated on the LB agar medium at 30 ◦C for 48 h. The culture was used for the amplification of bacterial 16S rRNA gene by PCR. Two universal 16S rRNA gene primers (F27:5'-AGTTTGATCMTGGCTCAG-3' and R1492: 5'-GGTTACCTTGTTACGACTT-3') were employed. Culture samples of 25 µL were prepared and each sample was composed of 0.5 µL of bacterial culture as the template DNA, The PCR procedure was carried out and the PCR products were detected on 1.5% agarose gel to confirm its purity and size. The PCR products were further sent for sequencing.

The 16S rRNA gene sequences were compared with other 16S rRNA gene sequences available in Genbank by using the Basic Local Alignment Search Tool (BLASTN) program and aligned with similar sequences by using multiple sequence alignment software (Thompson *et al.,* 1994). The phylogenetic tree was constructed by applying the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) 7.0 program based on Kimura-2 parameters with 1000 replicates of bootstrap values (Saitou and Nei, 1987).

Physical and biochemical characteristics of antibiotic degrading bacteria

According to the test results of isolated strains of azithromycin degrading ability, evaluation index for bacterial strains in different culture time. The growth temperature of the highest degradation rate of antibiotic strain, the growth of pH, peptone and yeast extract concentration characteristics were studied to determine the optimum growth conditions (Zhang *et al.,* 2017).

Initial temperature test

The effect of different temperature on bacterial growth was determined by incubation of bacterial cells with 100 ml base culture in 250 ml conical flask with different initial temperature values (25°C, 30°C, 35°C and 40°C), 120 rpm and 7.0 pH. The growth was monitored by determinations of OD_{600} from 2 to 24 h as blank with basic medium.

Initial pH test

The effect of different temperature on bacterial growth was determined by incubation of bacterial cells with 100 ml base culture in 250 ml conical flask with different initial pH values (5.0, 6.0, 7.0 and 8.0) at 37 °C, 120 rpm. The growth was monitored by determinations of OD_{600} from 2 to 24 h as blank with basic medium.

Initial peptone concentration test

Peptone is the main nitrogen source and carbon source for microbial growth. The effect of different temperature on bacterial growth was determined by incubation of bacterial cells with 100 ml base culture in 250 ml conical flask with different initial peptone concentration (0.5%, 1.0%, 1.5% and 2.0%) at 37°C, 120 rpm and 7.0 pH. The growth was monitored by determinations of OD_{600} from 2 to 24 h as blank with basic medium.

Initial yeast extracts concentration test

Yeast extract is also a major source of nitrogen and carbon for the growth of microorganisms. The effect of different temperature on bacterial growth was determined by incubation of bacterial cells with 100 ml base culture in 250 ml conical flask with different initial yeast extracts concentration (0.5%, 0.8%, 1.0% and 1.2%) at 37°C, 120 rpm and 7.0 pH. The growth was monitored by determinations of OD_{600} from 2 to 24 h as blank with basic medium.

Results and Discussion

Isolation and purification of strains

Different antibiotic degrader's bacteria were isolated from contaminated soil of pharmaceutical waste and initially labelled as A, B and C. Isolate A was distinguished with rod, beige in color and Circular. Similarly Rods size, beige in color, Circular and positive gram stain features characterize isolate B. C isolates characterized by cylindrical rods size, white, Irregular large form and negative gram stain.

Molecular identification

The 16S rDNA gene was amplified via PCR using a universal bacterial primer set, which was obtained from the isolates. On the basis of the observation of the morphology, Gram staining, the determination of biochemistry as well as the subsequent analyse of 16S rDNA, the No. 2 bacteria was identified as *Bacillus cereus* strain. The Gram staining and phylogenetic tree of No. 2 bacteria showed in Fig. 1-3.

Physical and biochemical characteristics of antibiotic degrading bacteria

Optimum growth temperature

Temperature, as one of the important factors, affects the growth and reproduction of microorganisms, determine the high azithromycin degradation bacteria optimum growth temperature can provide the basis for better using the strain.

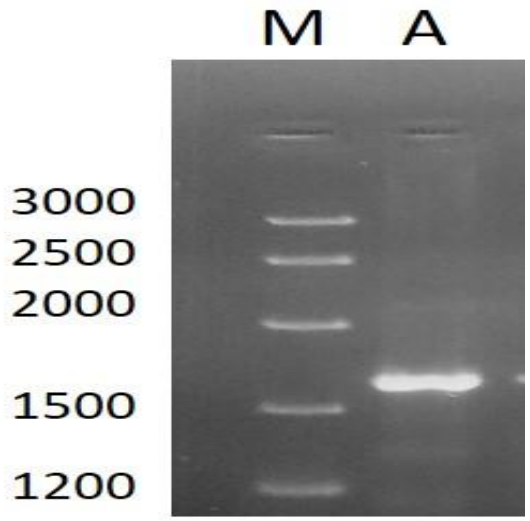
The growth curve of *Bacillus cereus* strain is shown in Fig. 4 at different culture temperatures, the biomass has obvious lag period at 5 h, entered the logarithmic growth phase, and then into the slowly stable after 8 h.

It was found that when the temperature was 25°C, 30°C, 35°C and 40°C, the growth curve of *Bacillus velezensis* strain were similar. However the biomass was the highest at 35°C, 35°C was chosen as the optimum temperature.

Strain optimum growth pH

As can be seen from Fig. 5, the growth of *Bacillus cereus* strain was inhibited at pH 5 and 6 after 8 h, while was increased at pH 7 and 8. And it was very slow due to the inhibition of the environment after 8 h at pH 6 and 7. It was found that the growth curve of *Bacillus cereus* strain were similar at pH 7 and pH 8. However the biomass was the highest at pH 7, pH 7 was chosen as the optimum pH.

Fig.1 Results of PCR amplification of bacterial isolate



(A)- *B. cereus*
M-marker (bp)

Fig.2 Phylogenetic analysis of isolated strain *B. cereus* in the neighbor- joining tree

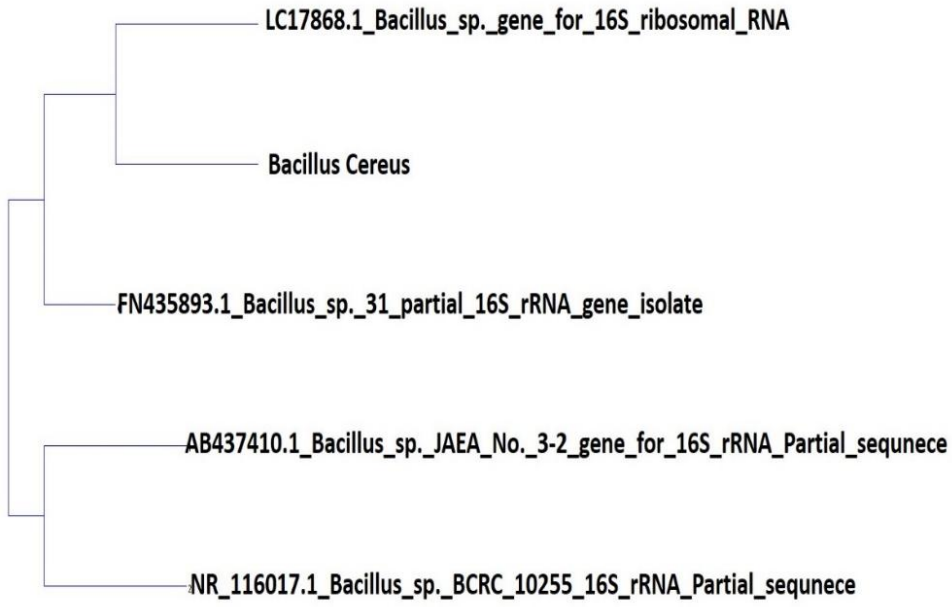


Fig.3 16S rDNA sequencing of bacterial isolate *B. cereus*

CGAAAATGCCCAAAAATTGCTATGCCCCACTGTCAGTTCCAT
ATCAGTTACCGGACGTCTGAATGGGTACGATCATTAGCAATC
CTCCACGATAGCTAAAACCTTAAGATATCGTGCAACGTACGC
ACGTGGCCAATTGCGTACAACGTAGCTGACGTCACGTCGAC
TACGATCGATCGATTACGACCATACATCTTCACGATAGCTAAC
CGTGCACGTATACGTGCAATTGCGTACACGATCATTAGCACA
TCTTTCCAACGAATTAGCTAAAACCTTTAATATCGTGCAACGA
ACGTACGTGGGCCAAAATTTGCTAGCTGACGTCACGTCGATA
GATCGATTACGTAGCTGATCGATACGATCGATCGATTACGTTAC
CGTGATCGGTTATCGGCATAGCTGCAGTCGCGAATTACTGCAC
TGCATCGTACGTAGTAGCGAATTCGAACGTAATTCGGAAACGA
CGATCGAT

Fig.4 Growth curves of *Bacillus cereus* strain at different temperatures

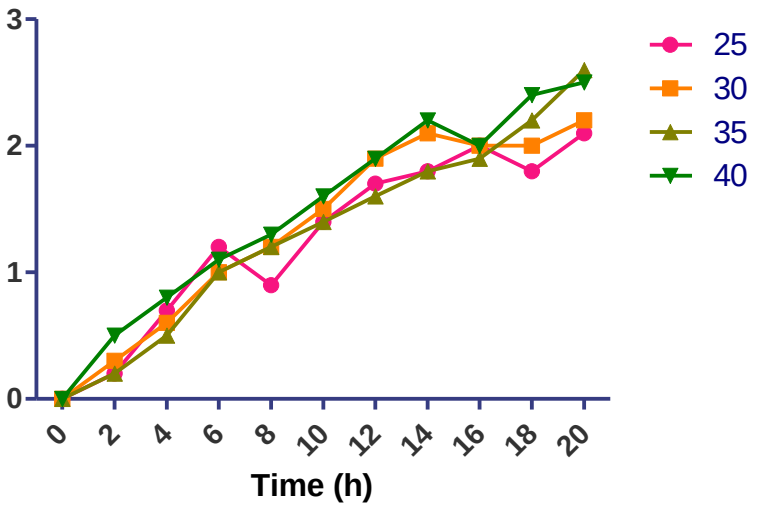


Fig.5 Growth curves of *Bacillus cereus* strain at different pH

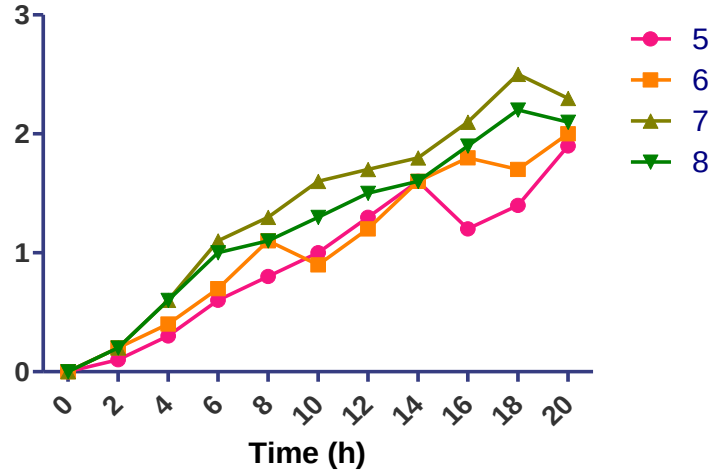


Fig.6 Growth of *Bacillus cereus* strain at different concentration of peptone

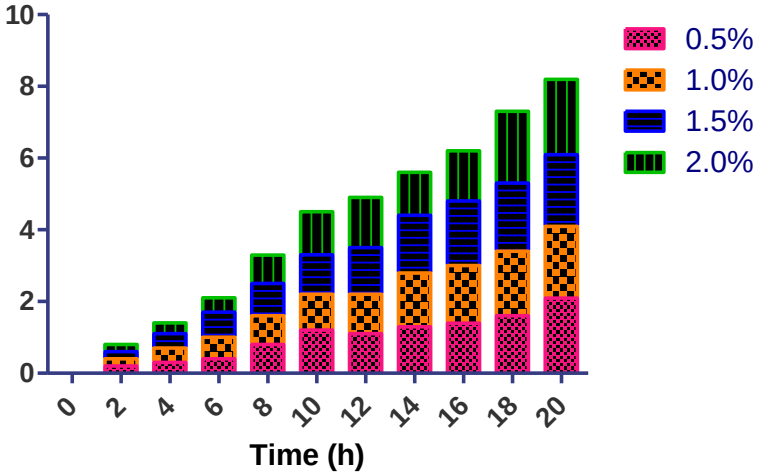
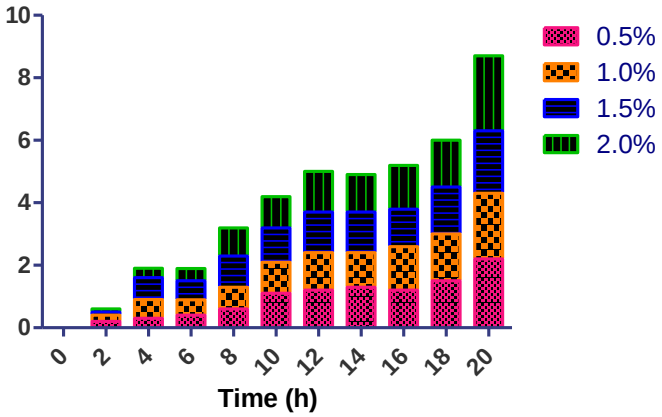


Fig.7 Growth of *Bacillus cereus* strain at different concentration of yeast



Effect of peptone concentration on the growth of the strain

Peptone is the main nitrogen source and carbon source for microbial growth. The effect of peptone with different concentration in culture medium on the growth of *Bacillus cereus* strain was studied in this paper.

The results are shown in Fig. 6, it can be seen that the growth curve of strain had no significant effect in a certain concentration range of peptone, but the biomass of *Bacillus cereus* strain is relatively high at the 2.0% peptone. Therefore, 2.0% peptone was chosen as the optimum peptone concentration.

Effect of yeast extracts concentration on the growth of the strain

Yeast extract is also a major source of nitrogen and carbon for the growth of microorganisms. The effect of yeast extract at different concentrations on the growth of *Bacillus cereus* strain was shown in Fig. 7. The growth curve of *Bacillus cereus* strain was affected by the concentration of yeast extract is not obvious, but the biomass of *Bacillus cereus* strain was higher than in other group in 2.0% concentration, so the culture concentration of yeast extract in the medium can choose 0.0% was chosen as the optimum fermentation yeast extracts concentration.

Environmental antibiotic pollution is a problem that is expected to gain more attention in the near future since antibiotic consumption is still increasing around the world. Rapid and efficient degradation of antibiotic is of great significance for environmental protection. It is reported that Bacillus has the degrade the antibiotics. In this study, the *Bacillus cereus* was identified for the colony morphology and the molecular characteristics by the enrichment and separation from pharmaceutical waste which was effectively degraded antibiotic.

Some scientific researchers were shown that *Bacillus cereus* can produce a variety of digestive enzymes such as amylase, protease, gelatinase, glucanase, and cellulase, and degradate strong organic matter decomposition. Moreover, *Bacillus cereus* can inhibit the growth and reproduction of harmful microorganisms, and can decompose organic substances, organic sulfides, and organic nitrogen. The growth of the *Bacillus cereus* was studied in terms of the effects of temperature, pH, peptone and yeast extract concentration characteristics, and as a result, Different conditions had great effects on the *Bacillus cereus*. The effects of temperature, pH, and the growth of pH, peptone and yeast extract concentration were studied to determine the optimum growth conditions (Zhang *et al.*, 2017; Tan *et al.*, 2016; Meng and Hao, 2017; Rodgersvieira *et al.*, 2015). Which were selected to do single factor test for further study of the optimum conditions for biomass, which was an ability to degrade antibiotic because of production-associated strain.

Moreover, Microbial immobilization technology was occure, this technology was beneficial to increase the number of microorganisms in the reactor, facilitate the solid liquid separation. As a result, they have received increasing attention in research on immobilized microorganism for antibiotic degradation. A high efficiency and stability biological carrier-immobilized *Bacillus cereus* strain system was constructed to remove antibiotic pollution based on biodegradation in the further research, the development and utilization of the genetic resources of *Bacillus cereus* should be further studied.

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