Journal of Life Sciences & Biomedicine, vol. 4(77), No 1, p. 40-45 (2022)

Study of the molecular mass of AZ-130 biomolecule and its stability at low pH

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Received: March 07, 2022; Reviewed: April 29, 2021; Accepted: May 26, 2022

The aim of this work was to determine the approximate value of the molecular mass of an exogenous biomolecule synthesized by the *Bacillus vallismortis* AZ-130 strain isolated from oil-contaminated soils of Azerbaijan and to determine the degree of its stability at low pH values. To achieve the goals, strain AZ-130 was cultivated for 24 hours on a shaker with constant shaking (180 rpm) at 32°C. Next, a supernatant was obtained from the culture solution, which, after the addition of trifluoroacetic acid and 10-fold concentration using 3K MWCO Amicon Ultra-15 Centrifugal Filter Devices centrifugal filter concentrators, was examined by the growth inhibition assay for the presence of activity against *S. aureus* ATCC 29213. Based on the obtained results, it was found that the bioactive molecule produced by the AZ-130 strain is stable at low pH values and has a molecular mass of more than 3000 Daltons.

***Keywords:** Antimicrobial activity, antibiotics, bioactive molecules, natural products, stability, pathogenic bacteria*

INTRODUCTION

Antibiotic-resistant bacteria have been on the rise in recent years, and unfortunately almost all commercially available antibiotics are slowly losing their effectiveness. This becomes a serious health problem (Ventola, 2015; Prestinaci et al.,2015). Of particular concern are six clinically pathogenic bacteria that Louis Rice summarized under the abbreviation "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacter spp.) (Rice, 2008). For bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* (CRAB), vancomycin-resistant *E. coli* (VRE), and multidrug-resistant (MDR) *Pseudomonas aeruginosa* (MDR) for treatment fewer antibiotics are available than 10 years ago (van Duin and Paterson, 2016; Minuta and Arias, 2016; Cetinkaya et al., 2000). The discovery of penicillin by Alexander Fleming in 1928 made a significant contribution to the treatment of infections caused by microorganisms (Fleming, 1944).

According to Newman and Cragg, 70% of antibacterial drugs on the market from 1981 to 2019 are natural products or their derivatives, 28% - synthetic drugs, 1% - imitation of natural products and pharmacophores (Schneider, 2021). Natural products and their structure play a significant role in the development of antibiotics (Smith, 2000; Harvey, 2008). Microorganisms are the most potential source for the production of natural antibacterial drugs (Wright, 2014). The number of bacteria that can theoretically be examined for the presence of new secondary metabolites is enormous. On the other hand, it is estimated that approximately 98% of microorganism species have not been identified to this day and are not subject to cultivation in the laboratory (Wade, 2002; Pham and Kim, 2012). Therefore, it is necessary to develop new, safe and effective antimicrobial compounds to fight pathogens. A recent success in the field of antibiotics was the discovery of

https://doi.org/10.29228/jlsb.76
Available online 30 June 2022

teixobactin, a novel antibiotic that was isolated using an iChip isolation chip approach in 2015 (Ling et al, 2015; Nichols et al., 2010). Cultivation strategies that aim to isolate bacteria from unexplored and unanalyzed sources allow the development of new natural products with completely new structures and biological activities (Challinor and Bode, 2015; Pidot et al, 2014).

The process of developing new drugs is very expensive and time-consuming (Ekins et al., 2019; Wright, 2018). In most cases, in the last stages of the development process, the bioactive molecule is eliminated due to the rediscovery of an already known compound (Atanasov et al., 2021). That is why it is very important to include in the development process the steps of dereplication of bioactive compounds (Schneider, 2021; Carrano and Marinelli, 2015). One of the tools for dereplication is to determine the molecular weight of a bioactive compound in the early stages of development. Identification of the producer strain at the molecular level and information about the approximate molecular weight of the compound of interest will help reduce the likelihood of rediscovery of a known molecule. Moreover, the process of discovering the antimicrobial compound from natural products includes several purification steps of the compound of interest from the cell culture supernatant. It's well known that the purification of antimicrobials involves the use of different buffers with different pH values. That's why, before any small- or large-scale purification, it is necessary to ensure that the compound of interest is stable at low pH.

Bacillus vallismortis strain AZ-130 could be a candidate that produces a novel antimicrobial compound (Агаева, 2019; Aghayeva et al., 2021). The goals of this study are to determine the degree of AZ-130 biomolecule stability at low pH and its approximate molecular mass.

MATERIALS AND METHODS

The object of study was AZ-130 antibacterial compound synthesized by the *Bacillus vallismortis* strain AZ-130 isolated from an oil-contaminated soil sample of Azerbaijan in 2014.

To determine the degree of stability of biomolecule at low pH, 100 ml of TB medium was inoculated with 1 ml of a bacterial suspension at OD_{600} = 0.5–0.6 and incubated at 180 rpm and 32°C for 24 h. After a 24-hour incubation, the culture was centrifuged at 10000 g for 15 min at 4° C and the supernatant was purified from the cell culture by filtration through a 0.22 μm PES membrane. Next, trifluoroacetic acid (TFA, a final concentration of TFA - 0.1%) was added to 1 ml of the supernatant, gently shaken, and centrifuged again at 10,000 g for 15 min at 4°C, followed by filtration through a 0.22 μm PES membrane. The resulting material was analyzed for the antibacterial activity against *S. aureus* ATCC 29213 by the growth inhibition assay. The screening was performed by the soft-agar overlay method as described by Hockett (Hockett and Baltrus, 2017; Balouiri et al., 2016) with some modifications. For screening, 10 μl of material was plated onto an agar plate confluent with the indicator strain - *Staphylococcus aureus* ATCC 29213. The plates were left to dry for 5 minutes under a hood and incubated at 37°C for 24 hours. The range of antibacterial activity (zone of inhibition (ZOI)) was expressed in millimeters as the diameter of the transparent zone (the zone where the growth of the test organism was suppressed). As a control, all the above steps were repeated with TB media.

To determine the approximate molecular mass of the bioactive molecule, the producer strain was inoculated into 50 ml of TSB medium and cultivated overnight at 32°C and 180 rpm. The next day, culture supernatants were clarified from the cell culture by centrifugation at 10000 g for 15 minutes at 4°C, then by filtration through a 0.22-μm polyethersulfone (PES) membrane. A 100 μl sample was taken from the collected supernatant for activity analysis and stored at 4°C. 15 ml of the supernatant was concentrated 10-fold (to 1.5 ml) using 3K MWCO Amicon Ultra-15 Centrifugal Filter Devices centrifugal filter concentrators. The concentration time was 67 minutes; the volume of the concentrate (retentate) is 1.5 ml. The pooled supernatant, retentate, and filtrate at various dilutions (undiluted (x), 2x diluted (x/2), 4x diluted (x/4), and 8x diluted (x/8)) were applied to the Petri dishes with TSA medium, confluent with soft agar containing the indicator strain and incubated for 20–24 h at 37°C. ZOI were measured.

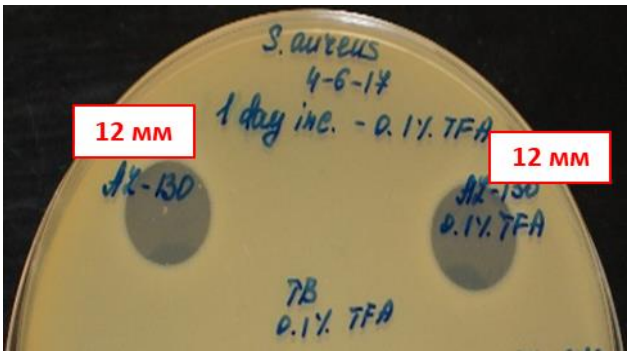

Fig. 1. Stability analysis of the supernatant of AZ-130 strain at low pH.

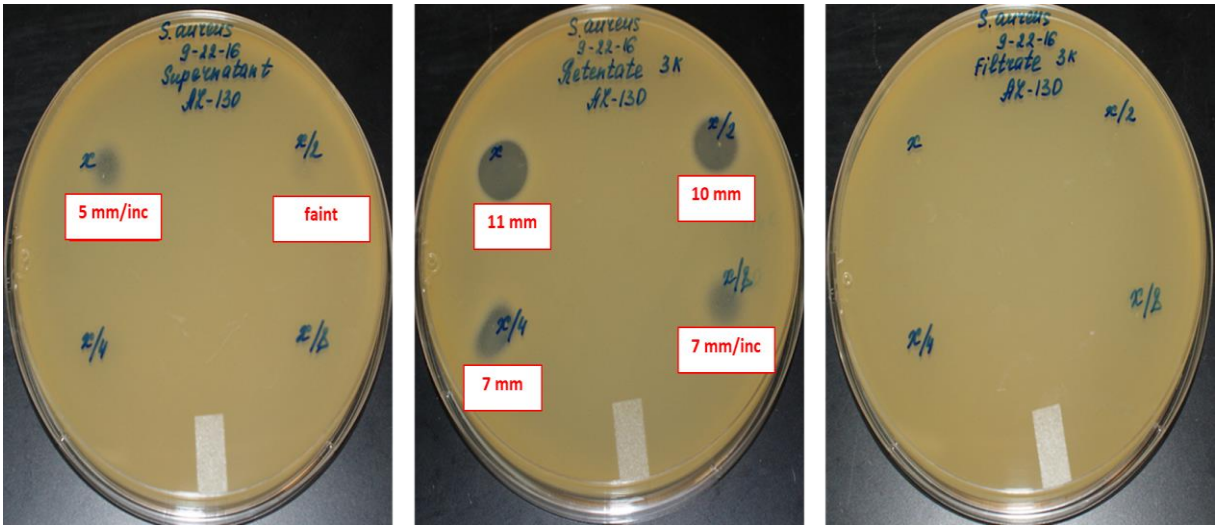

Fig. 2. The results of the analysis of the supernatant, retentate and filtrate in various dilutions against *S. aureus* ATCC 29213.

RESULTS AND DISCUSSION

For the characterization and purification of AZ-130 biomolecule, it was necessary to elucidate the degree of its stability at low pH and determine the approximate molecular mass. Figure 1 shows the results of the analysis of the AZ-130 supernatant at low pH. As can be seen from the figure, the activity of AZ-130 does not change before and after the addition of 0.1% TFA, being 12 mm in both and indicating the resistance of AZ-130 biomolecule to low pH. There was no activity in the controls (TB and TB+0.1% TFA).

Determination of the approximate molecular mass of AZ-130 biomolecule was carried out using 3K MWCO Amicon Ultra-15 Centrifugal Filter Devices centrifugal filter concentrators. As it can be seen in Figure 2, the activity of the undiluted supernatant, equal to 5 mm/partial, after 10-fold concentration increases to 11 mm. In addition, the 4-fold and 8-fold diluted supernatant had no activity, while the 4-fold and 8-fold diluted concentrate showed 7 mm and 7 mm/incomplete activity, respectively. There was no activity in the filtrate.

CONCLUSIONS

AZ-130 strain showed strong activity against gram-positive opportunistic *pathogenic S. aureus* and *E. faecalis* strains (Araeва, 2019) during initial and supernatant screenings. By 16S rRNA gene sequencing AZ-130 strain was identified as *Bacillus vallismortis*. It was found that strain AZ-

130 produced a single compound with antibacterial activity with the retention time at HPLC column 12.854 min (Aghayeva et al., 2021).

The AZ-130 bioactive molecule is stable at low pH values. This is very important for the correct choice of appropriate solutions in the processes of further purification of selected biomolecules from the cell supernatant. Moreover, the results of 10-fold concentration of the AZ-130 supernatant using Amicon Ultra-15 Centrifugal Filter Devices 3K MWCO centrifugal filter concentrators showed that the molecular mass of the AZ-130 bioactive compound is greater than 3000 Da. A search in the APD3 database revealed that there are currently no known antibiotics produced by *B. vallismortis* bacteria with molecular mass equal to or bigger than 3000 Da.

ACKNOWLEDGEMENT

This article was prepared based on the results of experiments carried out during the 2014-2018 years at the Fraunhofer USA Center for Molecular Biotechnology (currently Fraunhofer USA, Mid-Atlantic Center, Department of Biotechnology). The presented work is a part of the doctoral thesis to be submitted to the Institute of Molecular Biology & Biotechnologies of Azerbaijan NAS.

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AZ-130 biomolekulunun molekul kütləsinin və pH-ın aşağı qiymətində sabitliyinin tədqiqi

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Təqdim olunan işin məqsədi Azərbaycanın neftlə çirklənmiş torpaqlarından ayrılmış *Bacillus vallismortis* AZ-130 ştamının sintez etdiyi ekzogen biomelokulun aşağı pH-da stabillik dərəcəsinin və molekul kütləsinin təxmini qiymətinin müəyyən edilməsi olmuşdur. Qarşıya qoyulmuş məqsədlərə nail olmaq üçün AZ-130 ştamı 24 saat ərzində 180 rpm və 32°C-də kultivasiya edilmişdir. Daha sonra kultural məhluldan alınan supernatantın aktivliyi trifluoroasetik turşu əlavə edildikdən və 3K MWCO Amicon Ultra-15 Centrifugal Filter Devices istifadə edərək 10 dəfə qatılaşdırıldıqdan sonra böyümənin inhibə edilməsi metodu ilə *S. aureus* ATCC 29213 qarşı tədqiq edilmişdir. Əldə edilmiş nəticələrə əsasən AZ-130 ştamı tərəfindən istehsal olunan bioaktiv molekulunun pH-ın aşağı qiymətlərində stabil olması və onun molekul kütləsinin 3000 Daltondan çox olması müəyyən edilmişdir.

Açar sözlər: *Antimikrob aktivlik, antibiotiklər, bioaktiv molekullar, təbii məhsullar, sabitlik, patogen bakteriyalar*

Изучение молекулярной массы биомолекулы AZ-130 и ее стабильности при низком значении уровня pH

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Целью представленной работы было установление приблизительного значения молекулярной массы экзогенной биомолекулы, синтезируемой выделенным из нефтезагрязненных почв Азербайджана штаммом *Bacillus vallismortis* AZ-130 и определение степени ее стабильности при низких

значениях pH. Для достижения поставленных целей штамм AZ-130 в течение 24 часов культивировали на шейкере при постоянном встряхивании (180 об/мин) и температуре 32°C. Далее из культурального раствора был получен супернатант, который после добавления трифторуксусной кислоты и 10-кратного концентрирования с использованием центробежных фильтрующих концентраторов 3K MWCO Amicon Ultra-15 Centrifugal Filter Devices, был исследован методом подавления роста на наличие активности против *S. aureus* ATCC 29213. На основе полученных результатов установлено, что биоактивная молекула, продуцируемая штаммом AZ-130, стабильна при низких значениях pH и имеет молекулярную массу более 3000 Дальтон.

Ключевые слова: *Антимикробная активность, антибиотики, биоактивные молекулы, натуральные продукты, стабильность, патогенные бактерии*