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Evaluation of *norB* gene expression in ciprofloxacin resistant *Staphylococcus aureus* strains

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Introduction:

Staphylococcus aureus is one of the most important causes of hospital and community-acquired infections. Staphylococcus aureus has become one of the most important health problems in the world due to its potential pathogenicity and increasing resistance to antibacterial agents. Today, methicillin-resistant Staphylococcus aureus (MRSA) isolates are considered as the most dangerous pathogenic strains. The use of these antibiotics against gram-positive pathogens such as MRSA isolates can lead to their resistance to these antibiotics. Efflux systems, especially efflux pump NorA, NorB, NorC, cause the antibiotic to be removed and decrease the intracellular concentration, which results in a decrease in the performance of medicinal compounds. Considering that Staphylococcus aureus infection, especially MRSA strains, is an important challenge in infection control in hospitals due to the lack of quick and appropriate treatment, the lack of proper efficacy and the high cost of available drugs, in this research, the frequency of resistant Staphylococcus aureus strains isolated from patients to common antibiotics, especially fluoroquinolone antibiotics, and the expression level of norB

gene as one of the genes involved in the occurrence of resistance among sensitive and resistant strains to Fluoroquinolone antibiotic was evaluated.

Materials & Methods:

Samples

The study population includes all strains of *Staphylococcus aureus* obtained from clinical samples of hospitalized patients as well as samples suspected of *Staphylococcus aureus* in the laboratories of hospitals and clinics in Qom city. Samples from hospital were collected using sterile swabs after cleaning the wound surface from the deep parts of the wound and immediately inoculated into tubes containing sterile brain and heart fluid medium (BHI). The tubes were incubated at 37°C for 24 h, and then one loop of the medium was cultured on brain and heart agar medium. The obtained clones were evaluated by gram staining, catalase, tube coagulase, growth on mannitol salt agar, growth on blood agar medium, DNase test.

Amplification of norB, femA and rpoD genes using Multiplex PCR

In this study, three pairs of primers were used for femA, norB and rpoD genes. The femA gene primers were selected for the molecular identification of *Staphylococcus aureus* as the strain identification gene. Primers related to norB genes were designed using oligo7 software. The rpoD gene was also selected as a reference gene.

Determination of antibiotic sensitivity and minimum inhibitory concentration (MIC)

Antimicrobial susceptibility testing was performed based on the modified Kirby-Bauer disk diffusion method using Muller-Hinton agar and according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Antibiotic discs used were Polymyxin B, ciprofloxacin, ofloxacin, norfloxacin and levofloxacin. The standard strain of Staphylococcus aureus ATCC 25923 was used to control the tests to determine the sensitivity to antibiotics. MIC determination test was performed according to the method described by Andrews and CLSI M07-A9 guidelines.

Evaluation of norB gene expression

Bacterial isolates of resistant *Staphylococcus aureus* and standard strain of *Staphylococcus aureus* were cultured in two BHI culture media containing sub-MIC concentration of ciprofloxacin antibiotic and without antibiotic until OD of about 0.6. RNA extraction was done by RNX-plus extraction kit (Cinagen). After preparing the RNA, cDNA was made from it using

the 2-steps RT-PCR Kit (vivantis). The real-time PCR reaction was performed for rpoD and norB genes using EvaGreen and reaction mixture (Pisgam Company). The data obtained data were analyzed using REST software.

Results & discussion:

In the present study, out of 240 suspected strains, they were investigated using common biochemical tests and molecular methods. Based on the results of biochemical and culture tests, including catalase, coagulase, culture in mannitol-salt-agar medium, hemolysis in blood agar medium and DNase properties, out of 240 suspected strains, 82 isolates were identified as *Staphylococcus aureus*.

Staphylococcus aureus carries a set of highly conserved genes that can be used for rapid diagnosis of this pathogen. The conserved gene femA has been used as the identification gene of *Staphylococcus aureus*. All 82 isolates confirmed by biochemical tests were investigated using Multiplex PCR method. Among these, 40 isolates (48.78%) containing all three genes (femA, norB and rpoD) were detected. Rosy Chikkala and colleagues investigated the identification of genes related to the diagnosis of *Staphylococcus aureus* (mecA, femA, 16srRNA, nuc, fem-A1) and after comparing these genes, they stated that due to the heterogeneity of the femA gene, the usefulness of this gene as a specific marker for *Staphylococcus aureus* species has limitations.

Resistance to fluoroquinolone antibiotics is increasing, and one of the resistance systems to these types of antibiotics is the efflux pump mechanism. For this reason, the pattern of antibiotic resistance of *Staphylococcus aureus* to 4 types of fluoroquinolone antibiotics was investigated. The results obtained in the antibiogram pattern showed that all the isolates obtained in this research are resistant to polymyxin B (100%), which has the highest percentage of resistance among the investigated antibiotics. Also, the lowest level of resistance was determined with 26 samples (65%) related to ciprofloxacin antibiotic.

The rate of resistance to ciprofloxacin determined in this research is lower than the rate of resistance reported by Rezazadeh (85.7%) and Rahimi (95%). Ciprofloxacin resistance in this research compared to the study of Ali Gholi (38%), Adebayo O Shittu (29%), Sultan Dallal (3%) and Pirmoradian (4.7%) shows an increase. The increased use of antibiotics has contributed to the development of multidrug-resistant strains and the circulation of resistance

among bacterial species. As seen in this research and other studies, *Staphylococcus aureus* has been able to become resistant to most antibiotics used in treatment.

Quantitative measurement of norB gene expression in fluoroquinolone-resistant strains showed that the expression level of this gene increased 60 times on average after the strains were exposed to the ciprofloxacin. The results of a study conducted by Yanpeng Ding et al showed that the expression level of this gene increased 171 times in the presence of antibiotics. Investigations show that resistance to quinolones is caused by chromosomal mutations that may cause overexpression of efflux pumps norA, norB, norC, and tet38. Efflux systems, especially norA, norB and norC efflux pumps, cause antibiotic excretion and decrease intracellular concentration, which results in a decrease in membrane permeability to pharmaceutical compounds. NorB induces resistance to some NorA substrates such as hydrophilic fluoroquinolones (norfloxacin and ciprofloxacin), biocides and biocides (tetraphenylphosphonium and cetrimide) and ethidium bromide dye, and also induces NorA resistance to some of its non-substrate compounds like hydrophobic fluoroquinolones and sparfloxacin and tetracycline.

Conclusion:

The obtained results show that the level of resistance of *Staphylococcus aureus* to ciprofloxacin, which is one of the commonly used antibiotics, is increasing, and the most important mechanism can be the increase in the production of the efflux pump norB.

Keywords:

Staphylococcus aureus, efflux pump, gene norB, ciprofloxacin, Real-Time PCR

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Declaration of conflict of interest:

The authors declare that they have no conflicts of interest.

Statement on ethics:

the authors declare that this work has not been published elsewhere nor submitted to another publication simultaneously.

Optimization of uranium biosorption process by autoclaved Micrococcus Luteus biomass using response surface methodology

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Introduction

Toxic heavy metal contamination of industrial water is a significant universal problem. They accumulate in living tissues throughout the food chain which has humans at its top. These toxic metals can cause accumulative poisoning, cancer and brain damage. Uranium is one of the most seriously heavy metals because of its high toxicity and radioactivity. Excessive amounts of uranium have found

their ways into the environment through the activities associated with the nuclear industry. Conventional methods for removing uranium from wastewaters include; precipitation, evaporation, ion exchange, membrane processing and adsorption. Nevertheless, these methods have several disadvantages, such as high installation and operating costs, requirement of preliminary treatment steps, difficulty of treating the subsequently generated solid waste, and low efficiency at low metal concentration (o 100 mg/L). Owing to increase in environmental awareness, there has been an emphasis on the development of new environmental friendly ways to decontaminate waters using low-cost methods and materials. In this endeavor, microbial biomass has emerged as a complementary, economic and eco-friendly device for controlling the mobility and bioavailability of metal ions. The present work evaluates the performance of the *Micrococcus luteus* biomass to remove uranium ions from aqueous solutions. The effect of pH, temperature, initial concentration, and sorbent dose on biosorption capacity is studied. The results showed that the factor of initial uranium concentration, sorbent dose and pH statistically (p-value‹ 0.05) affect the uranium biosorption process. In contrast, temperature factor (p-value› 0.05) statistically have no effect on uranium removal by M. luteus.

Materials & Methods

Materials: *Micrococcus luteus* bacteria used in this research with PTCC No. 1408 was purchased from the Scientific and Industrial Research Organization of Iran. Uranyl nitrate salt ($UO_2(NO_3)_2.6H_2O$) was obtained from the Research Institute of Nuclear Sciences and Technologies. Nutrient agar culture medium, sulfuric acid and sodium hydroxide and other materials used in this research were supplied from Merck Company.

Preparation of uranium solutions and biomass: A stock solution containing 1000 mg L-1 of U(VI) was prepared of UO2(NO3)4.6H2O. The working solutions were prepared daily from stock solutions.
In this study, the biomass of *Micrococcus luteus* bacteria was heat treated in an autoclave at a temperature of $121^{O}C$ for 15 minutes at a pressure of 1.5 atmospheres. Then the parameters of temperature, pH, initial concentration of uranium and amount of biosorbent were optimized using the response surface method in Design Expert software.

Experimental design and batch biosorption studies: The design of the experiment was done using the response surface method by Design Expert software. Four variables, including initial uranium concentration (10-100 mg/l), temperature (25-40 C°), pH (2-5) and biosorbent dose (5-25 g/l), in five levels α-, -1, 0, +1, α+, 1 were used to design the experiment. Therefore, 27 experiments were presented using a central composite design. Uranium biosorption experiments were performed by adding specified amounts of bacterial biomass in 20 ml Erlenmeyer flasks containing uranium solution with the concentration and pH corresponding to each experiment, with the specified temperature in the Shaker. After 90 minutes, each sample was centrifuged at 4500 rpm for 15 minutes at 4°C. Then, the remaining uranium in the solution was measured by ICP (Perkin Elmer/Optima 7300DV). The percentage of uranium removal (R) was calculated by equation 1:

$$R(\%) = \frac{(C0-Cf)}{C0} * 100 \qquad (1)$$

Where C_0 and C_f are the initial and the final concentrations of the metal ion solutions (mg/l), respectively.

Results & discussion

By using the RSM-CCD method, the optimization of the biosorption process was carried out. The experimental results based on each point of the experimental design. Then, using analysis of variance (ANOVA), the obtained results were evaluated.
The equation obtained for the biosorption efficiency of uranium by *Micrococcus luteus* is expressed as follows:

Removal= +381.00267-15.47727* C(ppm)-45.12825 * pH+0.62243* T (^{0}C)-7.08198* M (g/l) +4.10347 *C (ppm) * pH+7.56296E-003* C (ppm)* T (^{0}C) +0.18786* C (ppm)* M (g/l)-0.10822* pH * T (^{0}C) +2.14135* pH * M (g/l)-0.036100* T (^{0}C) * M (g/l) +0.10988* C (ppm)^2-8.84880* pH^2-0.047385* C (ppm) * pH * M (g/l)-0.030184* C (ppm)^2* pH

The F-value and p-value of the proposed model are equal to 12.19 and 0.0001, respectively, reflecting the accuracy of the proposed model. This model with R^2 equal to 0.93 shows that the proposed model can well predict the experimental values.
The results showed that the factor of initial uranium concentration, sorbent dose and pH statistically (p-value‹ 0.05) affect the uranium biosorption process. In contrast, temperature factor (p-value› 0.05) statistically have no effect on uranium removal by *Micrococcus luteus*. With increasing uranium concentration from 10 mg/l to 100 mg/l, the removal decreases from %100 to %99/6. The increase in absorption efficiency at low uranium concentrations indicates that the bacterial biosorbent used in dilute metal solutions is efficient. On the other, one of the most important effective parameters in biosorption is the pH of the solution. with increasing the pH from 2 to 4/25, the removal increased from %49/18 to %100. Because the number of biosorbent binding sites decreases at low pH due to the protonation of functional groups. But with increasing the pH from 4/25 to 5, the removal decreased to %99/65 due to the formation of uranyl complexes. Also, with increasing the of biosorbent dose due to the increase in the area of the biosorbent surface, which enhances the number of grafting sites, the absorption percentage can be increased. Therefore, the results showed that the removal of uranium increases from %89/82 to %98/83 by increasing the amount of biosorbent from 5 g/l to 25 g/l.

Conclusion

In this research, he results indicated that the pre-treated biomass under the conditions suggested by Design Expert software (19.75 g/liter of biomass, temperature 32.14 ^{O}C and pH 3.33) is able to remove approximately 99.98 percent of uranium from the contaminated area is 26.11 mg/liter of uranium, which shows its valuable potential in bioremediation applications of uranium from acidic wastewaters contaminated with low concentrations of uranium.

Keywords: Biosorbent, Design–Expert, Radionuclide, Bioremediation

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Declaration of conflict of interest:
The authors declare that they do not have any conflict of interest.

Statement on ethics:
The authors declare that this work has not been published elsewhere and has not been submitted to another publication at the same time.