RESEARCH ARTICLE

An Integrated Suggestion to Decolourize Allura Red AC Dye in the Wastewater of Pharmaceutical Industries Using a Local Bacterial Isolates

Ahmed D. Homady, Mohammed A. Fayidh*

Department of Biology, College of Education for Pure Science Ibn Al-Haitham, University of Baghdad, Baghdad, Iraq

Received: 02nd August, 2022; Revised: 22nd August, 2022; Accepted: 03rd September, 2022; Available Online: 25th September, 2022

ABSTRACT

Many types of dyes used in pharmaceutical and food industries such as (Allura Red AC) might be thrown out into the environment, leading to contaminating the natural water sources and its concentration in some tanks. To support the biological treatment, designed this project was to isolate and identify the bacteria from the biological treatment tanks, able to decolourize some industrial dyes waste used in the pharmaceutical industry in Iraq. Streaking on Tryptone soy agar with Allura red dye 0.001-mg/L, more than 11 single bacterial colonies were separated and tested for their ability on decolorization. In this paper, only Two isolates will be in focus for decolourizing. The Absorbance of Allura red at wavelength 510 nm of spectrometer demonstrated its clearly decreased within the incubation time 6–24 hours and depended on the initial concentration of the dye, which was done in MSM media. The isolates were identified by the VITEK-2 XL compound system as well as the 16S rRNA gene. The identification results showed that these isolates were related to *Escherichia coli* and *Klebsiella pneumoniae.*

Keywords: Allura Red AC, Biological treatment, Decoloruziation, *Escherichia coli, Klebsiella pneumonia*.

International Journal of Drug Delivery Technology (2022); DOI: 10.25258/ijddt.12.3.46

How to cite this article: Homady, AD, Fayidh, MA. An Integrated Suggestion to Decolourize Allura Red AC Dye in the Wastewater of Pharmaceutical Industries Using a Local Bacterial Isolates. International Journal of Drug Delivery Technology. 2022;12(3):1196-1200.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Dyes are an important class of synthetic organic compounds, widely used in the Pharmaceutical industry, textile, leather, plastic, cosmetic and food industries are therefore common industrial pollutants.[1] Allura Red AC (E 129) is an Azo dye, chemically represented as R–N=N-R', where –N=N- is the azo group, and the R or R' can be either aryl or alkyl compounds and it's used in pharmaceutical products and released the excess of it to the environments.[2,3]

Pharmaceutical industry effluent released from industries is a complex mixture of many polluting substances such as organic chlorine-based pesticides, heavy metals, pigments and dyesl, and must be treated before being discharged into the environment because of their recalcitrant nature and potential toxicity to animals and human.[4-6] Dyes also obstruct light penetration and oxygen transfer, affecting water bodies.[7]

Biological treatment offers a cheaper and environmentally friendly alternative to dye decolorization and wastewater remediate and reuse in other industrial processes.[8,9] The general approach for the bioremediation of textile effluent is to improve the natural degradation capacity of the indigenous microorganism that allows degradation and mineralization of dyes with a low environmental impact and without using potentially toxic chemical substances, under mild pH and temperature conditions.[10]

Naturally, these environments (fluids or sludge) contain a lot of bacterial genera, which have many enzymatic capabilities that may not be available in other isolates of contaminated environments. The investigation and detection of such isolates are the main reasons for their applications in the field of biodegradation, including the decomposition and decolourization of dyes to reach less polluted environments.[11,12]

This paper proposed a protocol to obtain the environment of bacterial isolates in the best way to classify them to overcome all the difficulties of researchers in the classification of such isolates, besides the possibility of obtaining bacterial isolates or strains which have not been elected or registered before.

MATERIALS AND METHODS

Allura Red AC Sources

Allura Red AC (E 129) was used in this paper and obtained from Samarra Drug and Pharmaceutical Industry (SDI) as a colored agent used in Pharmaceutical products (Syrup and Tablets). 0.001-mg/mL was dissolved and mixed with MSM and other media for decolorization analysis. Allura Red is azo dye with the molecular formula $C_{18}H_{14}N_2Na_2O_8S_2$.

*Author for Correspondence: mohammedcbt66@gmail.com

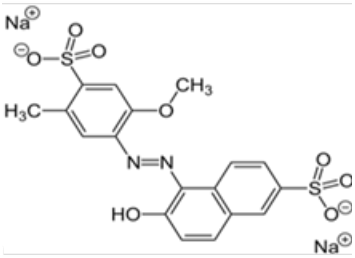

Fig 1: The structural formula of Allura Red AC

It has a molecular weight of 496.43 and CAS Registry Number 25956-17-6. The full chemical name is disodium 2-hydroxy-1-(2-methoxy-5-methyl-4-sulphonatophenylazo) naphthalene-6-sulphonate and has the structural formula shown in Figure 1.

Dye Concentration

Mineral Salt Media (MSM) was used as follows: Glucose: 3 g/L; $(NH_4)_2SO_4$: 2 g/L; KH_2PO_4: 1 g/L; K_2HPO_4: 10 g/L; $MgSO_4.7H_2O$: 0.1 g/L and NaCl: 5 g/l.13

1-mg of dye was dissolved in 1000 mL of deionized water as stock concentration and kept it at dark place of room temperature. For Preparing MSM media with dye, 0.1-mL of dye stock was added to 99.9 mL of MSM media than sterilized at 12°C, 1.5 atm for 15 minutes and used as standard media for decolourizing assay by the bacterial isolates.

Culture Media

Nutrient Broth and Nutrient agar from Oxoied (Oxoid company -500 g) was prepared due to the instruction protocol and used for growth of bacterial isolates and adjusted at pH 7.4 ± 0.2 at 25°C. For biochemical bacterial tests purpose, selective agar medium was used such as Macconkey agar, Blood agar (Blood was added to NA media after autoclaving), Salmonella-Shigella agar ''SS agar '', Eosin Methylene Blue agar, Triple Sugar Iron –'' TSI slant agar''. All media was disinfected by autoclave at 121°C, 1.5 atm. for 15 mins, distributed into disposable plates and test tubes, and kept at the refrigerator.[14] Other enzyme tests such as Urease, Oxidase, Lactose fermentation, Catalase and Indole Production were prepare their media and indicators due to Ali *et al.*[15]

Isolation of Bacteria from Wastewater Samples

Microbial isolations were carried out by serially diluting the sample collected from the pool engineering inspection treatment department in SDI company. Effluent samples were inoculated in serial sterile distilled water, transferred, and mixed 0.1 mL from the last 4th dilution tubes to nutrient agar plates. The plates were incubated at 37 ± 2˚C for 24 hours Growth colonies with distinct morphology were picked up and purified by regular sub-culturing using streaking on NA agar. The strains were kept under-minimized media with 20% glycerin sterile solution.[14]

Decolourization Assay

The density of the suspension of bacterial cells was applied with all initial inoculation compared to the McFarland turbidity standard (0.5 McFarland turbidity standard tube, approximately cell density (1.5×10^8 CFU/mL).[15,16]

Decolorization activity expressed in terms of percentage was determined. The decrease in absorbance was monitored at λ_{max} (501 nm) for a particular dye each 6 hours. Decolorized sample (5 mL) was withdrawn periodically and centrifuged at 10000 rpm for 15 minutes. The inoculated dye-free medium was used as blank and the un-inoculated dye as standard. All assays were performed in triplicate.[15] The color removal efficiency of bacterial isolates was expressed as the following equation:

$$\%\text{Decolorization} = \frac{(\text{Initial absorbance} - \text{Final absorbance})}{\text{Initial Absorbance}} \times 100$$

Molecular Identification of Bacterial Isolates

DNA Extraction

Genomic DNA was isolated from the most efficient isolates decolorization of the Allura Red AC dye. The protocol of ABIO pure Extraction by Tanner *et.al.,*[17] as summarized in the following steps:

For pellet cells, 1-mL of overnight culture for 2 min at 13000 rpm. The supernatant was then discarded. The cell pellet was re-suspended completely in 200 µL of Buffer CL. For protein digestion and cell lysis, 20 µL of Proteinase K solution (20 mg/mL) was added to 200 µL of Buffer CL and cell pellet, then the tube was mixed vigorously using vortex and Incubated at 56˚C for 30 min, for further lysis it was incubated 30 minutes at 70˚C.

After incubation, 200 µL of Buffer BL was added to the sample then the tube was mixed vigorously using vortex and Incubated at 70˚C for 30 minutes . From absolute ethanol 200 µL was added to the sample, pulse-vortex to mix the sample thoroughly.

All of the mixtures were transferred to the mini-column carefully, then centrifuged for 1 min at 6000 x g above (> 8000 rpm), and the collection tube was replaced with a new one. From Buffer BW 600 µL was Added to the mini-column, then centrifuged for 1-minute at 6000 x g above (> 8,000 rpm) and the collection tube was replaced with a new one. From Buffer TW 700 µL was applied, centrifuged for 1-minute at 6000 x g above (> 8000 rpm). The pass-through was discarded and the mini-column was reinserted back into the collection tube.

The mini-column was Centrifuged at full speed (> 13000 x g) for 1-minute to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 mL tube. From Buffer AE 100 µL was added and Incubated for 1-minute at room temperature, then centrifuged at 5000 rpm for 5 minutes.

Primers Preparation

For molecular identification of decolorized isolates, Two universal primers supplied by Macrogen Company as a lyophilized form were used for isolation of specific 16S rRNA gene.[18] Lyophilized primers (27F with sequence 5'AGAGTTTGATCCTGGCTCAG-3'and 1492R with sequence 5'TACGGTTACCTTGTTACGACTT-3'sequences), both were dissolved in a nuclease free water to give a final concentration of 100 pmol/µL as a stock solution. A working solution of these primers was prepared by adding 10 µL of primer stock solution

Table 1: The most important phenotypic characteristics, growth form, and some results of enzymatic reactions of the bacterial isolates.

			Growth on the medium				*Enzyme production*					
Sample id	*Gram stain*	*Microscopic shape*	*Nutrient agar*	*Macconkey agar*	*Tsi agar*	*Emb agar*	*Msm with dye*	*Urease*	*Oxidase*	*Lactose*	*Indole production*	*Catalase*
a	+	Short rods	++	-	A/A H_2S^-	No growth	+++	+	-	-	-	+
B	-	Rod-shape	+++	+	k/A G^+ H_2S^-	Pink /with metallic sheen	+++	-	-	+	+	-
C	-	Rod-shape	++++	Purple mucoid	A/A G+ H_2S^-	Pink /no metallic sheen	++++	-	+	+	-	+
E	+	Coccus, tetrad-arranging	++	Round shape with yellow pigment	K/K $G^-H_2S^-$	No growth	++++	+	+	+	-	+
G	-	Rod-shape	++++	+	k/A G^+ H_2S^-	Pink /with metallic sheen	+++	-	-	+	+	-
J	-	Rod-shape	+++	Lilac mucoid	A/AG^+ H_2S^-	Pink /no metallic sheen	++	-	+	+	-	+

A= Acid, K= Alkaline, G=Gas,+,++,+++ =Positives results, - = Negative results

(stored at freezer -20°C) to 90 µL of nuclease-free water to obtain a working primer solution of 10 pmol/µL.

Agarose Gel Electrophoresis for DNA Sequences Analysis

After PCR amplification, 1.5% agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria.[19] For analyzing the PCR product later, its send to Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation–company–Korea. For the identification of bacterial isolates, the Fasta sequencing were compared with the similar registered sequences at GenBank of NCBI-USA.

RESULTS AND DISCUSSION

Isolation of Bacteria from Wastewater Samples

By the serial dilution method that was adopted to obtain bacterial isolates from the neutralized basin in Samarra drug Company, it was obtained 11 distinct and heterogeneous isolates in their phenotypic characteristics as the shape and color of colonies, their edges, their internal structure, their spread on the solid medium. This research was focused on six isolates that showed the best decolorize for Allura red dye (Figure 2), two isolates are interaction Positive for Gram stain while the other four are Negative with different characteristic smells. On the other hand, these 6 isolates showed a difference in their enzymatic ability to decolourize the Allura red dye, and it can be considered the best among the other isolates and these isolates had been initially given marks (names) as capital English letters before identification by their 16S rRNA Gene.

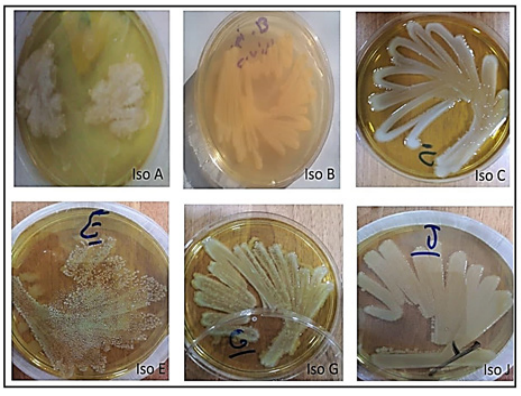

Figure 2: Growth of the best bacterial isolate on Nutrient Agar were isolated from neutralize basin at SDI company

In addition, the six isolates differed in the most important phenotypic traits and their growth on some culture media, as well as were observed vary in important enzymatic reactions such as the production of Urease, Oxidase, Lactose, Indole Production, Catalase, all these results were summarized and shown in Table 1.

On the other hand, most of the results of approved biochemical tests do not give results similar to what is proven for common pathological isolates. It showed some of these results, different results at the level of bacterial genus and/or species, so resorting to the use of diagnostics based on the 16S rRNA gene, is the best method for the classification of environmental isolates, as well as achieving unique properties for these isolates, and that was done in this research.

Decolorization Assay

By adopting the serial dilutions method, bacterial isolates distinguished by their ability to decolorize the Allura red dye in the minimum salt medium (MSM)were obtained. Six of the best isolates were applied in experiments to reduce the color of the red dye, with the initial concentration of dye (0.001 mg/L) and initial bacterial inoculates of 1.5*108 CFU/mL. The shortening speed was evident in the first hours of incubation at a temperature of 35°C, and the shortening process was clear

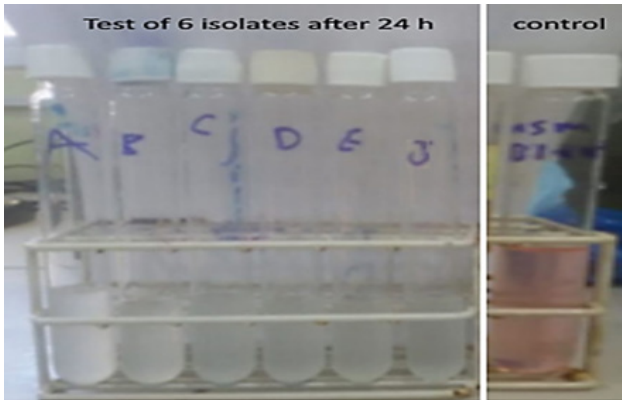

Figure 3: Shown the Decolorization of Allura Red in MSM broth after 24 hours incubation media by the best 6 isolates compared with control.

after 24 hours compared to the color stability of the dye in the non-inoculated MSM medium (control medium), as shown in Figure 3.

The UV spectrophotometer reading at 501 nm for MSM media inoculated with the best isolates is shown in Figure 4(1). The results showed that almost all of the isolates had the same initial absorption reading (0.34) and decreased during the isolate's incubation time within 24 hours. These results indicate performed for decolorizing the dye and consuming it by the study's bacterial isolates.

According to chemical information, Allura Red AC dye is soluble in water and slightly soluble in 50% ethanol. Spectrophotometry maximum wavelength is approximately 501 nm, so can determine the absorption spectrum of the sample solution dissolved in water using UV-visible spectrophotometer.

From Figure 4 (2), the % Decolorization of were calculated for all six bacterial isolates at three period time (6, 12 and 24 hours) of incubation. The results show that the best and faster isolates, its isolate C and E with 99.1 and 98.7%, then Iso B and A respectively after 24 hours incubation, while the low decolorizing and less growth of isolate by Iso J and G isolates with 60.1 and 50.5% after 24 hours These results indicate that these isolates differ in their ability of decolorization and their bionomic taxonomy, as shown later.

Molecular Qualitative Diagnosis of Isolates Using 16S rRNA Gene Precursors

By using specific universal primers for isolation of 16s rRNA gene as shown in DNA extraction parts, all six isolates which have a good ability to decolourize Allura red dye were chosen for extraction of their DNA and for molecular identification by 16s rRNA gene.

The fragments of the 16s rRNA gene for Unknown bacterial species was amplified by PCR then electrocuted using 1.5% agarose gel electrophoresis stained with Ethidium Bromide under 100 volts for 60 mints with ladder marker (M: 100-1500bp) (Figure 5).

The results showed that DNA concentration values were detected proximately 99.5%, and yielding of PCR products of 16S rRNA gene using a primer of 27F and 1492R; the amplified fragments gene was at least 1,300 bp or more as shown in sequencing data Table 2.

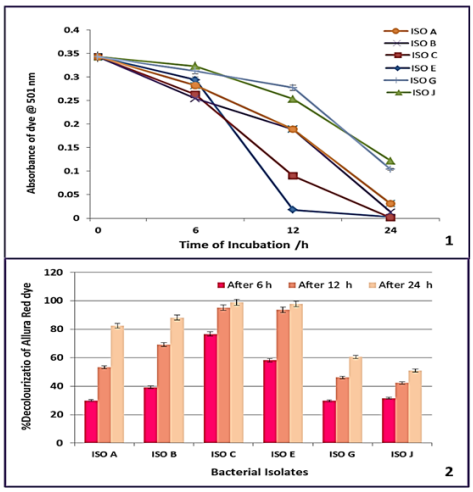

Figure 4: Decolorization pattern under optimized conditions of 35°C and neutral pH, (1) Initial Absorbance at 501 nm of Allura red dye into MSM medium inoculated by the six isolates and changes over time. (2) Percentage of decolorization is included by elected study isolates during 3 time periods of incubation.

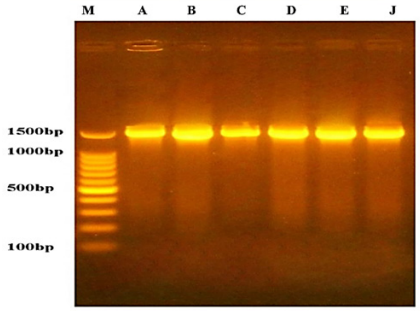

Figure 5: DNA electrophoresis of the six isolates under study using the specific primers of the 16S rRNA gene, which shows the alignment of the amplified DNA segments at around molecular weight 1500 bp.
Notes:- *The migration conditions are at a voltage of 100 volts for a period of 60 minutes,
**The letter M indicates the Volumetric guide 100 - 1500 bp, while the capital letters A to J indicate the amplified DNA paths of bacterial isolates.

PCR product were analyzed by Sanger sequencing using ABI3730XL, automated DNA sequences, by Macrogen Corporation–Korea. The results were received by email then analyzed using geneious software. Analysis on sequences and confirmation of microorganism's alignment and homogeny data with 16S rRNA database of GnBank of (NCBI) after amplification of Bacterial isolates.[20,21] All the information collected from alignments of the sequences of 16S rRNA of the elected bacterial study were summarized in Table 2.

In the Table 2, the bacterial were isolated shown that similar to four different genera (*Kurthia gibsonii, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus*) with 2 strains of *E. coli, K. pneumoniae* for each. These results hint that there are many isolates that might be isolated from the polluted environments and can be adapted for bioremediation of ecology at low cost and high safety.

Table 2: sequences of 16S rRNA of the elected bacterial study

Initial Bacterial Sample ID	Forward Length of Sequences Gene bp (S/GB)*	16SrRNA Description of Bacterial Sample in Library of NCBI	Per. Ident	Accession no.@ GenBank	Registration year
A	1,391/1,480	*K. gibsonii* strain KH2	100%	MN453416.1	Sep -2019
B	1,336/1,396	*E. coli* strain NF73_9	100%	MT649857.1	Jun-2020
C	1,395/1,398	*K. pneumoniae* strain OKF08	100%	KC969081.1	Jan-2014
E	1,393/1,400	*M. luteus* strain AsT5	99.93%	KX866674.1	Sep -2016
G	1,354/1,408	*E. coli* strain RCB862	100%	KT261074.1	Sep.-2015
J	1,356/1,473	*K. pneumoniae* strain OKF08	100%	KC969081.1	Jan-2014

*(S -length of sample study/ GB-length at GenBank Library)

CONCLUSION

In this research, it was possible to isolate many bacterial isolates from the effluents or sewage environment of a pharmaceutical company or any other company before discharging them to environments. The ability of Elected study isolates to decolorize Allura red AC dye was tested, and it was found that they were able to do it within a shortened time of 12–24 hours. Through the results, these isolates can be adopted as one of the biological treatment methods in purifying these environments by increasing the total number of these isolates and providing appropriate growth conditions such as pH, temperature, and others, in addition to the possibility of using genetic engineering techniques to develop the ability of these isolates on decolourizing of this dye or other dyes.

REFERENCES

1. Saraswathi K, Balakumar S. Biodecolourization of azo dye (pigmented red 208) using Bacillus firmus and Bacillus laterosporus. J. Biosci. Tech. 2009;1(1):1-7.
2. Khalid A, Arshad M, Crowley DE. Accelerated decolorization of structurally different azo dyes by newly isolated bacterial strains. Applied Microbiology and Biotechnology. 2008 Feb;78(2):361-9.
3. Dhanve RS, Shedbalkar UU, Jadhav JP. Biodegradation of diazo reactive dye Navy Blue HE2R (Reactive Blue 172) by an isolated Exiguobacterium sp. RD3. Biotechnology and Bioprocess Engineering. 2008 Feb;13(1):53-60.
4. Levine WG. Metabolism of azo dyes: implication for detoxication and activation. Drug metabolism reviews. 1991 Jan 1;23(3-4):253-309.
5. Hildenbrand S, Schmahl FW, Wodarz R, Kimmel R, Dartsch PC. Azo dyes and carcinogenic aromatic amines in cell cultures. International Archives of Occupational and Environmental Health. 1999 Dec;72(3):M052-6.
6. Martins MA, Queiroz MJ, Silvestre AJ, Lima N. Relationship of chemical structures of textile dyes on the pre-adaptation medium and the potentialities of their biodegradation by Phanerochaete chrysosporium. Research in microbiology. 2002 Jul 1;153(6):361-8.
7. Elisangela F, Andrea Z, Fabio DG, de Menezes Cristiano R, Regina DL, Artur CP. Biodegradation of textile azo dyes by a facultative Staphylococcus arlettae strain VN-11 using a sequential microaerophilic/aerobic process. International Biodeterioration & Biodegradation. 2009 Apr 1;63(3):280-8.
8. Dos Santos AB, Cervantes FJ, Van Lier JB. Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Bioresource technology. 2007 Sep 1;98(12):2369-85.
9. Mondal, P. K., and Ahmad, R. "Aerobic Biodegradation and Adsorption of Industrial Sludge Containing Malachite Green by Sequential Batch Reactor." Proceedings in International Conference on Energy and Environment (2009).March 19-21.
10. Kapdan IK, Erten B. Anaerobic treatment of saline wastewater by Halanaerobium lacusrosei. Process Biochemistry. 2007 Mar 1;42(3):449-53.
11. Moosvi S, Keharia H, Madamwar D. Decolourization of textile dye Reactive Violet 5 by a newly isolated bacterial consortium RVM 11.1. World Journal of Microbiology and Biotechnology. 2005 Jul;21(5):667-72.
12. Pandey, A., Singh, P., and Iyenger, L. "Bacterial Decolorization and Degradation of Azo Dye." International Journal of Biodeterioration and Biodegradation (2007) vol.59: 73-84.
13. Mabrouk ME, Yusef HH. Decolorization of fast red by Bacillus subtilis HM. J Appl Sci Res. 2008;4(3):262-9.
14. Cappuccino, James G. and Sherman, Natalie.Instructor Guide to Microbiology A Laboratory Manual (2011)- 9th edition Paperback. Benjamin Cummings.
15. Ali N, Hameed A, Ahmed S. Physicochemical characterization and bioremediation perspective of textile effluent, dyes and metals by indigenous bacteria. Journal of hazardous materials. 2009 May 15;164(1):322-8.
16. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, Eds. Manual of clinical microbiology. (1999).7th ed. Washington, DC: ASM,.
17. Tanner MA, Goebel BM, Dojka MA, Pace NR. Specific ribosomal DNA sequences from diverse environmental settings correlate with experimental contaminants. Applied and environmental microbiology. 1998 Aug 1;64(8):3110-3.
18. Fayidh MA, Kallary S, Babu P, Sivarajan M, Sukumar M. A rapid and miniaturized method for the selection of microbial phenol degraders using colourimetric microtitration. Current Microbiology. 2015 Jun;70(6):898-906.
19. Rantakokko-Jalava K, Jalava J. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. Journal of clinical microbiology. 2002 Nov;40(11):4211-7.
20. Al-Rubaye MR, Yildiztugay E, Uysa A, Mohammed TK, Abdullah HN. Molecular detection of virulent exoU mutation of Pseudomonas aeruginosa isolated from wound and burn samples. EurAsian Journal of Biosciences. 2020 Aug 1;14(2).
21. Ibrahim HK, Mahdi MS, Ameen RS. Study the Ability of the Newly Isolated Thermophilic Bacterium (Geobacillus Thermoleovorans) Strain Ir1 (Jq912239) in Decolonization and Degradation of Azo Dyes (Methylene Blue and Acid Orange G). Int. J. Drug Deliv. Technol. 2021;11:429-33.