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Extraction & Purification of Plasmid from Some Spices *of E. coli* in Different Ways

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ABSTRACT

This study involved the use of multiple methods to separate plasmids from bacterial cell DNA for some isolates of pathogenic E. coli through several steps, starting with the analysis of the bacterial cell using lysozymes to remove the outer wall, followed by centrifugation to isolate plasmids found in the solution from the rest of the proteins and other forms of DNA. Many sequential methods were used to separate plasmids. The first method used was the basal denaturation sodium hydroxide-based, which led to the denaturation of the chromosomal DNA without affecting the plasmid DNA, followed by the addition of sodium acetate, which led to the preservation of the shape and structure of the plasmid DNA. Second, using cesium chloride gradient density to isolate the protein cell components and the rest of the DNA forms. The different densities of these components led to the appearance of sequential bundles depending on their different molecular weights. Ethidium bromide, which gave the plasmid bundles a fluorescent dye, was added using ultraviolet rays. The last purification method was using the boiling method using a water bath. Plasmid samples extracted from the previous methods were taken to perform the purification and separation process using the high electrophoresis method. Akarose gel was used to separate the high molecular weight protein fragments. Standard proteins and plasmids were migrated to determine the volumes of purified plasmids.

Keywords- Extraction, Purification, Plasmid, *E. coli.*

I. INTRODUCTION

Plasmids are genetic elements outside the chromosome in addition to the chromosomes of the bacterial cell. Plasmids are circular-shaped structures that can replicate independently of the chromosome, it also reproduces in separate pieces from the DNA of the chromosome (1,2). Plasmids are usually not necessary for the life of the host, but their presence may enable it to live under exceptional conditions. Among the most important characteristics of plasmids are antibiotic resistance, hemolysin production, and resistance to heavy metals (3). Plasmids are divided into two parts: 1. Conjugative plasmids, which contain the carrier genes, which are called ultra-genes, stimulate the process of bacterial conjugation, and their sizes range between 60-120 kb (4). 2. Non-conjugative plasmids, which do not contain conjugation genes, therefore, are unable to initiate the conjugation process, and their sizes range between 1.5-5 kb (5). Plasmids are also divided according to their replication ability into 1. Relaxing plasmids of which there are many copies within the cell (6). Restriction plasmids that existin specific copies (6,7), Plasmids are used nowadays in chlorosis experiments as gene carriers because they are small in size, the sequence of nucleotides in them is known, and the ease use of restriction enzymes, as well as their rapid reproducibility (1,2). Plasmids are transferred from the donor cell to the recipient cell via cytoplasmic bridges, and this process is common in Gram-negative bacteria. Among the common plasmids are resistance plasmids that are responsible for encoding resistance to some antibiotics (2,3). Most of the purification methods depend mainly on the difference in the physical formula

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of each of the plasmid DNA and the DNA of the bacterial cell, in terms of size and shape, as the sizes of plasmids are very small compared to the size of the chromosome of the bacterial cell (7) and that the largest plasmid constitutes only 1.8 of the size of the bacterial chromosome of E. coli *(8).* This is helped by their different shape in the cell in a super-coiled manner, and they are called covalently closed circular molecules when they exist continuously without cutting (9), while it is called the open circular molecule in the case of any part of the two threads being cut, and the cutting is called linear cutting if one of the two threads is cut more than once in the same place (10).

II. LITERATURE SURVEY

Modern and advanced separation methods have been used to track and identify plasmid DNA bundles and separate them from the rest of the DNA forms present in the mixture. The use of agarose gel, which is an interlocking chain of complex polysaccharides consisting of galactose and its derivatives, is linked together by hydrogen bonds to form a complex network. The polyacrylamide gel is the result of linking crystallized acrylamide with bass-acrylamide to form a complex network with holes smaller in diameter than agarose gel. Therefore, polyacrylamide gel is more suitable for separating small pieces of DNA that are less than 4 kilobytes in length. Agarose is used to separate large pieces of up to 200 kilobytes in length (11).

III. MATERIAL AND METHOD OF WORK

3.1 The solutions used

The following solutions were prepared in the process of separating and isolating the plasmid DNA:

1. Isozyme enzyme solution: Prepared immediately upon use at a final concentration of 10 mg/ml, by adding 10 mg of the enzyme to 1 ml of Tris-HCl at a concentration of 10 mM.
2. A 2.5 molar sodium acetate solution is prepared by dissolving 34.02 g of sodium acetate in 50 ml of distilled water and then completing the volume to 100 ml, adjusting the pH to 4.8.
3. Cesium chloride solution: 5 mg of Caesium chloride was added to 10 ml of distilled water to a concentration of 0.5 µg/ml.
4. Ethidium bromide dye: was prepared as a buffer solution by dissolving 5 mg in 10 ml and used at a final concentration of 0.5 µg/ml.
5. STET buffer solution: prepared from a mixture of 8% sucrose, 50 mmol of EDTA, 10 mmol of Tris-HCl, 0.5 of Trilon-X-100, pH adjusted to 8, sterilized by autoclave, and kept at 4 °C until use.
6. TE buffer solution: 10 mM Tris-HCl was prepared with 1 mMEDTA, pH was adjusted to 8, autoclaved, and stored in the refrigerator until use.
7. TBE-solution was prepared at a final concentration by adding 89 mM Tris-base to 89 mM boric acid with 2 mMEDTA, adding the volume to 100 mM, adjusting the pH to 8, sterilizing with an autoclave, and storing at 4°C until use.
8. Acarose gel (concentration 0.7%) was prepared by dissolving 0.7 gm of agarose in 100 ml of TBE buffer.
9. Loading buffer was prepared by dissolving 0.25% bromophenol blue, 0.25% xylene, and 40% sucrose then stored at refrigerator temperature until use as mentioned in (12).

3.2 The method of work

1. Antibiotic susceptibility test: isolates of E.coli bacteria were used after culturing and purifying them on EMB medium (Eosin Methyl Blue), which is the optimal medium for their growth. The isolates were incubated at 37 °C for 48 hours. The sensitivity of the isolates to antibiotics was tested on a Nutrient agar medium (13). The isolates were incubated at 37°C for 48 hours, and the sensitivity of the isolates to antibiotics was tested on the Nutrient agar (13).
2. Bacterial cell replacement method: Starting, the Nutrient broth medium was inoculated with colonies of E.coli cells growing on the solid culture. It was left for an hour before starting the plasmid DNA isolation process from the host cells containing it by breaking the cell wall using the isoenzyme prepared in paragraph 1, on the preparation of solutions. Large chromosome DNA and small plasmids were isolated using centrifugation at a speed of 5000/rpm. Large chromosome segments are deposited with the rest of the cell components, while plasmids and small chromosome segments remain in cleared lyses (14).

3.3 Separating plasmid DNA 3 methods

a. Basal denaturation method: It is one of the common methods for separating the plasmid from the clear solution. This method is based on denaturing pieces of non-high-wrap DNA (plasmids) in a narrow range of 12-12.5 after adding sodium hydroxide, which led to the breakage of chromosome DNA. Plasmid DNA is not affected by this treatment. After that, acidic sodium acetate was added to change the pH to 7 and neutralize the denaturation effect, then the solution was centrifuged at 7000 rpm to obtain plasmid DNA later by precipitation with absolute alcohol (ethanol) (15).

b. The method of centrifugation in a density gradient cesium chloride solution in the presence of ethidium bromide: this method is used to obtain a highly purified plasmid to be used later as vectors in chlorination processes. First, the clear solution resulting from the process of cracking bacterial cells is withdrawn and the harmful cesium chloride solution is added to it in step (3) of the solution preparation section, then the ethidium bromide that was prepared in step (4) is added. The mixture was subjected to centrifugation at a speed of 700 rpm. The precipitated DNA segments were determined by exposing the tube to ultraviolet rays at a wavelength

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of 180 nm, where the bundles appeared in a bright color due to the presence of bromide dye after determining the locations of the bundles where they are separated by instilling a medical syringe on the walls of the centrifuge tube, where the desired bundles are heated without mixing them with the rest of the separated parts. Ethidium bromide is removed by washing the plasmid DNA several times with butanol until the red color of the bromide disappears. Then the remaining cesium chloride solution was removed by dialysis, which led to obtaining high purity plasmids, then concentrated using absolute ethyl alcohol, and they were kept until purification was performed (16,17).

c. Boiling method: (5) ml of the bacterial culture was inoculated with the azithromycin antibiotic, to which all the isolates under study showed resistance to it by a single colony. The medium was incubated in a rocking incubator at 37 °C for 24 hours, 1.5 ml of the cultured bacteria was transferred after the end of the development period to a sterile Eppendorf tube, then the bacterial cells were precipitated by the microcentrifuge for one minute, then the tube containing the bacterial precipitate was left at 4 °C for 24 hours. The filtrate was neglected and the granule was left to dry completely. Then, it was suspended with 350 microliters of the buffer in step (5) of the solution preparation section, with 25 microliters of the isozyme enzyme solution prepared in (1) of the solution preparation section. The mixture was mixed well for 3 minutes and was placed in a water bath at 100 °C for 40 seconds and precipitated for 10 minutes using a micro-centrifuge at room temperature. The sticky granule formed with wooden sticks was withdrawn, and 40 microliters of the remaining solution were added through the sodium prepared in (2) of the solution preparation section with 240 microliters of isopropyl alcohol. It was mixed and kept at -20°C for 24 hours, the mixture was discarded by a micro-centrifuge for 15 minutes at 4°C. The filtrate was neglected and the alcohol was left to evaporate at room temperature. 50 µl of the buffer was added to the mixture in (6) of the solution preparation section, which is added to dissolve the plasmid DNA deposited on the walls of the tube (18), the sample becomes ready for the next step of purification.

3.4 Purification method

The method of separation was adopted with agarose gel prepared in (8) in the section on preparing solutions. The gel was first heated to a boiling point and then left to cool to 50-45 °C, 10 microliters of ethidium bromide dye prepared in paragraph (4) of the solution preparation section were added to it. Pour the agarose softly to prevent air bubbles in the support plate, after fixing the comb at one end to make the holes prepared for loading the samples. Leave the gel to solidify for 30 minutes at room temperature, raise the comb quietly and fix the plate in the horizontal electrophoresis unit represented by the basin used for electrophoresis. The basin is filled with the buffer prepared in step (7) of the solution preparation paragraph so that the buffer covered the surface of the gel. Transferring 10 liters of the prepared sample to be loaded from each separation process that was previously carried out with three samples, where each sample was placed separately in an Eppendorf tube after adding 3-5 microliters of the loading buffer prepared in step (9) of the solution preparation section and mixed with the sample calmly and loaded into the holes designated in the gel. Pass the electric current with a force of 5 volts/cm for (1-2) hours until the dye reaches the second side of the gel. The locations and sizes of the separated pieces were determined by staining the pieces with ethidium bromide dye. The gel was soaked in a buffer solution containing ethidium bromide dye at a concentration of 0.5 µg/ml for 45 minutes, and after the dye was attached to the DNA bundles, the dye was removed by soaking the gel in the same buffer solution (without dye), the samples were examined under U.V at a wavelength of 366 nm and the results were fixed (11).

The absorption measurement method uses the Spectrohomometer, where this method was adopted by reference to the degree of absorption of the standard plasmid. As each of the samples was taken separately after the purification stage was completed, the degree of absorption was measured at a wavelength of 600 nm and the readings were fixed (19).

IV. RESULTS AND DISCUSSION

The results showed the resistance of the developing bacterial cells to doxycycline antibioticsas shown in Table (1).

Isolation no.	antibiotic name	antibiotic concentration	response to antibiotic
1	Doxycycline	(10mcg) Do	S
2	Metronidazole	(30mcg) Met	R
3	Erythromycin	(10mcg) E	R
4	Bacitracin	(10 u) B	S
5	Ampicillin	(25mcg) Am	R
6	Clindamycin	(10 mcg) DA	R

Therefore, doxycycline was selected in the experiment to test the isolates by boiling method, as shown in Figure (1).

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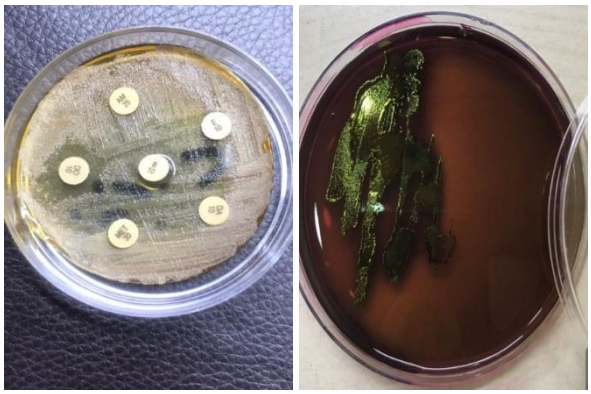

Figure 1: An experiment to test the sensitivity of isolates to antibiotics

Besides, the results indicated that the addition of acid sodium acetate by basal denaturation method to the equation of the denaturation effect caused by the addition of sodium hydroxide, led to the return of the denatured strands to the connection, forming a complex insoluble network. The addition of sodium acetate and sodium dodecyl leads to the precipitation of protein and RNA molecules of high molecular weight and the survival of the circular plasmid DNA molecules suspended in the Cleared lyses**.**

The separation method using cesium chloride gradient density showed that the mixture turned into a density gradient form, where the density increases as we go down to the bottom of the centrifuge tube. Depending on the density of the separated bundles as they were distributed along the length of the tube, Buoyant density, and since the density of DNA is equal to the density of cesium chloride, it is separated in the middle of the tube and the proteins appeared in bundles to the top of the tube because its density is lower and the RNA was deposited at the bottom as shown in Table (2).

Material	Concentration g/cm3
Low Density Proteins	1.2
High Density Proteins	1.45
DNA	1.6
RNA + protein	1.75
RNA	1.8

By applying the curve of the relationship between the relative motility and the concentration of standard proteins, it appeared as in the following diagram, where the molecular weight of the proteins of the bacterial cell and the L-molecule was estimated in its chromosome and plasmid forms by reference to the standard curve for proteins.

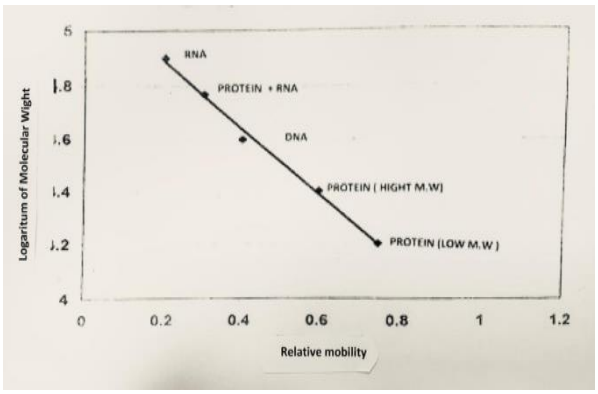

Figure 2: Standerd curve of proteins

The addition of ethidium bromide to the cesium chloride solution affectedthe separationof the closed plasmids from the rest of the forms of L, as it is known that ethidium bromide is a chelate material and can bind to a molecule by inserting its molecules between the nitrogenous bases at the first coil of the helix. Also, the closed circular molecule does not contain free ends, the dissociation of the helix filament will be difficult, while at the same time, the circular filamentous plasmid DNA molecule with open ends is allowed to enter this material because it contains free ends, and this was confirmed by Michael, and the more ethidium bonded to the DNA molecule, the lower its density. Therefore, the closed plasmid molecules will be denser than the rest of the DNA forms in the cesium chloride mixture containing ethidium bromide. After centrifugation at a very high speed, the closed plasmids were deposited to the bottom, leaving other bundles of DNA in suspension. After exposing the bands and forming separately to ultraviolet rays, the bands bound to ethidium appeared in a bright color (red) even if the DNA bands were few and low in focus, as shown in Figure (3) (20).

Figure 3: Exposing the plasmid bundles to ultraviolet rays

4.1 Purification methods results

1. The results of purification with agarose gel showed that the DNA segments moved after the current passed towards the positive electrode because they are negatively charged at a speed inversely proportional to their molecular weights, as the small pieces separate after longer distances and thus each group of similar pieces of length will have specific bundles in a specific

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location on the Gel. The intensity of the brilliance of the dye is directly proportional to the amount of DNA in each separated bundle. The method of gel separation also determines the physical nature of the DNA molecule, where the covalently closed molecule migrates fastest, followed by the filamentous molecule and then the open circular molecule. This also depends on the concentration of the gel, the intensity of the electric current, the molecular weight of the DNA, and the type of buffer used. (11).

Figures (4), and (5) show the separation of plasmid DNA fragments from other protein forms in the samples under study.

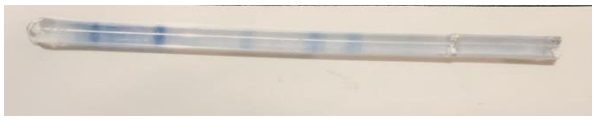

Figure 4: Separation of the plasmid pieces from the protein forms of the model

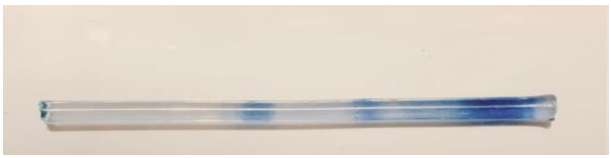

Figure 5: Separation of plasmid pieces from protein

To investigate plasmid pieces in the three samples that were taken from the previous purification methods, the samples were successively exposed to the spectrophotometer for the investigation to match the wavelengths of the samples with the wavelength of the standard plasmid. Figure (6) shows the absorption of the standard plasmid at a wavelength of 600 nanometers (18,21), while the subsequent samples showed that they contained the plasmid sample. Figure (7) shows the sample taken from this basal denaturation method, which showed the presence of a region identical to the uptake of plasmid DNA, and this also appeared in the two subsequent samples. Figure (8) shows the plasmid sample taken from the centrifugation method, while figure (9) refers to the sample taken from the boiling method. Despite the presence of other accompanying protein materials that are close to the degree of absorption of plasmid DNA, this is because other materials may appear close to the degree of absorption of the sample but at very low concentrations. Daniel (18,22, 23) confirmed that.

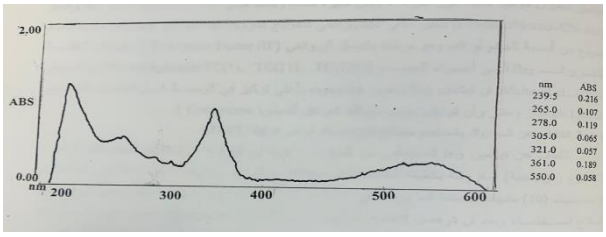

Figure 6: Standard plasmid uptake

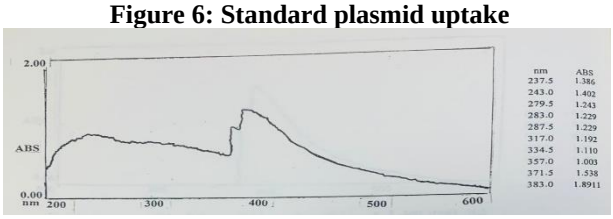

Figure 7: Uptake of the plasmid sample taken from the basal denaturation sample

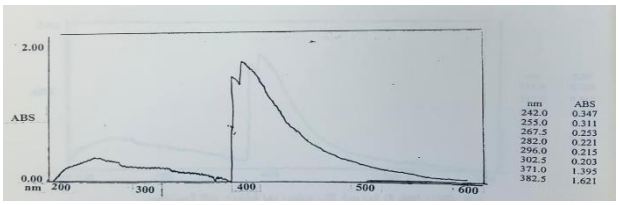

Figure 8: Plasmid uptake by centrifugation

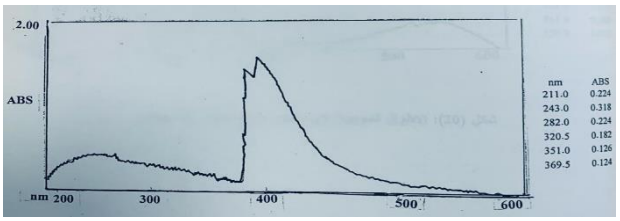

Figure 9: plasmid sample uptake taken by boiling method

V. CONCLUSION

The best methoid for extraction of plasamid from pathogenic species *of E.coli* is the addition of cisium chloridafter purefication of samples anbd show the absorbtion under UV light, so we recommend to use this method for the work in the same field in future,

REFERENCE

[1] DavidP.Clark,MichelleR.McGehee.Plasmid.Inmole cular biology(third edition) 2019

[2] Jawad.R.Al-Zaidi:Ahmed.S.Mohammed.Firset Record of dissemination of BLBLi *resistant Enterobacter cloacae* from public hospitals in Bachdad-Iraq.Identifies and Pagination Vol:16(2022)

[3] Silve.S,Misra.T.K.Plasmid-mediated heavy metal resistance.Annual review of microbiology.vol.42;717-743(volume publication date October 1988)

[4] Waleed Haj Saeed A .Kreyi,Samira Yowni Yousif, Mahde Assafi.Antibiotic resistant & plasmid conjugation study of *Salmonella typhi*. Journal Teknologi Laboration,V7:2.134.

[5] John.H,Nijkamp and Edward Veldkamp.Genetic organization and expression of the non-conjugative plasmid. Molecular biology pathogenicity and ecology of bacterial plasmids.2007 pp247-258

ISSN: 2583-4053

Volume-2 Issue-2 || April 2023 || PP. 161-166

https://doi.org/10.55544/jrasb.2.2.23

[6] Michael.A.Lovett,Donald.G.Guiney&Donald.R.He linski.Relaxation complexes of plasmids ColE1 and CoE2;unique site of the nick in the open circular DNA of the relaxed complexesproenat-acad.Sci USA ,Vol-71, No.10pp.3854-3857(1974)

[7] Herma.A.et.al.Differential replication of plasmids during the stringent and relaxed response of *Escherichia coli*. plasmid, 1994

[8] Huda ALdogheither&Munazza Gull. Plasmid as genetic tools and their application in ecology and evaluation,metric overview .2019Richard

[9] Tania.S.Darphom,KeshiaBel.Antibiotic resistance plasmid composition and architecture in *Escherichia coli* isolates from meat.Scientific reports 11 articlesnumber:2136(2021)

[10] Kenneth Timmis,F.ELipeCapello&Staniley.E.Y.N.COHen.covale ntly closed circular DNAmolecules of low superhelix density as the intermediate form in plasmid replication. Nature 261, 512-516(1976)

[11] Stephen.D.Levene and Bruno .H.Zimm. Separations of open circular DNAusingpulsed-fieldelectrophoresis.Journal article,vol.84.No.12:pp4054-4057(1987)

[12] Anne.M.Livesley, Stephen.N.Smith,Richard.A.Armstrong. Analysis of plasmid profiles of Aeromonassalmonicida isolates by pulsed-field gelelectrophoresis.FILMS.Microbiology.1 letters 146(1997)297-301

[13] NoboraSasagawa. Plasmid purification.Metriesoverview (2018)

[14] Amanda.K.Kidsly,Sam.Abraham,Jan.M.Belt.Anti microbial susceptibility *of Escherichia coli* and *Salmonella.* Spp.Isolates from healthy pigs in Australia:Results of a pilot national survey original research article,Front.Microbial(2018)

[15] Jose.Manuel.Borrero,Cristian. Hidalgo.Anoval programmable lysozyme-basedlysis in *Pseudomonas*putida for biopolymer production, scientific report,7,Article number:4373(2017)

[16] GeraldinFargo,Niels.Larens.Protein sliding and DNA organization by the human mitochondria transcription factor, ANature communication 3,Article number;1013(2012)

[17] Victoria,HermandesEquilibrium Density Gradient Centrifugation in cesium chloride stahlsolutions.Developed by Mathew Meselon&Franklin(2017)

[18] Ali.A .A-Nashaat: Azhar.N.Hussein:Adnan.H,.Al-Hamadani.Curing of plasmids contents of proteins spp.isolated from urinary tract infection in Aldiwaniyah city.Al-Qadisiah MedicalJournal,2007,Vol2, (160-169)

[19] SarahDebiny,Richard.Murphy& Fiona,Walsh.A comparison of methods for the extraction of plasmid capable of conferring antibiotic resistance in a human pathogen.Front. Microbial.,(2018)

[20] DanielR.Albert,Michael.A.Todt&Floud.H.Davis.A low-cost Quantitativeabsorption spectrophotometer.J.chen.Educ.article.No11.Vol.89,pp:1 432-1435(2012)

[21] Madhumita.Roy,Dona.sinha,Sutapa.Mukherjee&Ja ydip.Biswas.Curcumin prevents DNAdamage and enhances the repair potential in a chronically arsenic-exposed human population in West Bengal- India. European journal of cancer prevention.Vol,20,No.2(2011)

[22] Jacobebeal, Netalie .G.farny.Robust estimation of bacterial cell count from optical density. Communication Biology.Artical,3.No,512(2020)

[23] Mohammed AdilJaafari:Abdul kareem Al kazzaz and Ali Sadiq Mohammed. Plamid role in crystals protein production in locally isolated *Bacillus thuringiensis*. Iraqi Journal of science, Vol:5.No2(301-306)