A novel method for isolation new strain of *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.) and other strain(sensitive for Cloxacillin acillin and resist to Ampicillin.) *and* check it's transformation with isolated plasmid and it's stability by serial plating method

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Abstract

Bacterial strains (*E. coli*) were collected from two different places , The first strain was collected from central Laboratory – Baghdad university , meanwhile the second strain was collected from pathogenic laboratory – Baghdad university

Replica plating and gradient plate method technique were used to prepare strains with different multi trails

Replica plating was used to select strain resist to specific antibiotic with low concentration (sensitive for other type of antibiotic) then go head subsequently with gradient plate method to adapt the strain to resist (high concentration)

Replica plating was used to select first strain resist to Ampicillin. (100 Mg- ml) and (sensitive to Cloxacillin ..) then gradient plate method was used to adapt first strain to resist Ampicillin. (500 Mg- ml) therefore. first strain was resistant to Ampicillin. (500 Mg- ml) and sensitive to Cloxacillin (500 Mg- ml) , on other hand ,by using same methods , second strain was resistant to Cloxacillin (500 Mg- ml) and sensitive to Ampicillin.. (500 Mg- ml) Replica plating was used to select third strain sensitive to Ampicillin. and Cloxacillin .(500 Mg- ml) for each type of antibiotics and it is consider as competent cell . To visualize if (Ampicillin. resist trail and Cloxacillin resist trail) carried on plasmid , first , second and third strains were transformed by using transformation technique

The Transformant strain was resistant to (1000 Mg- ml) by which resist to Ampicillin. and Cloxacillin . (500 Mg –ml) for each antibiotic .

The efficiency of transformation was determined as percentage of transformation , it was so high a round (9%)

The Transformant strain was checked for stability of it's plasmid by using sub – culturing method . It was stable for 12 times of sub culturing of transformant growth

Concentration of the protein for *E. coli* (resist for cloxacillin and sensitive to Ampicillin.) and other strain(sensitive for Cloxacillin acillin and resist to Ampicillin.) was **0.065** and **0.116** meanwhile transformant was **0.190**

Finally , . using replica plating , gradient plate and transformation process consider as a novel technique to prepare strains with multi trails which resist very high concentration of antibiotics and efficient transformation process .

Introduction :

Transformation is a process whereby ‘cell-free’ or ‘naked’ DNA containing a limited amount of genetic information is transferred from one bacterial cell to another. Cells that are in a state in which they can be transformed by DNA in their environment are said to be competent. In a significant number of bacteria, entry into the competent state is encoded by chromosomal genes and signaled by certain environmental conditions. Such bacteria are said to be capable of undergoing natural transformation. Many other bacteria do not become competent under ordinary conditions of culture but they can be made competent by a variety of highly artificial treatment such as exposure of cells to

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high concentrations of divalent cations. Such systems of transformation have been termed as artificial transformation (1,2)

The DNA is taken in through the cell wall and the cell membrane of the recipient cell. The molecular size of the DNA affects transformation. The number of transformants increases linearly with increase in concentration of DNA. However each transformant results from the transfer of a single DNA molecule of double stranded DNA.(3)

Different methods employed for transformation can be categorized as:Chemical methods:E. coli cells washed in cocktails of simple salt solutions achieve a state of competence during which DNA molecules maybe admitted to the cell. Currently used methods for bacterial transformation are based on the observation made by Mandel and Higa (1970) who showed that bacteria treated with ice-cold solutions of CaCl2 and then briefly heated to 37°C or 42°C, could be transfected with bacteriophage λ DNA. This simple and robust method generates 105-106 transformed colonies of E. coli per μg of supercoiled plasmid DNA. Different methods have been tabulated below with their respective transformation efficiencies.(4,5)

Physical methods:It includes methods such as electroporation technique that was originally developed to introduce DNA into eukaryotic cells and was subsequently adapted for transformation of E. coli and other bacteria by plasmids. Exposure to an electrical charge destabilizes the membranes of E. coli and induces the formation of transient membrane pores through which DNA molecules can pass. Transformation efficiencies in excess of 1010 transformants/ μg of DNA can be achieved by optimizing various parameters. Plasmid size ranging from 2.6 – 85 kB can be introduced with efficiencies ranging from 6 X 1010 to 1 X 107 transformants/μg of DNA respectively. This is 10-20 times higher than can be achieved by chemical methods.(6,7)

The aims of research

1 . It is a novel method to isolate strains with multi trails

2 . Transformant strain was resist to very high concentration of multi antibiotics with (1000 Mg / ml) meanwhile most Transformant strain resist not more than 500 Mg / ml .

3 . The efficiency of transformation was so high in spite of the transformation was done with multi trial which lead to reduce efficiency of transformation

4 . The stability of plasmid was very high

5 . Finally the using replica and gradient are efficient method to isolate strains with multi trails and very efficient transformation with resist strains for curing agent .

Material and methods

Material :

1 - First and second strain of *E.coli* form different sources

2 - Sterile N.A plates.

3 . Luria-Bertani broth and agar medium.

4 . Ampicillinicillin and Cloxacillin . stock solution (500 mg/ml).for each one

5 . CaCl2.2H2O solution (1M).

6 . Glycerol CaCl2 stock solution.(8 , 9)

Method :

1 . Prepare over night agar growth of (*E. coli*.) by adding 0.2 ml bacterial suspension into plate and incubate for 24 hours at 37 C .

2 . After incubation (master plate) , colonies are picked up with filter paper carrier .

3 . Ampicillinicillin agar with 100 Mg / ml is incubated with carrier at 37 C for 24 hours to isolate the ampicillinicillin resistant colony

4 . Gradient plate method was used to prepare strain resist to 500 Mg-ml .

5 . Pick up ampicillinicillin resistant colony and make suspension by N. B. , it is considered (suspension 1)

6 . Prepare over night agar growth of (*E. coli*.) by adding 0.2 ml bacterial suspension into plate and incubate for 24 hours at 37 C .

7 . After incubation (master plate) , colonies are picked up with filter paper carrier .
8 . Cloxacillin . agar with 100 Mg / ml is incubated with carrier at 37 C for 24 hours to isolate the Cloxacillin resistant colony
9 . Gradient plate method was used to prepare strain resist to 500 Mg-ml .
10 . Pick up Cloxacillin resistant colony and make suspension by N. B. , it is considered (suspension 2)
11 . Prepare over night agar growth of (*E. coli*.) by adding 0.2 ml bacterial suspension into plate and incubate for 24 hours at 37 C .
12 . After incubation (master plate) , colonies are picked up with filter paper carrier .
13 . Cloxacillin . agar with 500 Mg / ml is incubated with carrier at 37 C for 24 hours to isolate the Cloxacillin sensitive colony then transfer this colony to Ampicillin. agar with 500 Mg-ml to isolate Ampicillin. sensitive colony
14 . Pick up Cloxacillin and Ampicillin. sensitive colony and make suspension by N. B. , it is considered (suspension 3)
.15 . Lyse suspension 1 and suspension 2 by incubation for 15 min. at 60 C in water bath and release DNA components of cells .
16 . Take 2 ml of suspension 3 and centrifuge for 3000 rpm for 10 min . then add (100 mM CaCL2 with 2 ml) to pellet and keep it for 10 min. in ice . The suspension again is centrifuged for 3000 rpm for 10 min. (this step is required to prepare competent cells) .
17 . Mix (1 ml) suspension 1 , 2 and 3 then put the mixture at 0 C for 20 min.
18. Transfer the mixture to water bath at 42 C for 90 sec. then again transfer it to ice at 0 C for 2 min. to make shock in cell wall of suspension 2 (competent cells) and receive the DNA of suspension 1 .
19 . Incubate the mixture for 30 min. at 37 C then add it to ampicillinicillin - Cloxacillin acillin agar plate with 500 Mg/ ml for each type of antibiotics and incubate for 24 hours at 37 C .
Determine efficiency and frequency of transformation. (10 , 11)

Calculations:

$$\text{Percent transformation} = \frac{\text{TVC of transformants X 100}}{\text{TVC of competent cells.}}$$

EXTRACTION OF INTRACELLULAR PROTEINS

Materials required:

- Solution I - Bacterial Cell Lysis buffer
- Solution II - Lysozyme
- Bacterial culture (exposed to CoCl2 and Control)
- Micro-fuge tubes
- Centrifuge apparatus
- Micropipettes

Method :

- Take 25 ml of bacterial culture. Centrifuge at 8000 rpm for 10 min. at 4ºC. Decant the supernatant media.
- Add 500 µl of solution I, mix well. Add 15 µl of solution II, mix well.
- Incubate at 37ºc for 10 min.
- Add 100 µl of 20% SDS (pre-warmed at 37ºc). Mix gently for 5 min.
- Incubate on ice for 1 hour.
- Centrifuge at 13,000 rpm for 10 min at 4ºC to pellet down other cell organelle.
- Collect the supernatant in a fresh vial.

EXTRACTION OF DNA

Materials:
1-Culture of E.coli.
2-Sterile 50 ml Luria-Bertini broth.
3-1M Tris buffer.
4-10% sodium lauryl sulphate, SLS or SDS.
5-Phenol equilibrated with Tris.
6-Phenol- chloroform mixture (25:24).
7-Chloroform-isoamyl alcohol mixture (24:1).
8-5M Nacl or 3M NaAc.
9-95% chilled ethanol.
10-70% chilled ethanol.
11-T10 E1 buffer (pH: 8.5)

Methods:
1-Prepare an overnight culture of E.coli in 50ml LB broth (24hr. at 370C on a Shaker)
2-Pellet cells by centrifuging at 8000 rpm for 10 minutes at 4oC.
3-Wash pellet in T10E1.
4-Centrifuge at 8000rpm for 10 minutes at 4oC.
Harvest Pellet.
5-Add 200µl of T10E1 and transfer it into an eppendorf tube.
6-Add 10% SLS or SDS to a final concentration of 1%.Also add a pinch of lysozyme.
7-Keep at room temperature for about 30 minutes.
(If the solution becomes too viscous, dilute with more of T10E1).
8-Add equal volume of phenol-chloroform mixture. (24:1)
9-Spin at 8000 rpm for 10 minutes at room temperature.
10-Remove above aqueous layer carefully in a fresh eppendorf and again add equal volume of phenol-chloroform mixture.
11-Spin at 8000rpm for 10 minutes at 4oC.
12-Transfer aqueous layer into another fresh eppendorf and add equal volume of chloroform-isoamyl alcohol mixture.
13-Centrifuge at 8000 rpm for 10 minutes at 4oC.
14-Take aqueous layer in a clean DNase free eppendorf and add 5M NaCl to a final concentration of 0.15M.
15-Add 2 volumes of chilled ethanol to the above solution to precipitate DNA (12).

ESTIMATION OF PROTEINS

Concentration of the protein sampicillinle can then be calculated using the formula as under.

$$\text{Concentration of Proteins (µg / µl)} = 1.55 \times A_{280} - 0.76 \times A_{260}$$

Result

Replica plating was used to isolate strain with ampicillin resistant plasmid containing *E. coli* strain with 100 Mg / ml concentration as elucidated in figure (1)

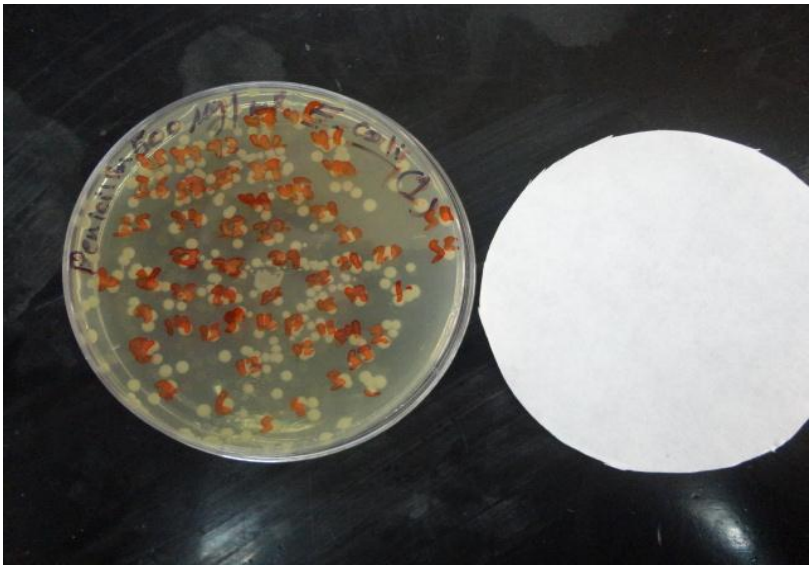

Figure (1) first strain resist to ampicillin (100 Mg/ml) which is selected by replica plating method

then by using gradient plate method , the ampicillin resistant plasmid containing strain was resistant to 500 Mg / ml and the heavy growth of bacteria have seen with large number of colonies as elucidated with figure (2)

Figure (2) first strain resist to ampicillin . (500Mg/ml) which is adapted to that concentration by using gradient plate method

ampicillin resistant plasmid containing *E. coli* strain was streaked out on plates containing cloxacillin.., The ampicillin Plasmid containing *E coli* strain was sensitive for cloxacillin.. (500 Mg/ml).thereby there was no growth in plate , Therefore by using Replica plating and Gradient plate method we isolated strain resist to 500 Mg /ml ampicillin.. and sensitive to 500 Mg / ml for cloxacillin .

Same method repeated on second strain but cloxacillin resistant plasmid containing *E coli* strain was selected and further sequential method to prepare strain resist to cloxacillin. thereby heavy growth

have seen and sensitive to ampicillin thereby no growth have seen With 500 Mg/ml as elucidated in figure (3)

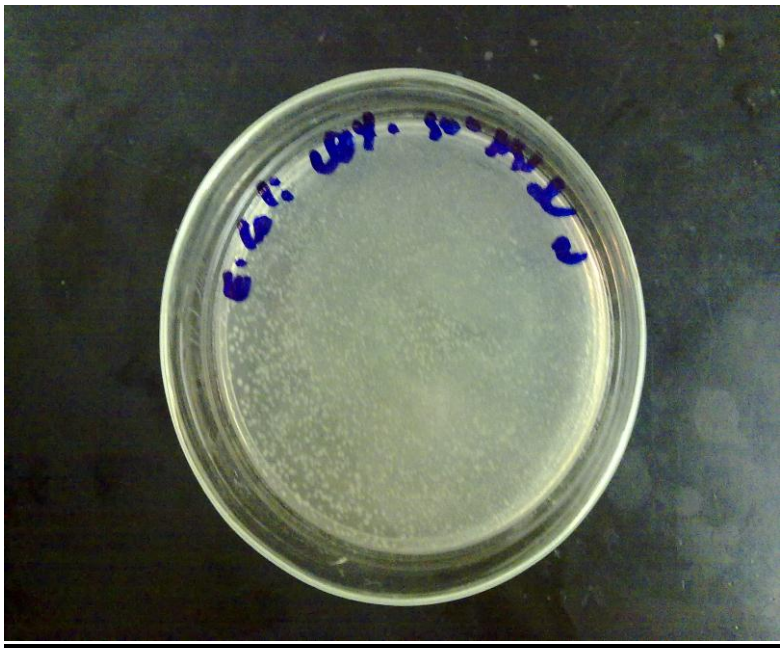

Figure (3) second strain Resist to cloxacillin (500 Mg/ml) which is adapted to that concentration by using gradient plate method

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The transformation technique was done for two strains with competent cell to get transformant can resist 1000 Mg/ml of ampicillin. , cloxacillin . as shown in figure (4)

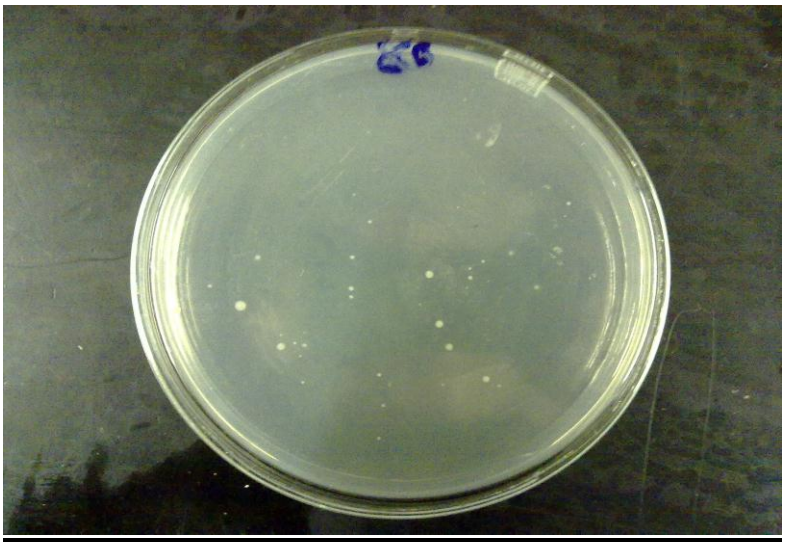

Figure (4) Transformant strain resist to clox..and ampicillin. (500 Mg/ml) for each antibiotic

Meanwhile strains before transformation process could not growth on plates containing ampicillin. , cloxacillin with 1000 Mg/ml and considered as control as elucidated in figure (5,6)

Figure (5) Cloxacillin resistant plasmid containing strain sensitive to ampicillin.and cloxacillin (500 Mg/ml) for each antibiotics before transformation process and considered as control

Figure (6) ampicillin resistant plasmid containing strain sensitive to ampicillin.and cloxacillin (500 Mg/ml) for each antibiotics before transformation process and considered as control

The Percent transformation was calculated for transformation process of three strains by using this formula

$$\text{Percent transformation} = \frac{\text{TVC of transformants X 100}}{\text{TVC of competent cells.}}$$

$$\text{Percent transformation} = \frac{25 \text{ X } 100}{257}$$

Percent transformation was 9%

The stability of plasmid in transformation strain was determined by using curing method , or by using sub – culturing method .

The stability of plasmid can be seen for 12 times of sub culturing method through growing the strain on Ampicillin., Cloxacillin .. with 1000 Mg-ml (500 Mg / ml for each)
Extracted protein from *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.) was estimated by measuring O.D. at 280 nm and it was 0.0936 meanwhile extracted protein from *E. coli* (sensitive for cloxacillin acillin and resist to Ampicillin.) was 0.1146
Extracted protein from transformant strain was 0.2217
Extracted DNA from *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.) was estimated by measuring O.D. at 260 nm and it was 0.1049 meanwhile extracted DNA from *E. coli* (sensitive for cloxacillin acillin and resist to Ampicillin.) was 0.0796
Extracted DNA from transformant strain was 0.2010
Concentration of the protein *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.)

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times A_{280} - 0.76 \times A_{260}$$

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times 0.0936 - 0.76 \times 0.1049$$

$$= 0.065$$

Concentration of the protein *E E. coli* (sensitive for cloxacillin acillin and resist to Ampicillin.)

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times A_{280} - 0.76 \times A_{260}$$

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times 0.1146 - 0.76 \times 0.0796$$

$$=0.116$$

Concentration of the protein in transformant was calculating by using the formula

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times A_{280} - 0.76 \times A_{260}$$

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times 0.2217 - 0.76 \times 0.2010$$

$$= 0.190$$

Strain	A260	A280	Proteins (µg / µl)
E. coli (resist for cloxacillin acillin and sensitive to Ampicillin.)	0.1049	0.0936	**0.065**
E. coli (sensitive for cloxacillin acillin and resist to Ampicillin.)	0.0796	0.1146	**0.116**
transformant strain	0.0796	0.1146	**0.190**

Discussion

Replica plating and gradient plate method were used to isolate strains with multi trails by using those technique , The transformation process can also used to prepare strain with addition trail through the transformed the different strains with different trails .
From this point , we tried to use Replica plating , gradient plate and transformation method to prepare strain with multi trails which can cope the different harmful condition and can survive in different environmental
This method is very powerful , if we are going to prepare bacterial strains with multi trails which have very efficient role in biological control and bioremediation and bioaccumulation by using this method we can prepare strain resist stress condition which have ability to degrade the polluted environmental .
Transformation technique was done for first strains (carry Cloxacillin . plasmid), second strain (carry Ampicillin.. plasmid) and third strain (sensitive to both Ampicillin. and Colx. Antibiotics) , the transformant strain has very efficient transformed with 1000 Mg / ml of antibiotics meanwhile most strains can make transformation with very low concentration from what is mentioned STANLEY N *et al* did transformation process with concentration of antibiotics less than 100 Mg / ml and the efficiency of transformation was less than 9% meanwhile The efficiency of transformation was calculated for transformation process of three strains , the efficiency was 9% . In spite of that the transformation was done with multi trails . Stability of plasmids also was determined by using curing method (sub – culturing method) and it was stable for 12 times of sub culturing of transformant growth

Concentration of protein extracted form transformant starin was **0.190** and it was higher than *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.) which equal to **0.116** and *E. coli* (sensitive for cloxacillin acillin and resist to Ampicillin.) . which equal to **0.065** . increasing concentration of protein in transformant strain indicate for transformation process was successful between *E. coli* (resist for cloxacillin acillin and sensitive to Ampicillin.) and *E. coli* (sensitive for cloxacillin acillin and resist to Ampicillin.)

The efficiency and stability of transformation for multiple strains , this approach open one way to new era to prepare probiotics strain with multi trails resist different antibiotics also have ability to make transformation with very high efficient ,stable and tolerant high concentration of antibiotics .

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