A novel method for isolation new strain of *E. coli (* resist for Ampicillin and sensitive for Gentamycin) and other *(*sensitive for Ampicillin and resist for Gentamycin)and check it's conjugation by solid media , liquid media 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 Amp. (100 Mg / ml) and (sensitive to Gen.) then gradient plate method was used to adapt first strain to resist Amp. (500 Mg-ml) therefore. first strain was resistant to Amp. (500 Mg / ml) and sensitive to Gen. (500 Mg / ml) , on other hand ,by using same methods , second strain was resistant to Gen. (500 Mg / ml) and sensitive to Amp.. (500 Mg / ml)

To visualize if (Amp. resist trail and Gen. resist trail) carried on plasmid , first and second strains were conjugated by using conjugation technique in solid media and liquid media .

The conjugant strain was resistant to (1000 Mg / ml) by which it resists Amp. and Gen. , (500 Mg / ml) for each antibiotic .

The efficiency of conjugant was determined on solid media and liquid media , it was so high a round 9% . The conjugant strain was checked for stability of it's plasmid by using sub – culturing method . It was stable for 25 times of sub-culturing

Concentration of the protein for ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** and other strain ***E. coli (*****sensitive for Ampicillin and resist for Gentamycin)** was **0.0021**and **0.0052** meanwhile conjugant was **0.106**

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

Introduction :

Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells.which is often incorrectly regarded as the bacterial equivalent of sexual reproduction or mating since it involves the exchange of genetic material. During conjugation the donor cell provides a conjugative or mobilizable genetic element that is most often a plasmid or transposon. Most conjugative plasmids have systems ensuring that the recipient cell does not already contain a similar element.

The genetic information transferred is often beneficial to the recipient. Benefits may include antibiotic resistance, or the ability to use new metabolites (1 , 2).

The prototypical conjugative plasmid is the F-plasmid, or F-factor. The F-plasmid is an episome (a plasmid that can integrate itself into the bacterial chromosome by homologous recombination) with a length of about 100 kb. There can only be one copy of the F-plasmid in a given bacterium, either free or integrated, and bacterium that possess a copy are called F-positive or F-plus (denoted F+). Cells that lack F plasmids are called F-negative or F-minus (F-) and as such can function as recipient cells.(3,4) the benefit of this study is that It is a novel method to isolate strains with multi trails and Conjugant strain was resist to very high concentration of multi antibiotics with high efficiency and the stability of plasmid was very high . Finally the using replica and gradient are efficient method to isolate strains with multi trails and very efficient conjugant 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 - Sterile N.A + Amp (500 µg/ml) plates.
4 - Sterile N.A + Gen. (500 µg/ml) plates.
5 - Sterile N.A + Amp + Kan plates.
6 - Sterile N.B.
7 – Sterile N.A.+ (Amp and Gen. with different concentration) plates

Method :

1 . Replica plating and gradient plate method

1 . Over night agar growth of (*E. coli*. strain 1) was Prepared by adding 0.2 ml bacterial suspension into plate and incubated for 24 hours at 37 C .
2 . After incubation (master plate) , colonies are picked up with filter paper carrier .
3 . Ampicillin agar with 100 Mg/ ml is incubated with carrier at 37 C for 24 hours to isolate the ampicillin resistant colony .

4 . Ampicillin agar with 300 Mg/ ml is incubated with (the ampicillin resistant colony for 100 Mg/ ml) at 37 C for 24 hours to isolate the ampicillin resistant colony for 300 Mg/ ml) .
5 . Ampicillin agar with 500 Mg/ ml is incubated with (the ampicillin resistant colony for 300 Mg/ ml) at 37 C for 24 hours to isolate the ampicillin resistant colony for 500 Mg/ ml) .

6. Ampicillin resistant colony was picked up and made suspension by N. B. then incubated with Gentamycin agar plate with 500 Mg/ml to isolate the Gentamycin sensitive colony .
7 . Ampicillin Resistance Gentamycin Sensitive colony was picked up and made suspension and this is considered (suspension 1)
8 . Over night agar growth of (*E. coli*. strain 2) was Prepared by adding 0.2 ml bacterial suspension into plate and incubated for 24 hours at 37 C .
9 . After incubation (master plate) , colonies are picked up with filter paper carrier .
10 . Ampicillin agar with 500 Mg/ ml is incubated with carrier at 37 C for 24 hours to isolate the ampicillin sensitive colony .

11 . Ampicillin sensitive colony was picked up and made suspension by N. B. then incubated with Gentamycin agar plate with 500 Mg/ ml to isolate the Gentamycin resistance colony .
12 . Ampicillin Sensitive – Gentamycin Resistance colony was picked up and made suspension and this is considered (suspension 2) .
13 . 1 ml suspension 1 and 2 was mixed and incubated , the mixture was at 37 C for 24 hours .
14 . r 0.2 ml from the previous mixture was poured and incubated it with ampicillin – Gentamycin agar with 500 Mg/ml for each , then checked the No. of colony which is represented the conjugated colony (5).

2 . conjugation process in solid media (Agar plate method)
1 - The donor and recipient cultures in sterile broth was Inoculated, and incubated overnight at 37ºC on shaker.
2 - The donor and recipient cultures was Steaked on sterile N.A plates in perpendicular fashion.
4 - The growth from the intersecting area was Picked up and streaked on the agar medium containing both the antibiotics.
5 - The growth of conjugants was Incubated and checked .

3 . conjugation process in Liquid media (Broth mating)
1 - The pure cultures of donor and recipient in separate broth tubes was inoculated, and incubated overnight at 37ºC.
2 - 1 ml of recipient was added to 5 ml of sterile broth and to it add 0.1 ml of donor culture.
3 – The growth was incubated overnight at 37ºC.
4 - The T.V.C of donor, recipient and conjugants. was determined
5 - The frequency of conjugation was calculated .(5)

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 :

• 25 ml of bacterial culture was taken and Centrifuged at 8000 rpm for 10 min. at 4ºC. the supernatant media was decanted .
• 500 µl of solution Iwas added and mixed well. 15 µl of solution Iiwas added and mixed well then incubated at 37ºc for 10 min.
• 100 µl of 20% SDS (pre-warmed at 37ºc).was added mixed gently for 5 min.then incubated on ice for 1 hour. After that centrifuged at 13,000 rpm for 10 min at 4ºC to pellet down other cell
organelle.finally the supernatant in a fresh vial was collected .(6)

ESTIMATION OF PROTEINS

Concentration of the protein sample 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 first strain with Amp. Resistant plasmid containing *E. coli* strain with 100 Mg – ml as elucidated in figure (1)

Figure (1) show first strain resist to Amp. (100 Mg-ml) which is selected by replica plating method

Then by using gradient plate method , the Amp. Resistant plasmid containing strain was resistant to 150 , 250 then 500 Mg – ml as elucidated with figure (2, 3, 4)

Figure (2) show first strain resist to Amp. (150 Mg-ml) which is adapted to that concentration by using gradient plate method

Figure (3) show first strain resist to Amp. (250 Mg-ml) which is adapted to that concentration by using gradient plate method

Figure (4) show first strain resist to Amp. (500 Mg-ml) which is adapted to that concentration by using gradient plate method

Amp. Resistant plasmid containing *E. coli* strain was streaked out on plates containing Gen., The Amp. Plasmid containing *E coli* strain was sensitive for Gen. (500 Mg-ml). Therefore by using Replica plating and Gradient plate method we isolated strain resist to 500 Mg – ml Amp. and sensitive to 500 Mg – ml for Gen.. Figure(5)

Figure (5) show first strain sensitive to Gen. (500 Mg-ml)

Same method repeatred on second strain but Gen. Resistant plasmid containing *E coli* strain was selected and further sequential method to prepare strain resist to Gen. and sensitive to Amp.. With 500 Mg-ml for each antibiotics figure (6 , 7)

Figure (6) show second strain sensitive to Amp. (500 Mg-ml)

Figure (7) show second strain resist to Gen. (500 Mg-ml) which is adapted to that concentration by using gradient plate method

The conjugation technique was done for two strains to get conjugant can resist 1000 Mg –ml of Amp. , Gen.. on solid media as shown in figure (8)

Figure (8) show Conjugant strain resist to Gen.and Amp. (500 Mg-ml) for each antibiotic

The efficiency of conjugation was calculated for conjugation process of three strain , the efficiency was ……

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

The stability of plasmid can be seen for 25 days through growing the strain on Amp., Clox. and Gen. with 1500 Mg-ml (500 Mg – ml for each)

Extracted protein from ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** was estimated by measuring O.D. at 280 nm and it was 0.0832 meanwhile extracted protein from ***E. coli (*****sensitive for Ampicillin and resist for Gentamycin)** was 0.1198

Extracted protein from conjugant strain was 0.2269

Extracted DNA from ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** was estimated by measuring O.D. at 260 nm and it was 0.1656 meanwhile extracted DNA from ***E. coli (*****sensitive for Ampicillin and resist for Gentamycin)** was 0.2365

Extracted DNA from conjugant strain was 0.3223

Concentration of the protein ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)**

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

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times 0.0832 - 0.76 \times 0.1656 = 0.0021$$

Concentration of the protein ***E. coli (*****sensitive for Ampicillin and resist for Gentamycin)**

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

$$\textbf{Concentration of Proteins (µg / µl)} = 1.55 \times 0.1198 - 0.76 \times 0.2365$$

$$= 0.0052$$

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

Concentration of Proteins (µg / µl) = 1.55 x A_{280} – 0.76 x A_{260}

Concentration of Proteins (µg / µl) = 1.55 x 0.2269**– 0.76 x** 0.3223

= 0.106

Strain	A260	A280	Proteins (µg / µl)
E. coli (**resist for Ampicillin and sensitive for Gentamycin)**	0.1656	0.0832	**0.0021**
E. coli (**sensitive for Ampicillin and resist for Gentamycin)**	0.2365	0.1198	**0.0052**
conjugant strain	0.3223	0.2269	**0.106**

Discussion

Replica plating and gradient plate method were used to isolate strains with multi trails, The strains with any trial can be prepared in this technique . In this study , strains resist to one antibiotic and second strain to other antibiotics were prepared .

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. We also can prepare strain resist for stress condition which have ability to degrade the polluted environmental .

Conjugation technique was done for those strains , strain containing plasmids were isolated , the conjugation strain has very efficient conjugation with 1000 Mg / ml meanwhile most strains can make conjugation with not more than 300 Mg – ml . In spite of that the conjugation was done with multi trail , the efficiency was high in liquid and solid media .

Stability of plasmids conjugation also was determined by using curing method (sub – culturing method) and it was stable for 25 days of sub culturing

Concentration of protein extracted from conjugant starin was **0.106** and it was higher than ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** which equal to **0.0021**and ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** .which equal to **0.0052** increasing concentration of protein in conjugant strain indicate for conjugation process was successful between ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)** and ***E. coli (*** **resist for Ampicillin and sensitive for Gentamycin)**

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

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طريقة جديدة لعزل سلالات أي كولاي المقاومة لأمبسلين وحساسة للجنتمايسين وسلالة اخرى مقاومة للجنتمايسين وحساسة لأمبسيلين وفحص الاقتران بالحالة الصلبة والسائلة وثباتيتها بواسطة طريقة سلسلة من المزوع البكتيري المتدرج

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الخلاصة

جمعت سلالات لبكتريا أي كولاي من مكانين مختلفين ، السلالة الاولى عزلت من مختبر المركزي لجامعة بغداد بينما السلالة الثانية جمعت من المختبر المرضية لجامعة بغداد . استخدمت طريقة البصمة وطريقة الطبق المتدرج لتحضير سلالات مع صفات مختلفة . استخدمت طريقة البصمة للأختيار سلالة مقاومة لنوع محدد من المضاد حيوي مع تركيز قليل (حساس لنوع الاخر من المضاد الحيوي) بعد ذلك استخدمت طريقة الطبق المتدرج لتطبع السلالة لمقاومة تركيز عالي . استخدمت طريقة البصمة لمقاومة مضاد الانبسلين بتركيز 100 ملغرام بالمل وحساسة لمضاد الجنتمايسين ، بعد ذلك استخدمت طريقة الطبق المتدرج لتطبيع السلالة الاولى لمقاومة امبسلين بتركيز 500 ملغرام بالمل لهذا السلالة الاولى كانت مقاومة للأمبسلين بتركيز 500 ملغرام بالمل وحساس لجنتمايسين بتركيز 500 ملغرام بالمل ، من جهة اخرى بأستخدام نفس الطريقة السلالة الثانية كانت مقاومة لجنتمايسين بتركيز 500 ملغرام بالمل وحساس للأمبسلين بتركيز 500 ملغرام بالمل . لأظهار صفة مقاومة الامبسلين والجنتمايسين محمولة على البلازميد او الكروموسوم السلالة الاولى والسلالة الثانية اقترنت باستخدام تقنية الاقتران بأستخدام الوسط السائل والوسط الصلب . السلالة المقترنة كانت مقاومة لتركيز 1000 ملغرام بالمل والتي هي تقاوم تركيز 500 ملغرام بالمل لكل من المضادين الحيوين . حددت كفاءة الاقتران على الاوساط الصلبة والاوساط السائلة وكانت جدا عالية حوالي 9% . فحصت السلالة المقترنة لثبات البلازميد باستخدام طريقة الزرع المتكرر وكانت ثابتة 25 مرة . تركيز البروتين لسلالة الاولى والثانية كانت 0.0021 و 0.0052 على التوالي بينما للسلالة المقترنة كان 0.106 واخيرا استخدام تقنيات البصمة والطبق المتدرج وعملية الاقتران كتقنية جديدة لتحضير سلالات مقاومة لصفات عديدة بكفاءة عالية .