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# Prevention of *Proteus mirabilis* Biofilm by Surfactant Solution

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## ABSTRACT

**Background:**

The crystalline biofilms formed by *Proteus mirabilis* can seriously complicate the care of patients undergoing bladder catheterization, the prevention of crystalline biofilms is important to avoid urinary catheter complications. The surfactant of *Lactobacillus acidophilus* can be interacting with attachment of many microorganisms.

**Aims of the study:**

1- To detect the biofilm formation ability of *Proteus mirabilis* isolates in urinary catheter.
2- To detect the growth and biofilm formation ability of *Proteus mirabilis* isolates on 96-wells microtiter plates.
3- To study the role of *Lactobacillus acidophilus* surfactant in biofilm formed by *Proteus mirabilis* isolates.

**Materials and methods:**

Since Jan/2011 to Jan/2012 a 48 isolates of *Proteus mirabilis* were isolated from the encrusted catheter of a patient undergoing indwelling catheterization in Al-Ramadi General Hospital while *Lactobacillus acidophilus* isolate was obtained from urogenital tracts of healthy woman by vaginal swab and surfactant was extracted from it. The biofilm formation on catheters, quantitative assays of biofilm formation and biofilm inhibition assay by surfactant were studied.

**Results:**

Fourty five 45 (93.75%) *P. mirabilis* isolates produced a biofilm in urinary catheter while 3 (6.25%) isolates were not produce biofilm while 48 (100%) isolates forms good biofilms results on 96-wells microtiter plate, in brain heart infusion broth with 0.2% glucose, and at 37°C. The growth of *P. mirabilis* isolates in 96-well microtiter plates were determine at OD630, the optimum growth of *P. mirabilis* isolates were recorded after 48 hours of incubation (OD630= 0.65). Biofilm kinetics of *P. mirabilis* isolates refers to that attached cells increased with time and the maximum biofilm formation ratio occurs at 48 hours of incubation (OD550= 0.70).

Surfactant show a good ability to inhibit biofilm formation, the increasing amounts of surfactant led to a decrease in the amount of biofilm formed by *P. mirabilis* isolates and that 6.0 µg/ml of surfactant was more than sufficient to completely abolish biofilm formation.

**Keywords**: biofilm, surfactant, *P. mirabilism, L. acidophilus*

## INTRODUCTION

Biofilms are aggregates of microorganisms, which are formed due to the attachment of cells to each other and/or to a host surface in an aqueous environment. Lynch, J. F, *et al*. (2003) Bacterial adhesion to surfaces is one of the initial steps that lead to biofilm formation. Donlan R.M. & Costeron J.W. (2002).

Biofilms are a matter of concern to many medical industries, since bacteria

can colonize medical devices, altering their properties. Furthermore, it may represent an important source of contamination, releasing pathogenic bacteria. Biofilms are more resistant to antimicrobial agents, impairing the control of this form of microbial organization, when compared to free cells. Costeron JW (2002) and Dunne MW (2002).

The crystalline biofilms formed by *Proteus mirabilis* can seriously complicate the care of patients undergoing bladder catheterization. The generation of alkaline urine by the bacterial urease causes calcium and magnesium phosphates to precipitate from urine and accumulate in the catheter biofilm, blocking the flow of urine from the bladder. They are capable of generating ammonia from urea and elevating the pH of the urine and biofilm. Under these conditions, crystals of calcium and magnesium phosphate precipitate in the urine and in the developing biofilm. Aggregates of crystals and bacteria form in the urine and these also become incorporated into the developing crystalline biofilm. Morris, N. S., *et al*. (1999)

Probiotic bacteria, such as lactobacilli, are well known to have a positive effect on the maintenance of human health. Merk K, *et al*. (2005) and Gupta V & Garg R.(2009). These bacteria, which constitute an important part of natural microbiota, are recognized as potential interfering bacteria by producing various antimicrobial agents such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, and adhesion inhibitors, such as surfactant. Gupta V & Garg R. (2009)

In particular, lactobacilli have long been known for their antimicrobial activity and capability to interfere with the pathogens adhesion on epithelial cells of urogenital and intestinal tracts. Ali, O. A. (2012) and Reid G, *et al*. (2001), and for their anti-biofilm production on voice prostheses. Rodrigues L, *et al*. (2006-a) and Rodrigues L, *et al*. (2006-b). The mechanisms of this interference have been demonstrated to include the release of surfactant. Gudina EJ, *et al*. (2010).

## MATERIALS AND METHODS

Since Jan/2011 to Jan/2012 a 48 isolates of *Proteus mirabilis* were isolated from the encrusted catheter of a patient undergoing indwelling catheterization in Al-ramadi General Hospital. The *Proteus mirabilis* isolates were characterized by Colony morphlogies, Grams stain, Catalase, Oxidase, Haemolysin production, Indole production, Voges-proskauer, Methyl red, Citrate utilization, Urease test, Mannitol fermentation and Motility tests and they were cultured on triple sugar iron (TSI) slant. Atlas, *et al*. (1995)

*Lactobacillus acidophilus* isolate was obtained from urogenital tracts of healthy woman. Vaginal swab was cultured on chocolate agar, blood agar, and MacConkys agar (as routine work), and incubated at 37◦C for 24-48 hours under 5% carbon dioxide conditions. The lactobacilli isolates were characterized according to Atlas, *et al*. (1995) by Grams stain, culturing the isolates in De man Rogosa Sharpe (MRS) broth media anaerobically, culturing the isolates in nutrient agar aerobically, catalase test, production of ammonia from arginine, production of acids from raffinose and mannitol, growing on 45◦C.

**Surfsactant Extraction:**

Surfactant solution extracted from *Lactobacillus acidophilus* isolate was done according to Walencka E, *et al*. (2008).

**Biofilm Formation on Catheters:**

A method for assaying biofilm formation of *P. mirabilis* isolates was based on Mireles J. R. *et al.* (2001) with minor modifications. Typically, 10 µl of overnight *P. mirabilis* culture was

inoculated into 500 µl of brain heart infusion broth medium and injected into foly urinary catheters. The catheters were capped at both ends and incubated at 37°C for 48 hours. The catheters were rinsed with Phosphate Buffer Saline (PBS) pH 7.2. After drying at room temperature for 15 min, 700 µl of crystal violet (1%) was added to the catheters for 20 min. The stained biofilms were rinsed several times with (PBS) pH 7.2 and allowed to dry at room temperature for 15 min before examination.

**Quantitative Assays of Biofilm Formation:**

20 µl of *P. mirabilis* overnight culture was used to inoculate microtiter wells containing 180 µl of brain heart infusion broth with 2% glucose. The covered microtiter dish was sealed with Parafilm during incubation at 37°C for deferent periods. Cultures were removed to determine the optical density at 630 nm (OD630), and the wells were rinsed with (PBS) pH 7.2. After drying at room temperature for 15 min, 200 µl of crystal violet (1%) was added to the wells for 20 min. The stained biofilms were rinsed three times with PBS pH 7.2, allowed to dry at room temperature for 15 min, and extracted twice with 200 µl of 95% ethanol. The OD550 was estimated using automatic microtiter plates reader. Mireles J. R. *et al.* (2001)

**Biofilm Inhibition Assay:**

Biofilm inhibition assays with the extracted surfactant solutions were performed in pre-coating and co-incubation experiments. Typically, in pre-coating experiments 96-well microtiter plates were filled with 200 µL of different concentrations of surfactant solution (ranging from 1.0µg/ml to10.0 µg/ml) and incubated for 24 hours at 4°C. Surfactant solutions were, then, removed and the plates carefully washed twice with PBS pH 7.2 to remove non-adhering surfactant. 200 µL of each *P. mirabilis* isolate suspension in brain heart infusion broth with 2% glucose were then added to each well and plates incubated at 37°C for 24 hours. Non-adherent cells were removed by gently washing twice the wells with PBS pH 7.2.

In co-incubation experiments, *P. mirabilis* suspensions in brain heart infusion broth with 2% glucose were added to 96-well microtiter plates together with different concentrations of surfactant solutions, (ranging from 1.0µg/ml to 10.0 µg/ml) and incubated at 37°C for 24 hours. Non-adherent cells were removed by gently washing twice the wells with PBS pH 7.2. Staining method was as above for quantitative assays of biofilm formation in 96-Well microtiter plates assay.

**Statistical analysis:**

Percentages of microbial biofilms were calculated as described in equation:

$$\% \text{ Microbial biofilm} = (Ac/Ao) \times 100$$

Where *Ac* represents the absorbance of the well with surfactant concentration c at OD550 and *Ao* the absorbance of the control well at OD550. This allows estimating the percentage of microbial biofilm in relation to the control wells, which were set at 100% indicating total cells adhesion in the absence of surfactant. Fracchia, L. *et al.* (2010).

## RESULTS

Since Jan/2011 to Jan/2012 a 48 isolates of *Proteus mirabilis* were isolated from the encrusted catheter of a patient undergoing indwelling catheterization in Al-ramadi General Hospital. Surfactants solution extracted from *Lactobacillus acidophilus* isolate that obtained from urogenital tracts of healthy woman.


Fig. 1: *P. mirabilis* biofilm in urinary catheters, -A- positive and –B- negative.

To investigate the biofilm forming ability in urinary catheter, all of *P. mirabilis* isolates were tested. 45 (93.75%) isolates produced a biofilm in urinary catheter while 3 (6.25%) isolates were not produce biofilm. (Fig. 1)

The biofilm assay used in this study monitors the ability of *P. mirabilis* to attach to the wells of microtiter plate. The biofilms quantitated by staining with crystal violet as described in Materials and Methods. Initial experiments showed that the 48 (100%) *P. mirabilis* isolates forms good biofilms results on 96-wells microtiter plate, in brain heart infusion broth with 0.2% glucose, and at 37°C. (Fig. 2 -A-).

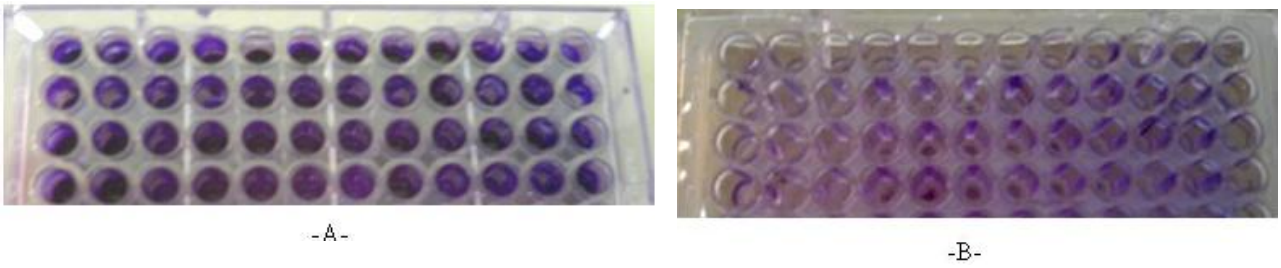


Fig. 2: *P. mirabilis* biofilm on microtiter wells,
-A- untreated (control) –B- treated with 5.0µg/ml surfactants solution.

The growth of *P. mirabilis* isolates in 96-well microtiter plates were determine at OD630, the optimum growth of *P. mirabilis* isolates were recorded after 48 hours of incubation (OD630= 0.65). Biofilm kinetics of *P. mirabilis* isolates refers to that attached cells increased with time and the maximum biofilm formation ratio occurs at 48 hours of incubation (OD550= 0.70). (Fig. 3)

To analyze the effect of surfactant solutions on biofilm formation, either the microtiter wells were coated with surfactant or surfactant solutions were included in the growth medium. Fig. 4 shows that increasing amounts of surfactant led to a decrease in the amount of biofilm formed by *P. mirabilis* isolates and that 6.0 µg/ml of surfactant was more than sufficient to completely abolish biofilm formation. The optical density (OD550) of untreated (control) microtiter wells were ranged from 0.50 to 0.70 while the optical density (OD550) of microtiter wells treated with 6.0 µg/ml surfactant solution ranged from 1.250 to 2.0. (Fig. 2) Surfactant treated sample decreased at a faster rate than that of the untreated sample

of biofilm formation, resulting in an approximately 70.0% decrease in total biofilm by the end of the experiment after 24 hours of incubation at 37°C.

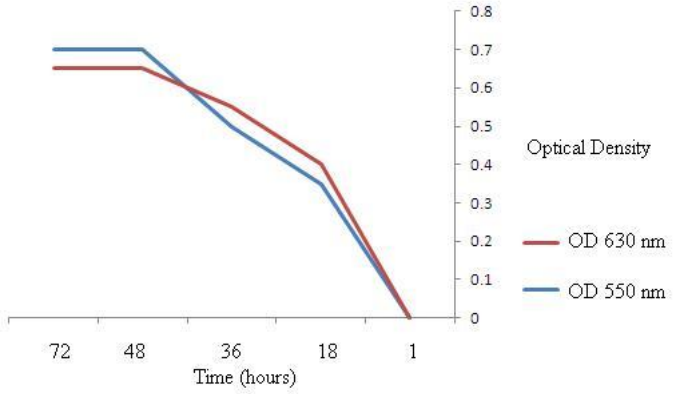


Fig. 3: Kinetics of biofilm formation by *P. mirabilis* isolates in 96-well microtiter plates containing brain heart infusion broth with 0.2% glucose, growth was determine at OD630 and biofilm formation was determine after crystal violet staining at OD550.

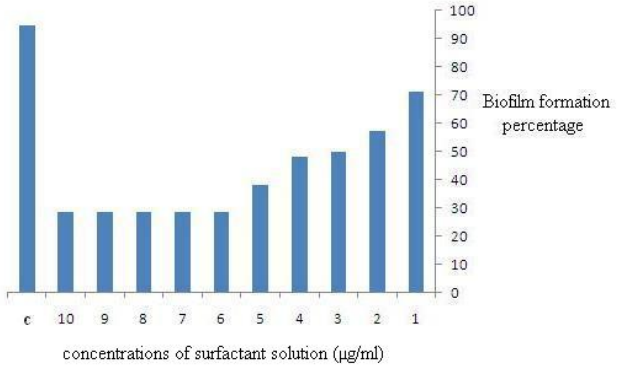


Fig. 4: Surfactants solutions inhibits biofilm formation by *P. mirabilis* isolates, c = untreated (control).

## DISCUSSION

Bacteria have a basic survival strategy: to colonize surfaces and grow as biofilm communities embedded in a gel-like polysaccharide matrix. The catheterized urinary tract provides ideal conditions for the development of enormous biofilm populations. Many bacterial species colonize indwelling catheters as biofilms, inducing complications in patients care.

*P. mirabilis* is urease producing bacteria colonize the catheter surfaces forming extensive biofilm communities, the results of current study shows that 45 (93.75%) of *P. mirabilis* isolates produced a biofilm in urinary catheter and the biofilm formation in the wells of microtiter plate by *P. mirabilis* isolates were measured, and the results shows that 48 (100%) produced a good biofilm in the wells (OD550= 0.70) while the optimum isolates growth were recorded after 48 hours of incubation (OD630= 0.65). (Fig. 3)

*P. mirabilis* is the third most common cause of complicated UTI and the second most common cause of catheter associated bacteriuria in catheterized patients. *P. mirabilis* have multiple virulence factors help it in colonization of urinary catheters, it have been shown to produce various fimbriae and hemagglutinins involved in the colonization of the urinary tract and possibly catheter surfaces, including MR/P fimbriae, that are perhaps the best-understood fimbriae expressed by *P.*

*mirabilis* strains during UTIs. Jacobsen, S. M. *et al*. (2008)

The flagellar motility and swarming are important during colonization of catheter. Swarming may play a role in the migration of Proteus strains on catheter materials; however, swarmer cells of Proteus species are capable of migrating across 1cm-long sections of Foley catheters consisting of either hydrogel-coated latex, hydrogel/ silver-coated latex, silicone-coated latex, and all silicone in vitro. Sabbuba, N. (2002) In addition to generating ammonia from urea and elevating the pH of the urine and biofilm, producing crystals of calcium and magnesium phosphate precipitate in the urine and in the developing biofilm. Morris *et al*., (1999). Under these circumstances, urine can leak around the outside of the catheter. Alternatively, urine is retained within the bladder and refluxed the infected urine to the kidneys Kunin, (1997). All available types of indwelling catheter are vulnerable to this problem Morris *et al*., (1999) and currently there are no effective procedures available for its control Kunin, (1997).

Different concentration of *Lactobacillus acidophilus* surfactant solutions were used to study the role of surfactant on biofilm formation, two experiments were used, either the microtiter wells were coated with surfactant once and surfactant solutions were included in the growth medium in another. The two methods showed approximately same results in view of the fact that increasing amounts of surfactant led to a decrease in the amount of biofilm formed by *P. mirabilis* isolates and that 6.0 µg/ml of surfactant was more than sufficient to completely abolish biofilm formation. (Fig. 4).

The number of adhering *S. aureus* and *S. epidermidis* cells after a 3-hours co-incubation with biosurfactants was reduced by 5–56 %. The *L. acidophilus* derived surfactants inhibit bacterial deposition rate and biofilm morphology showed that staphylococcal biofilms formed in the presence of *L. acidophilus* surfactant covered 1.3-2.4 times less surface area, their total biovolume decreased by 17–85 %, the mean biofilm thickness being 1.2-4.5 times lower. Biosurfactant isolated from *L. acidophilus* strain H-1 had the most potent antibiofilm activity in all tests. Walencka E, *et al.* (2008) Both biosurfactants of *Lactococcus lactis* 53 and *Streptococcus thermophilus* greatly reduced microbial numbers on prostheses and also induced a decrease in the airflow resistance that occurs on voice prostheses after biofilm formation. Rodrigues L, *et al*. (2004)

Fracchia, L. *et al*. (2010) recorded that, the anti-adhesive properties of the Lactobacilli surfactant against two *Candida albicans* biofilm producers suggesting that its potential use as an anti-adhesive product on medical devices (catheters, prosthesis, stents) to prevent *Candida albicans* infections. The ability of surfactants of *Lactobacillus sp.* to decrease bacterial attachment was observed by many other researches. Ali,O.A.(2012), Merk K, *et al*.(2005) Reid G, and Burton J. (2002), and Mireles J. R. *et al*. (2001)

The inhibitory effect of surfactant seems to be dependent on the type of surfactant, microorganism, and surface properties, Walencka E, et al. (2008) surfactant solution reduce hydrophobic interactions and consequently microbial adhesion. Hydrophobic surfaces have shown to be particularly colonized by microorganisms, probably because these surfaces facilitate the close approach between microorganism and solid substratum, favoring the elimination of interfacial water present in the interacting surfaces. Zeraik A. E. and M. Nitschke.(2010) Consequently, when a surface is conditioned with surfactant, it becomes more hydrophilic, with an

expected decrease of microbial attachment. Our results indicated a decrease in hydrophobicity on surfaces treated with surfactant and also a substantial decrease of bacterial attachment, in agreement with the aforementioned explanation.

*P. mirabilis* have other factors may contribute to this effect, surface charge of microorganism and substratum, the presence of fimbria, flagella and surface proteins, are features involved in the adhesion process, and surfactant may interact with these factors to inhibit biofilm formation

In conclusion, results of current study demonstrate that *P. mirabilis* have a high ability to produce biofilm in urinary catheter and 96-well microtiter plate and the biofilm formation can be decreased by using surfactant solution displayed from *Lactobacillus acidophilus*.

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## ARABIC SUMMARY

### منع تكوين الغشاء الحيوي لبكتريا *Proteus mirabilis* باستخدام محلول Surfactant

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**خلفية الدراسة:**

الغشاء الحيوي الذي تكونه بكتريا *Proteus mirabilis* يمكن ان يسبب مشاكل خطيرة للمرضى عند استخدامهم لانبوب قسطرة المثانة، منع تكوين الغشاء الحيوي مهم لتلافي المشاكل الصحية المرافقة لقسطرة المثانة. محلول surfactant لبكتريا *Lactobacillus acidophilus* له القدرة على منع التصاق عدة احياء مجهرية لذا يمكن استخدامه لهذا الغرض.

**اهداف الدراسة:**

1- تحديد قدرة بكتريا *Proteus mirabilis* في تكوين الغشاء الحيوي على انابيب القسطرة البولية.
2- تحديد قدرة بكتريا *Proteus mirabilis* في النمو وتكوين الغشاء الحيوي في اطباق الاليزا المختبرية.
3- دراسة دور محلول surfactant لبكتريا *Lactobacillus acidophilus* في تكوين الغشاء الحيوي لبكتريا *Proteus mirabilis*

**المواد وطرق العمل:**

منذ كانون الثاني/2011 ولغاية كانون الثاني/2012 تم عزل 48 عزلة لبكتريا *Proteus mirabilis* من مرضى يستخدمون انابيب القسطرة البولية وراقدين في مستشفى الرمادي العام في حين تم عزل بكتريا *Lactobacillus acidophilus* من الاعضاء التاسلية لنساء صحيات بواسطة المسحات المهبلية وتم استخلاص surfactant منها.كما درست قابلية بكتريا *Proteus mirabilis* على تكوين الغشاء الحيوي في انابيب القسطرة البولية وحددت قدرتها على تكوين الغشاء الحيوي كميا بالاضافة الى دراسة امكانية تثبيط بناء الغشاء الحيوي باستخدام surfactant .

**النتائج:**

45 (93.75%) عزلة من بكتريا *P. mirabilis* كونت غشاء حيوي في ابابيب القسطرة البولية و3 (6.25%) لم تنتج الغشاء الحيوي في حين 48 (100%) عزلة انتجت غشاء حيوي جيد على اطباق الاليزا المختبرية باستخدام وسط مستخلص القلب والدماغ المدعم بسكر الكلوكوز 2% عند درجة حرارة 37 درجة سيليزية.

افضل نمو لعزلات بكتريا *P. mirabilis* سجل عند 48 ساعة من التحضين (OD630= 0.65) في حين اظهرت تجارب انتاج الغشاء الحيوي زيادة في اعداد خلايا بكتريا *P. mirabilis* بزيادة الوقت الى حد 48 ساعة من التحضين حيث ظهر عند هذا الوقت افضل انتاج للغشاء الحيوي (OD550= 0.70) محلول Surfactant اظهر قدرة جيدة على منع بناء الغشاء الحيوي حيث ان زيادة تركيز المحلول تؤدي الى خفض كمية الغشاء الحيوي في حين كان التركيز 6.0مايكروغرام/مليليتر كافيا لمنع بناء الغشاء الحيوي.