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ISOLATION&IDENTIFICATION OF MICROORGANISM FROM PHARMACUETICAL PURIFIED WATER

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Abstract:-A typical purification water system in pharmaceutical provide the purified water must protected from microbial proliferation and also minimize the cross contamination. In pharmaceutical environment and water system bacterial population investigated for many days from different sampling point of different grade water including raw water, treated water, drinking water, purified water and water for injection (WFI). Purified water use for different purpose in Pharma industry like manufacturing of pharmaceutical product, active pharmaceutical ingredients (APIS), processing and formulation, production, storage, distribution system but in that chemical and microbiological quality are main concern [1]. Microorganisms found In pharmaceutical ingredients, finished product are frequently identified. This most important to isolate that kind of unwanted microbes because if exceed alert and/or action level take particular action against water system. Purified water sample microbial quality tested in routine by using membrane filtration and then it's cultured on the less nutrient growth medium R2A agar for 5 days. In that culture based methods and phenotypic characters identification of isolate include gram's staining, cellular morphology (rod, cocci etc.) and colony characteristics [12]. This type of information sufficient for routine purpose to confirm that bacteria found in the water sample are not change the water quality. If we taken research in depth perform orientation test (biochemical test) and API web identification system. The process of manufacturing the purified water in pharmaceuticals is reverse osmosis (RO) [2]. After that result act as a benchmark for pharmaceuticals and industrial microbiologists for determine or compare against the current water system, to present typical cultivable microorganisms recoverable from pharmaceutical water system.

Index Terms:- **Active pharmaceutical ingredients, Purified water, Microorganisms, Isolation, Identification**

1. INTODUCATION

water is used in pharmaceutical and life sciences operations. Water is widely used for raw material, ingredient, and solvent in the processing, formulation, and manufacture of pharmaceutical products, active pharmaceutical ingredients (APIs), hand washing, steam provide for autoclaves [8]. Maintain the quality of water during production, storage and distribution processes which is very difficult task. In that specially balancing the chemical and microbial quality are major concerns in pharmaceutical industry because it's related to health. Purified water production, storage and distribution system in pharmaceutics we have to designed, installed, qualified and maintained properly for reliable production of purified water with appropriate quality.

Each grade of water is a potential source of microbiological contamination, especially when the water is not properly controlled. In that case control is not only concern but also we have to found which type of microbes isolate from the pharmaceutical water system. In that case we have to changes in to the water trends and to understand if they are indicators of more serious problems like biofilm or if they present a special risk to products so that time take immediate action for water system[3].

Mostly pharmaceutical water systems are controlled, if the microorganisms be present inlow numbers.

1.1 Purified Water

Purified Water is used in the production of solvent preparations, cleaning of equipment's, sterilization etc. Purified Water meet the requirements for ionic and organic chemical purity and so it's must be protected from microbial contamination. The source of water for the production of Purified water is Drinking Water. It should also be protected from recontamination and microbial proliferation [12].

In pharmaceutical plant quality of purified water and microbial monitoring of purified water is performed by using TAMC (Total Aerobic Microbial Count) and specified microorganism test(like Pathogen test: E.coli., Salmonella, Pseudomonas, S. aureus) [13]. In those kind of two test has some acceptance criteria established for Total Aerobic Microbial Count (TAMC) in purified wateri.e.100cfu/ml. if any microorganisms repeatedly observe that time isolate & identification of that microbes shall be perform and also check its pathogen city by pathogen test. Identification of microorganisms performs by using Gram's staining, morphology & biochemical tests (orientation test).

1.2 Microbiological test for water

Microbial test found that number of microorganisms in a water sample, so we ensure that bacterial loads don't more which mention in USP. Microbial tests for water include the estimation of the number of viable aerobic bacteria present in given purified water. The method for testing is membrane filtration in that process uses the cellulose acetate 0.45 µm filter [18]. This filter place onto R2A agar plate and incubate at 30-35 °C temperature for five days for the purified water sample.

For obtain proper result of purified water select optimal microbiological cultural condition like most appropriate temperature, time for incubation period and culture media for growing. This tests showed that R2A developed higher counts when its incubated for 5–7days at 20°C or 30°C [19].

In this R2A medium needs the larger sample volumes that's why we using the membrane filtration method. If R2A provide the optimal incubation conditions so we get the better counts but do not grow the full range of microorganisms present.

2. METHODOLOGY

2.1 Isolation of microbial culture

a. Observe the R2A plate & identify the obtained microbial colony from water source.

b. Marked the isolated colony.

c. Verify the colony morphology characteristics of microbes on respective R2A Petri plate as per table and record the characteristics. Different types of bacteria will produce different looking colonies, some colonies may be colored, some colonies are circular in shape, and other is irregular. A specific terminology is used to describe common colony types.

d. Prepare a suspension of suspected colony of R2A plate in a 10ml of sterile normal saline.

e. Take a loop full of prepared suspension on pre - sterilize and pre- incubated SCDA plate for streaking.

f. Streak the suspension in such way to get a isolated colonies by four flame method.

g. Incubate the plate at30-35cor24hrs.

h. After getting pure culture of microbial isolated colony perform gram staining from single isolated colony and mention the shape of bacteria.

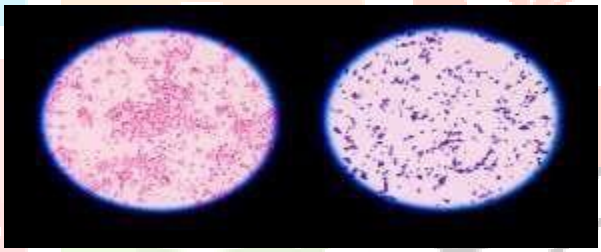
2.3 Gram's staining

This test differentiates the bacteria into Gram Positive and Gram Negative Bacteria. When bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol.

Procedure:

a. Taken a clean, grease free slide. b. Prepared the smear of suspension on the clean slide with a loopful of sample. c. Air dried and heat fixed. d. Then stained with crystal violet for about half to 1 minutes and rinse with water. e. Then applied gram's iodine for 1 minute and wash with water. f. Then, washed with 95% alcohol for about 10-20 seconds and rinse with water. g. Added safranin for about 1 minute and wash with water. h. Air dried and Observe under Microscope with oil immersion objective.

Interpretation



Those bacteria appear purple or violet color is referred as Gram positive, those appearing pink color cells are described Gram Negative.

2.3 Biochemical Screening Test System

Principle of orientation test:

According to morphology of colonies and gram staining for decision of next procedure of microbial identification quick tests are perform like oxidase, catalase, coagulase tests are called orientation tests. Hence, after performing gram staining and morphological features these orientation test shall be perform and based on the results the appropriate API strip shall be selected.

2.3.1 Catalase test

This test indicate the presence of catalase enzyme that release oxygen from hydrogen peroxide (H2O2). It is used to differentiate those bacteria that produce an enzyme catalase, such as Staphylococci, from non- catalase producing bacteria such as Streptococci.

Procedure:

a. Used sterile loop for transfer small amount of colony growth in the surface of a clean, dry glass slide. b. Placed a drop of 3% H2O2 in the glass slide. c. Observed the evolution of oxygen bubbles.

Interpretation:

(a) Catalase positive: Copious bubbles produced, active bubbling

Examples: Staphylococci, Micrococci, Listeria, E.coli, Enterobacter, Klebsiella, Shigella, Proteus, Salmonella, Pseudomonas, Mycobacterium tuberculosis, Aspergillus.

(b) Catalase negative::No or very few bubbles produced.

(c) Examples: Streptococcus and Enterococcus spp.

2.3.2 Coagulase test

Coagulase test is used to differentiate Staphylococcus aureus (positive) from Coagulase Negative Staphylococcus (CONS). Coagulase is an enzyme produced by S.aureus that converts (soluble) fibrinogen in plasma to (insoluble) fibrin. (23).

Procedure:

a. Emulsified a Staphylococus colony in drop of water on clean & grease free glass slide. b. Made a similar suspension of control positive and negative strains to confirm the proper reactivity of the plasma. c. Dipped sterile wire loop into the undiluted plasma at room temperature, withdraw, and stir the adhering traces of plasma into the staphylococcus suspension on the slide. Flame the wire and repeat for the control suspensions. d. Positive result indicates by shown visible clumping with our naked eye within 10 seconds and negative result indicates by absence of clumping. (14)

Interpretation:

(A) Coagulase positive: Macroscopic clumping in10 seconds.
Ex. S.aureus

(B) Coagulase negative: No clumping in either drop

2.3.3 Oxidase test

The oxidase test is used in microbiology to determine any bacterium have a cytochrome coxidase system that will catalyse electrons transport chain. oxidase test is particularly use for the rapid identification of Neisseria gonorrhea& Pseudomonas.

Procedure:

a. A strip of filter paper is soaked with a little freshly made 1% solution of the reagent. b. Sterile loop full culture was rubbed on it with a sterile loop. c. A positive reaction is indicated by intense deep-purple blue, appearing within 5-10 seconds, a "delayed positive" reaction by coloration in 10-60 seconds, and a negative reaction by absence of coloration in 60 seconds.

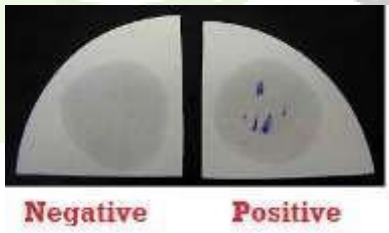
Interpretation:

(a) Oxidase positive:

Develop deep purple-blue color indicates oxidase production within 5-10 seconds.

(b) Oxidase negative:

No purple-blue color / No color change.

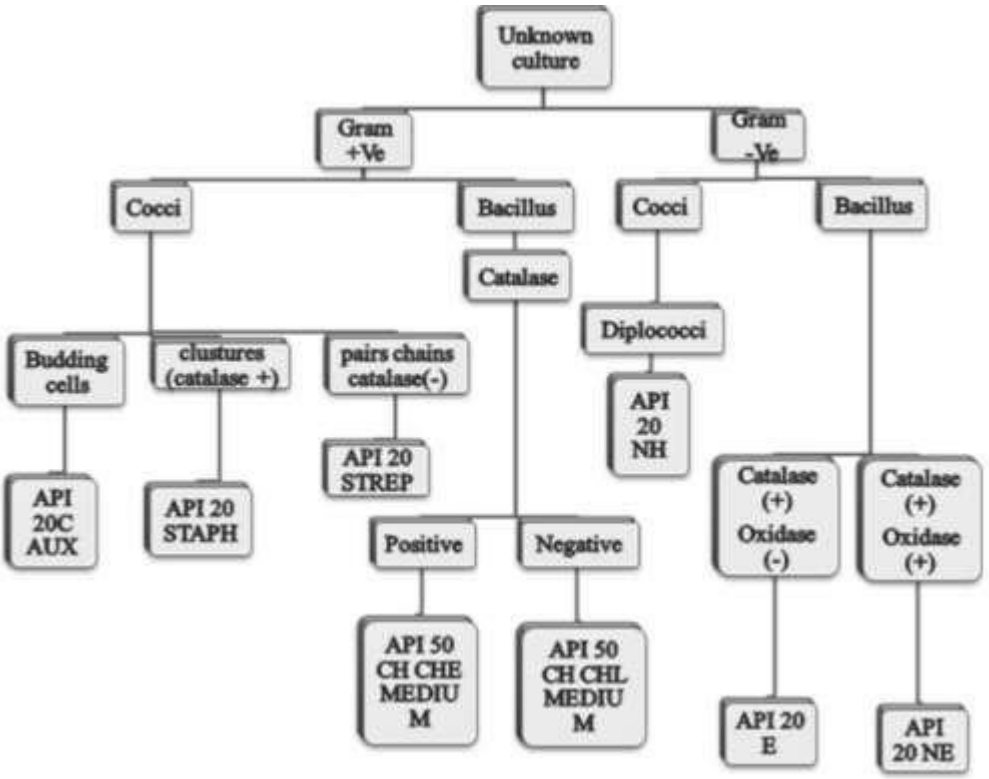


E.g. Unknown isolate from purified water sample point (S02) Gram's staining result–Gram positive

Shape – cocci in pairs and tetrads

Orientation test result Coagulase test – Negative Oxidase test – Negative Catalase test–Positive

Based on gram staining and orientation test result. API strips selection shall be as per below mentioned chart.



3. API WEB MICROBIAL IDENTIFICATION

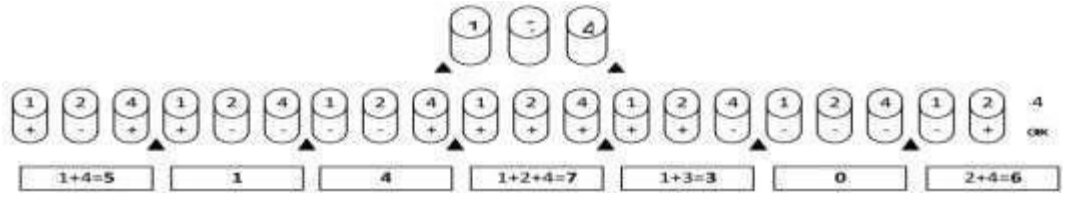
Principle:

API test strips consists of microtubes (cupules) containing dehydrated substrates to detect the enzymeatic activity or the assimilation/fermentation of sugars by the inoculate organisms. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. A positive result is indicated by growth. Test results are enter dint online database to determine the bacterial identify. [21]

Method:

API strips should only be used to identify pure cultures of an unknown organism. Confirm Gram stain (plus catalase and oxidase if appropriate) before inoculating a test strip.

Profile calculation and interpretation



a. Add any reagents as described for the type of strip used. b. Mark each test as positive or negative on the lid of the tray c. The wells are marked off into triplets by black triangles, for which scores are allocated as follows & Add up the scores for positive wells only in each triplet. The highest score possible for a triplet is 7 (the sum of 1, 2 and 4) and the lowest is 0. [5] d. The profile for this combination of reactions is therefore 5147306 e. Identify the organism using API web f. Start Internet Explorer or Firefox web browser. g. Go to: https://apiweb.biomerieux.comi. Select the correct test (e.g. API 20E). j. Enter the numerical profile to obtain the identity. k. Record identity along with comments (% ID and T value) on results sheet.[1]

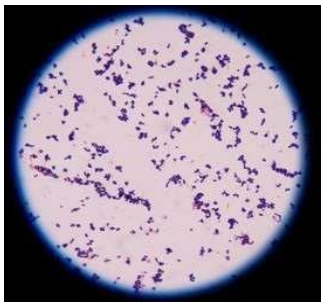
4. RESULT&DISCUSSION

A. Types of organisms: Water isolates B. Location: SP-12

C. Colony characteristics & Isolated organism plate:

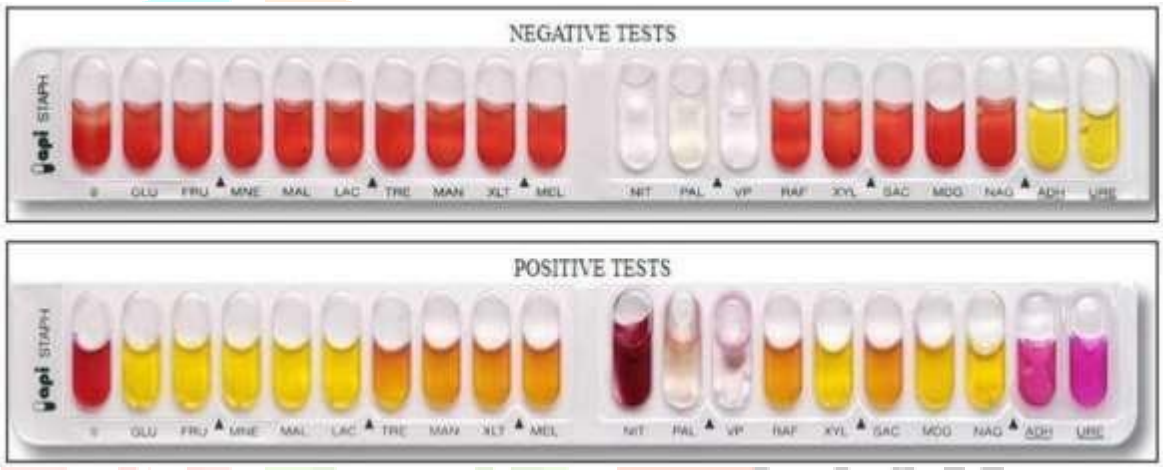
Shape	Round
Size	Medium
Color	Yellow
Margin	Entire
Surface	Smooth
Opacity	Opaque
Elevation	Convex
Consistency	Buttery

D. Gram's staining: by performing gram's staining we observed gram positive, non motile, purple colored organisms are in cocci shaped& arranged in cluster form shown against color less background.

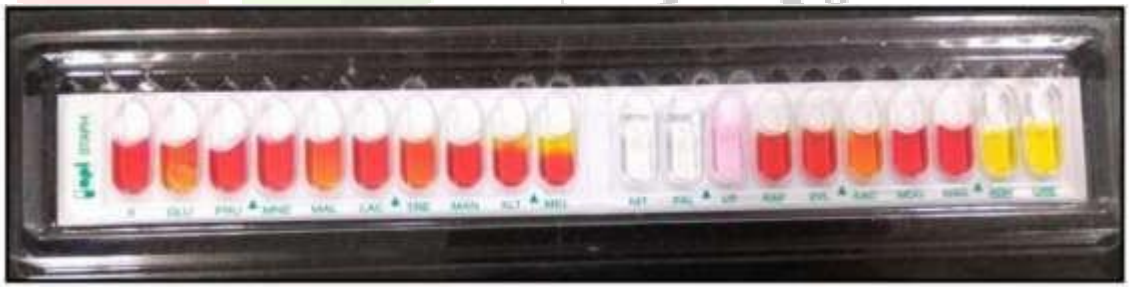


Gram staining result with gram +ve, purple colored, cocci & clusture form arranged bacteria

API web identification result:

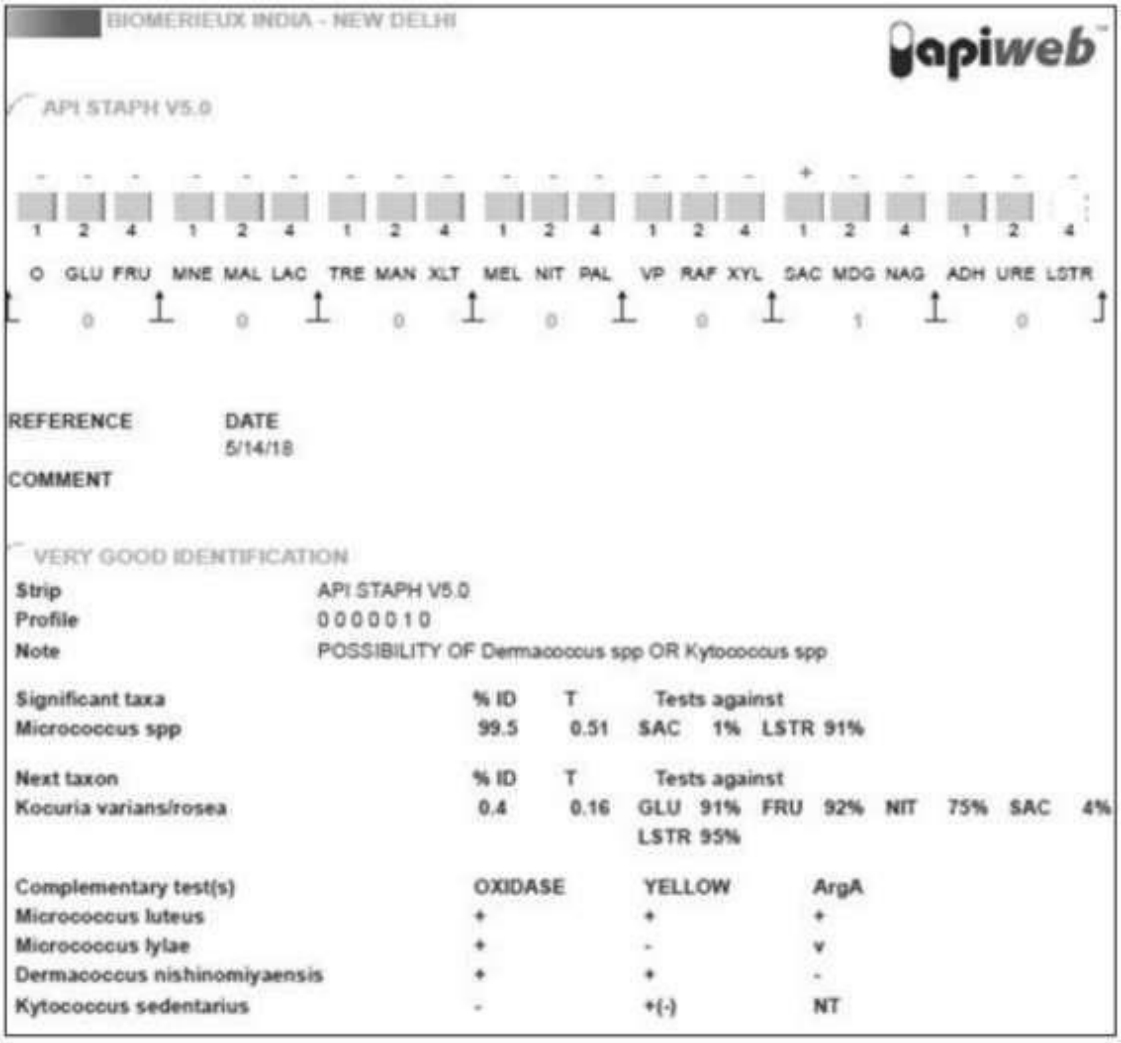


Standard identification strip



From the API web result we found that above isolated colony of Micrococcus luteus. Colony from the R2A agar plate of S-02 sample point of purified water and its morphological characteristics and gram's staining was performed followed by microbial identification using API web microbial identification system. From the identification results it its observed that the isolated organism is Micrococcus luteus. Microorganisms found in pharmaceutical purified water are frequently identified to assist in the product investigation. This is especially common if their members exceed alert or action levels for the materials or process environment. The presence of gram positive bacteria in pharmaceutical purified water implicates human interventions as a one of the reason for purified water contamination.

The concentration of microorganisms in water distribution system is after attributed to factors such as unhygienic water source, poor water, inadequate treatment conditions, and stagnation. The habitat of the Micrococcus luteus is soil, dust, water and air and as a part of normal flora of mammalian skin. Micrococcus luteus is frequently being observed in the purified water system hence it is considered as normal flora of the purified water system. Micrococcus luteus may be entered in the purified water from any of the source mentioned above. More over Pathogen city of this bacteria is checked against FDA Bad bug book and found it is non pathogenic and non objectionable.



BIOMERIEUX INDIA - NEW DELHI

apiweb

API STAPH V5.0

1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
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O	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE	LSTR
	0		0		0		0		0		0		0		1			0		

REFERENCE DATE 5/14/18

COMMENT

VERY GOOD IDENTIFICATION

Strip	API STAPH V5.0
Profile	0 0 0 0 0 1 0
Note	POSSIBILITY OF Dermacoccus spp OR Kytococcus spp

Significant taxa	% ID	T	Tests against
Micrococcus spp	99.5	0.51	SAC 1% LSTR 91%

Next taxon	% ID	T	Tests against
Kocuria varians/rosea	0.4	0.16	GLU 91% FRU 92% NIT 75% SAC 4% LSTR 95%

Complementary test(s)	OXIDASE	YELLOW	ArgA
Micrococcus luteus	+	+	+
Micrococcus lylae	+	-	v
Dermacoccus nishinomiyaensis	+	+	-
Kytococcus sedentarius	-	+(-)	NT

5. CONCLUSION

From the result it is concluded that the isolated microorganism from purified water sampling point-S02 is Micrococcus luteus which common flora of pharmaceutical purified water system. This is not pathogenic or any harmful for human.

6. INFERENCE

The process for manufacturing purified water in pharmaceutical by reverse osmosis. Here, we are using deionized water for membrane filtration process. The isolated organism from this purified water was identified as Micrococcus luteus .which is common flora of purified water system and it is nonpathogenic as its pathogen city reviewed against Bad bug book and it is found as non objectionable organism. So by this identification method we found that which type of micro flora in pharmaceutical water system and whether it's beneficial or harmful.

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