CHEMICAL AND MICROBIOLOGICAL ANALYSIS OF WATER.

An Industrial Training Report submitted

for the partial fulfillment of the Degree of Master of Science

By

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[M.Sc. (Microbiology), Semester IV]

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2020-2021

C E R T I F I C A T E

This is to certify that this training report entitled **"Chemical and Microbiological Analysis of water"** was successfully carried out by Miss **Sidpara Jeny Kantibhai** towards the partial fulfillment of requirements for the degree of Master of Science in Microbiology of Atmiya University Rajkot. It is an authentic record of his/her own work, carried out by her under the guidance of Ms. Bhavika Chandrani for a period of **1st February 2021 to 15th April 2021** during the academic year of **2020-2021** The content of this report, in full or in parts, has not been submitted for the award of any other degree or certificate in this or any other University.

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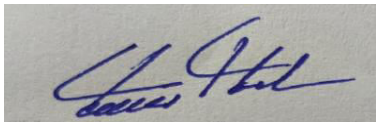

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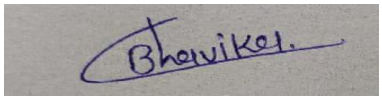

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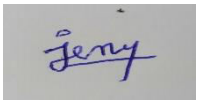
DECLARATION

I hereby declare that the work incorporated in the present training report entitled **"Chemical and Microbiological Analysis of Water"** is my own work and is original. This work (in part or in full) has not been submitted to any University for the award of any Degree or a Diploma.



04\05\2021 Jeny Kantibhai Sidpara

Date (Name and signature of Student)

Acknowledgement

As always said ," Success is not the job of one person, but it requirs support of all."

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Abbreviations

TDS	Total dissolved solids
MFT	Membrane filtration technique
MLT	Microbial limit test
SOP	Standard Operating Procedure
NMT	Not more than
SEM	Scanning Electrone microscope
B.R.	Burette reading
CFU	Colony forming unit
PPM	Parts per Million

Abstract

Water is the most common raw material used to manufacture medicine or nutritional products in pharmaceutical industries. Therefore it is important to employ water quality monitoring system. The present study investigated the Chemical and Microbiological quality of process water used in pharmaceutical industry. Chemical parameters like pH, total hardness and TDS were monitored and Microbiological analysis included Total Bacterial Count and Specified pathogen testing. The pH, total hardness and TDS values were within permissible limits. Results of Specified pathogen testing performed for *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* and *Staphylococcus aureus* were negative for all the samples of process water. Chemical and Microbiological analysis of process water indicated that water used for manufacturing and other purposes in pharmaceutical industry was of good quality. Also it indicated that Evaqua water system employed for water processing is working properly.

Introduction

1.0 Company profile

Orchev Pharma Pvt. Ltd. established in 1989 as a single site operation, Orchev has a reputation for manufacturing Ranitidine as per the latest USP/EP/BP/IP. Its growth has been fuelled by relationships with global market participants that want to conduct business with a company that maintains high levels of quality.In 2020, ORCHEV has taken next step by introducing few more APIs which are Famotidine, Levetiracetam and Allopurinol. Orchev manufactures under (fully complies with) the standards of USFDA, CEP and WHO-GMP. (https://orchev.com/).The focus of our company remains to provide high-quality APIs to both regulated and non-regulated markets, which helps us to offer our products at economical rates. Orchev Pharma Pvt. Ltd. is an ISO 9001:2015 company.

❖ Different Departments

1. QA Department : Documentation handling.
2. QC Department : Chemical analysis (HPLC and GC).
3. Microbiology Department : API products Testing
4. Store room

5. Production Department : Production of Ranitidine, Famotidine, Levetiracetam, Telmisartan, Allopurinol.
6. PA Department
7. Engineering Department
8. ETP (Effluent Treatment Plant) : Discard the waste materials for Discardation they strictly follow the rule of government.

❖ **Microbiology Department**

1. Standard Operating Procedure training
   - General SOP
   - Operation SOP
   - Method of Analysis SOP
   - Preparation SOP
   - Calibration SOP
   - Validation SOP
   - Cleaning SOP
   - Others SOP

2. Some Microbial test performed like

a) Growth Promotion test (GPT)

- The growth promotion test is a quality control requirement that confirms the ability of a new batch of media to support growth of a predetermined selection of representative microorganism.

b) Environment Monitoring

- Environmental monitoring and testing equipment are used to test for surface and airborne contaminants within pharmaceutical clean rooms and other controlled environments. The data is often used for regulatory compliance and manufacturing protocols for safety and QA.

c) Personnel Monitoring

- Surface and personnel monitoring is a critical tool in the health and pharmaceutical industry where many measures are taken to keep production plants clean and minimize the risk of contamination.
- Personnel monitoring is done by the two methods :
- Finger dab method
- Gown swab method

d) Validation

- Validation is an essential part of good manufacturing practices (GMP). Validation checks the accuracy and reliability of a system or a process to meet the predetermined criteria.
- Validation of Autoclave
- Validation of laminar air flow

e) Calibration

- The aim of the calibration procedure is to establish the accuracy of the equipment being used. This helps to control for errors and uncertainties, ensuring that the data collected is reliable and accurate.
- Calibration of pH. There are three types of pH are calibrated (4,7,10).
- Calibration of analytical balance (limit : 500 mg to 150 gm).

f) Finish Good Analysis

- This analysis is a critical step in the manufacturing of pharmaceuticals and ensures that the product is suitable for its intended use.
- Products like Ranitidine, Famotidine, Levetiracetam, Telmisartan, Allopurinol.

- Microbiological analysis of API by two methods :-
- Microbial limit test (MLT)
- Specified pathogen testing

g) Water analysis

- Chemical analysis of process water
- Microbiological analysis of process water

h) Staining

- Lactophenol cotton blue stain :- *Aspergillus brasiliensis*
- Gram's Staining :- *Escherichia coli, Salmonella abony ,Bacillus subtitlis, Pseudomonas aeruginosa , Staphylococcus aureus ,Candida albicans.*

1.1 Instruments which are used in water analysis:-

1. Water bath
2. Weighing balance
3. pH meter
4. TDS meter
5. BOD incubator
6. Laminar air flow
7. Autoclave
8. Dynamic pass box
9. Refrigerator
10. Microscope
11. Colony counter

1.2 Introduction of water

- Water quality refers to the chemical, physical, biological, and radiological characteristics of water.
- It is a measure of the condition of water relative to the requirements of one or more biotic species and or to any human need or purpose.
- Water is widely used in pharmaceutical manufacturing – either as a raw material, as an ingredient, or as a final product. Water is also used for rinsing equipment or for the preparation of disinfectants and detergents.
- These applications require pharmaceutical- grade water to be used, which is water that has been through a chemical purification step.
- Process water is a key component in the manufacturing of virtually all pharmaceutical products.
- In pharmaceutical industry we are concerned with three primary types of water:

1. Potable water
2. Process water
3. Water for injection (WFI).

❖ **Chemical parameters**

Table : 1 chemical parameters of process water.

Parameters	Process water standards as per SOP(permissible limits)
Color	Transparent
Taste	Normal

pH	7.0 to 8.5
Conductivity	NMT 2.10 µS/cm
Total hardness	NMT 5 ppm
TDS	NMT 10 ppm

1.3 Objectives of study

- Collections of sample of process water.
- To investigate the chemical analysis of process water,

1. pH
2. Total dissolved solids (TDS)
3. Hardness

- To investigate the microbiological analysis of Process water,

1. Total bacterial count
2. Detection of Pathogenic organisms
   (a) *Escherichia coli*
   (b) *Pseudomonas aeruginosa*
   (c) *Salmonella spp.*
   (d) *Staphylococcus aureus*

1.4 REVIEW OF LITERATURE

Microorganisms are the smallest living organisms on Earth but at the same time they are the most abundant ones as they have occupied the entire biosphere. They are also the most diverse and in their majority unknown to

scientists. Thy can be found in every macro or micro environment from the surface and the vast depths of oceans to the skin and digestive system of the humans and animals. The morphology (eg shape, size) taxonomy (classfication) and biology (eg metabolism reproduction) of microorganisms are studied by scientific disciplines as microbiology and molecular biology.

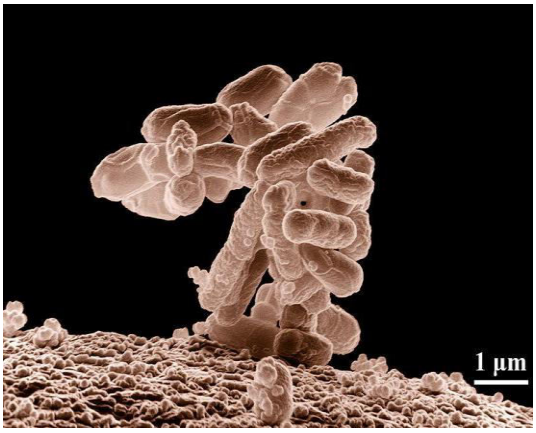
But microbes been abundant and in extremly large numbers not only influenced but also influence their environment by participating a mediating important geochemical reactions which allow the composing of organic molecules from simple elements (eg CO2, N2) and the decomposition of complex ones as the remains dead organisms Microbial ecology and particular the water microbial ecology studies the relationship among microorganisms and the way they influences water environments by using principles and methods from microbiology, chemistry, physics, ecology, mathematics and computer science.

Water microorganisms are capable of flourishing in all water habitants from several kilometers below the sea floor to the first millimeter of a shallow trunch created by rain water and into acidic lakes. Three major domains compose water microorganisms- *Eukaryote ,Archean* and Bacteria - as well as viruses. Their omnipresence impacts the entire biosphere as they are the main producers of energy and carbon flow to other organisms. Some of the microbes decompose organic matter and thus they recycle nutrients in complex manner inolved in various geochemical cycles as the nitrogen the phosphorus and the carbon cycle (A. Alexopoulos 2010).

1.5 Microbial pathogens in water

1) Escherichia coli :-

Escherichia coli was first described by Theodore Escherich in 1885, is a member of family enenterbacteriaeace. It is a Gram negative, motile, non-sporing bacillus, produced rose pink colonies on MacConkey Agar. The



species can be differentiate from other members of enterobacteria by biochemical reactions, being members of enterobacteriaceae. It is present as normal flora in the lower intestine of both humans and animals. However, some strain on causes gastrointestinal illness ranging from mild to cholera-like diarrhea and may lead to potentially fatal complications, such as hemolytic uremic syndrome (HUS) and thrombotic thromobo cytopanic purpurea in human beings (Edward's, 1972).

Fig.1 : SEM view of *E.coli* (Barr, 2018)

2) Pseudomonas aeruginosa :-

Pseudomonas aeruginosa is a common biofilm forming Gram negative bacterium often found in soil and ground water, which is implicated in diseases as special of the lungs. It is an opportunistic pathogen, but rarely affects healthy

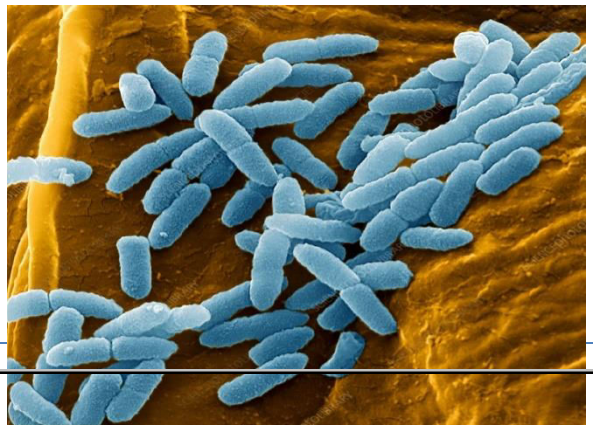


Fig.2 : SEM view of P.aeruginosa(Berger)

individuals. It can however cause a wide range of infections in almost any organ or tissue, particularly in proteins with a weakened immune system, such as cancer patients, new borne and those with severe bumps, diabetes or cystic fibrosis (DH, estates and facilities, 2013).

3) Salmonella spp.:-

The genus Salmonella incorporate Gram negative, Facultative anaerobic rod shaped bacilli characterized by O, H, and Vi antigens (Giannella, 1996). They are members of the family Enterobacteriaceae. Salmonellae are ubiquitous human and animal pathogens, and salmonellosis, a disease that affects an estimated 2 million American each year, is common throughout the world. Typhoidal serotypes can only be transferred from human to human, and can cause food-borne infections, typhoid fever, and paratyphoid fever (Ryan I KJ, 2004).

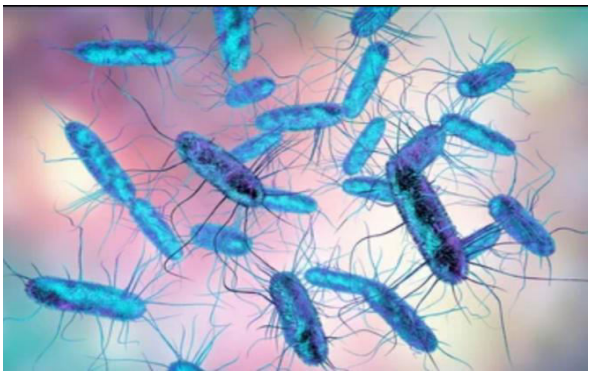
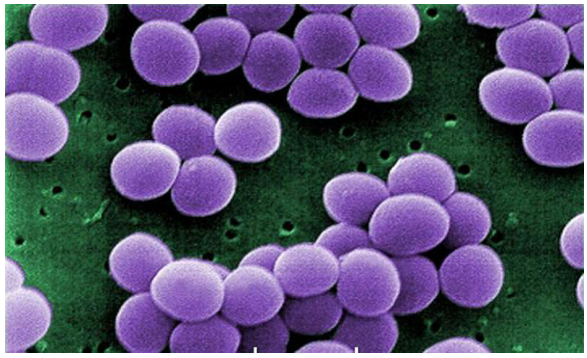


Fig.3: SEM view of Salmonella (Kan, 2016)

4) Staphylococcus aureus :-

S.aureus are Gram-positive bacteria, with diameter of 0.5 – 1.5 μm and characterized by individual cocci, which divide in more than one plan to form grape-like clusters. They are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation (



Kloos WE, 1994). Colonization with S.aureus is an important risk factor for subsequent S.aureus infection (Wertheim, 2004).

Fig.4: SEM view of S.aureus

1.6 Chemical parameters of water

1.6.1 pH

Looking after pH is one of the most important attributes of any aquatic system since all the biochemical activities depend on pH of the surrounding water. It was established that the pH of water were slightly alkaline (6.0 to 8.5) and were within the maximum limit set for domestic use as per APHA. High value of pH may result due to waste discharge, microbial decomposition of organic matter in the water body (Patil S.G., 2012).

1.6.2 Hardness

Hardness is a significant parameter in declining the toxic effect of noxious elements. The hardness was found to be NMT 250 ppm. It is within

standard limit. The hardness of water proliferate in the polluted waters by the deposition of calcium and magnesium salts(Bhatt L.R.,1999).

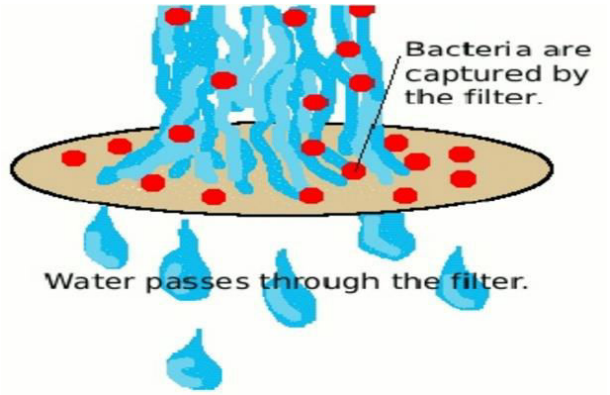
1.6.3 Total Dissolved Solids (TDS)

The most notable observation of examination was the alarmingly high level of total dissolved solids(TDS). The TDS of all the samples were NMT 500 ppm. High level of TDS in water used for drinking purposes leads to many diseases which are not water borne but to excess salts (M.P., 1995).

1.7 Microbiological analysis of process water

1.7.1 Membrane Filtration technique

Membrane filtration have a known uniform porosity of predetermined size (generally 0.45 μm) sufficiently small to trap microorganisms. Using the membrane filter technique, sample is passed through the membrane using a filter funnel and vacuum system. Any organisms in the sample are concentrated on the surface of the membrane. The membrane, with its trapped bacteria, is then placed in a special plate containing a pad saturate with the appropriate medium. The passages of nutrients through the filter during incubation facilities the growth of organisms in the form of colonies, on the upper surface of the membrane. Discrete colonies thus formed can be easily transferred to confirmation media.



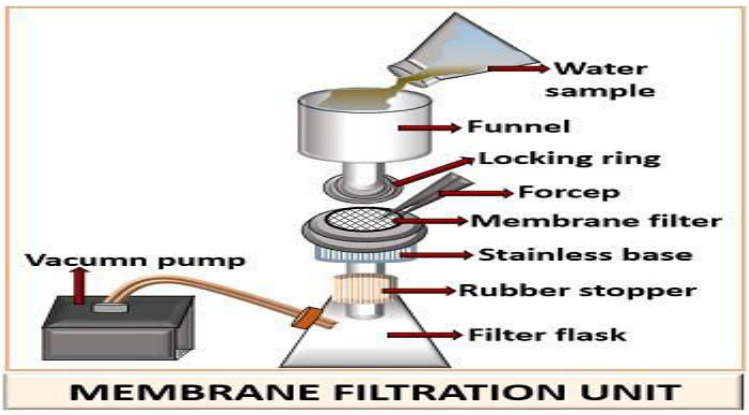


Fig.5 : MFT process **Fig.6** : MFU used for water testing.

2.0 Materials and Methods

+`

2.1 Materials

2.1.1 Glassware and apparatus

1.Airlock glass bottles(100 mL)
2.Sterile forceps
3.Membrane filter holder
4. Membrane filters
5.Petri plates(100 mm>45 mm)
6.Burette(50 ml)
7.Conical flask (250 ml)
8.Beaker(10 ml & 50 ml)

2.1.2 Equipment/ Instruments

1.Hot air oven
2. Horizontal Autoclave
3.Biosafety cabinets/ Laminar air flow hood
4.Membrane filtration system
5.Incubator
6.Colony counter
7.pH meter
8.Conductivity meter
9.TDS analyzer

2.1.3 Requirements

1. 70% Isopropyl alcohol
2. EDTA solution
3. Erichrome Black-T indictor
4. Buffer solution

2.1.4 Media

1. Reasoner's 2Aagar(R2A)
2. Soyabean casein digest broth(SCDM)
3. MacConkey agar(MA)& MacConkey broth(MB)
4. Cetrimide agar(CA)
5. Mannital salt agar(MSA)
6. Xylose lysine deocxycholate agar(XLDA)
7. Rappaport vassiliadis Salmonella enrichment broth(RVB).

2.2 Sampling

Process water (storage tank) sample were collected daily.

Procedure

1. Previously sterilized airlock glass bottle.
2. 70% IPA spray on sampling point valve.
3. Before water samples were collected, the water was allowed to run for few minutes.
4. The water samples were collected into Airlock glass bottle.
5. The bottles were filled to the neck to avoid air bubbles and then bottles were sealed.
6. Sample for microbial analysis were collected in a sterilized and phosphate free bottle.
7. The bottle were labeled (Date, Sampling point, sample no.).

8. Sample were immediately taken into laboratory for analysis.

2.3 Chemical analysis of water

2.3.1 pH

- 50-100 mL of sample was taken and Dip the electrode of pH meter in beaker.
- pH was measured using calibrated pH meter.
- The range of pH was 7.0-8.5 for both Sampling Point (S7 and S9).

2.3.2 Hardness

- 100 mL of water sample is pipettes out into a conical flask and add 10 mL Ammonia solution.
- Few drops of Erichrome Black-T indicator was added.
- Water sample is titrated against 0.01 M EDTA(Ethylene Diamine Tetra Acetic acid) solution till the pink color changes to purple which is the end point.

- Hardness = B.R. X 10 (ppm)
  = _____ X 10
  = _____ ppm (range- NMT 5 ppm).

2.3.3 TDS

- The conductivity was measured at 25°C with a calibrated conductivity meter and the value was converted in TDS by the following formula.(range-NMT 10 ppm).

  - TDS = $\frac{\text{Conductivity X 100}}{}$

2.4 Microbiological analysis of water

2.4.1. Membrane filtration technique (for process water samples)

- 1 ml of process water sample was added into 100 ml sterile buffer.
- With the help of sterile forceps membrane filter was removed from sterile package.
- The membrane filter was placed into the filtration assembly.
- Process water sample was poured into the membrane filter holder.
- Membrane filtration system was turned on and the sample was allowed to draw completely through filter.
- The membrane filter was removed from filteration assembly using sterile forceps.
- Membrane filter was placed onto prepare R2A plate.
- R2A plates were incubated at 32.5 +/- 33.5 °C for 5 days.
- Results were noted after 5 days incubation period.

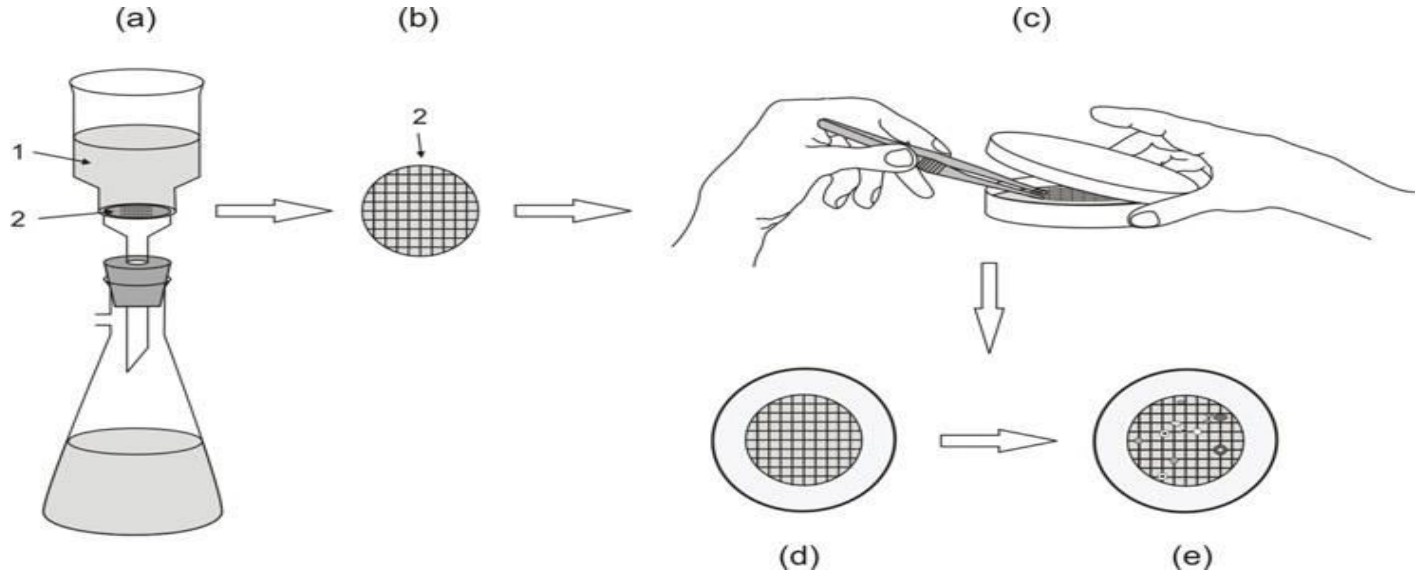


Fig.7 : Steps of membrane filtration technique

2.4.2 Specified pathogen testing

- 1 mL of process water sample add into 100 mL SCDM medium and incubate at 32.5 °C for 24 hrs.
- 10 mL of water sample add into 100 ml SCDM and incubate at 32.5 °C for 24 hrs (specific for *Salmonella abony*).

A) Escherichia coli

- 1 mL of sample from SCDM was added into 100 mL of MacConkey broth.
- Incubate MCB at 42-44°C for 24-48 hrs.
- Streak on MacConkey Agar plate.
- MCA plate were incubated at 32.5°C for 72 hrs.
- Light pink colonies over MCA plate shows positive results.

B*) Pseudomonas aeruginosa*

- A loopful from SCDM was streaked on Cetrimide Agar plate.
- Cetrimide agar plate were incubated at 32.5 °C for 48 to 72 hrs.
- Yellowish green colonies on Cetrimide agar plate shows positive results.

C) *Staphylococcus aureus*

- A loopful from SCDM was streaked on Mannitol Salt Agar plate.
- MCA plate were incubated at 32°C for 48 to 72 hrs.

- Yello colonies on MCA plates followed by showing color change (red to yellow) in MSA plate which indicates the fermentation of Mannitol by *S.aureus*. Hence, positive results.

D) *Salmonella spp.*

- 0.1 mL of sample from SCDM was added into 10 mL R.V. broth.
- Inoculated R.V. broth was incubated at 32.5°C for 24 hrs.
- Streak on XLDA plates
- XLDA plate were incubated at 32.5°C for 48 to 72 hrs.
- Red colonies with/without black centers on XLDA plates shows positive results.

3.0 Results and Discussion

3.1 Chemical analysis of water

Table 2. Results of chemical analysis of process water

Sr.No.	Sampling point	Test	Specification	Result
1	S7	pH	7.0 to 8.5	7.15
		Total hardness	NMT 5 ppm	02 ppm
		TDS	NMT 10 ppm	06 ppm
2	S9	pH	7.0 to 8.5	7.13
		Total hardness	NMT 5 ppm	02 ppm
		TDS	-	-

Process water samples from two sampling points were analyzed chemical analysis were showing range of results that are as per standards of SOP and indicates that water can be used for manufacturing and other purposes.

3.2 Microbiological analysis of water

Table 3. Results of Microbiological analysis of water

Sr. No.	Sampling point	Test	Specifications	Results
1	S7	Total bacterial count	NMT 100 CFU/mL	22 CFU/mL
		E.coli	Should be absent	Absent
		P.aeruginosa	Should be absent	Absent
		S.aureus	Should be absent	Absent
		S.abony	Absent/10 mL	Absent
2	S9	Total bacterial count	NMT 100 CFU/ mL	21 CFU/mL
		E.coli	Should be absent	Absent
		P.aeruginosa	Should be absent	Absent
		S.aureus	Should be absent	Absent
		S.abony	Absent/10 mL	Absent

Process water samples from two sampling point were analyzed Microbiological analysis were showing range of results that are as per standards of SOP and indicates that water can be used for manufacturing and other purposes. Both sampling points are tested for total bacterial count and the results were in the range of 00 to 22 CFU/mL. These results were in

the range as per the standards of SOP.Specified pathogen testing for four microorganisms was performed for two sampling points, both results were negative for all pathogen.

4.0 Conclusion

- Chemical and Microbiological study of process water samples collected have been presented.
- All the parameters for each process water sample are within the permissible range.
- The process water pH was found within the range of 7.0 to 8.0 which indicated that it can be used as a manufacturing ingredient.
- The microbiological study of process water revealed that total bacterial count was found to be NMT 100 CFU/ mL.
- Specified pathogen testing results of process water sample were negative. Regular checkup of the process water can help in maintaining water quality.

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ANNEXURE :- 1

Preparation of media and reagents

- **Soyabean casein digest medium (SCDM)**

Ingredients	Gms / Litre
Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500
Final pH (at 25°C)	7.3±0.2

- **MacConkey Agar (MA)**

Ingredients	Gms / Litre
Proteose peptone (meat and casein	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm
Crystal Violet	0.001 gm

Agar	13.5 gm
Distilled Water	Add to make 1 Liter
Final pH (at 25°C)	7.3±0.2

❖ **Cetrimide Agar (CA)**

Ingredients	Gms / Litre
Pancreatic Digest of Gelatin	20.0 gm
Potassium Sulfate	10.0 gm
Magnesium chloride	1.4 gm
Cetyltrimethylammonium Bromide	0.3 gm
Glycerine	10.0 ml
Agar	13.6 gm
Distilled water	1000 ml
Final pH (at 25°C)	7.2 +/- 0.2

❖ **Mannitol Salt Agar (MSA)**

Ingredients	Gms / Litre
Pancreatic Digest of Casein	5.0 gm
Peptic Digest of Animal Tissue	5.0 gm

Beef extract	1.0 gm
Sodium Chloride	75.0 gm
D – Mannitol	10.0 gm
Phenol Red	0.025 gm
Agar	15.0 gm
Distilled water	1000 ml
Final pH (at 25°C)	7.4 ± 0.2

❖ **Xylose lysine deoxycholate agar (XLDA)**

Ingredient	Gms / Litre
Lactose	7.5 gm
Sucrose	7.5 gm
Sodium thiosulphate	6.8 gm
L- lysine	5.0 gm
Sodium chloride	5.0 gm
Xylose	3.75 gm
Yeast extract	3.0 gm
Sodium Deoxycholate	2.5 gm
Ferric ammonium citrate	0.8 gm
Phenol red	0.08 gm
Agar	15.0 gm
Final pH (at 25°C)	7.4 ±0.2

❖ **Rapport Vassiliadis salmonella enrichment broth (RVSEB)**

Ingredient	Gms / Litre
Soya peptone	4.500
Sodium chloride	8.000
Dipotassium hydrogen phosphate	0.400
Potassium dihydrogen phosphate	0.600
Magnesium chloride,hexahydrate	29.000
Malachite green	0.036
pH after sterilization (at 25°C)	5.2 ± 0.2

❖ **R2A Agar**

INGERDIENTS	Gms/Litre
Tryptone	**0.25**
Peptone	**0.25**
Acicase	**0.50**
Yeast Extract	**0.50**
Glucose(Dextroes)	**0.50**
Starch Soluble	**0.50**
Dipotassium Hydrogen Phosphate	**0.03**
Magnesium Sulphate, Heptahydrate	**0.50**

Sodium pyruvate	**0.03**
Agar	**15.00**
Final pH	**7.2**

ANNEXURE 2

Instrument	Company Name
Water Bath	THERMOLAB
pH meter	EUTECH
Colony Counter	AVM SCIENTIFIC
Microscope	OLYMPUS (CH20i)
Analytical balance	SHIMADZU
Vortex mixture	Eltek
Centrifuge	REMI MOTORS LTD.
Incubators	THERMOLAB
TDS meter	Toshcon industry PVT. LTD.
Autoclave	LEQUITRON
LAF	Klenz flo
Refrigerator	VIDEOCON