Faculty of Resource Science and Technology

Isolation and Identification of Biosurfactant Producing Bacteria from Fats, Oils and Greases (FOGs)

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Isolation and Identification of Biosurfactant Producing Bacteria from Fats, Oils and Greases (FOGs)

Soonali A/P Wasdewa

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours

(Resource Biotechnology)

SUPERVISOR: DR. NGIENG NGUI SING

Programme of Resource Biotechnology
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UNIVERSITI MALAYSIA SARAWAK
2022

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Isolation and Identification of Biosurfactant Producing Bacteria from Fats, Oils and Greases (FOGs)

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ABSTRACT

Foods contain fats, oils, and greases (FOGs) are commonly washed down into the kitchen sink and sticks to the inner walls of kitchen pipes, drains and sewer pipes and forming clogs which causes the blockage that restrict the flow of wastewater. Waste FOGs material sometimes flows directly to the mainstream and forms an oil layer which give threat to aquatic organisms by reducing the dissolved oxygen level. The biosurfactant producing bacteria act as biodegradation agent to emulsify the FOG materials from clogging and prevent blockage in the drains and sewage system. This study therefore aims to isolate and identify the biosurfactant producing bacteria. The oil spreading assay and emulsification index (E24) test were used to determine the presence of biosurfactant and *Bacillus paramycoides* and *Bacillus cereus* were identified via comparison of the isolates'16S rRNA with deposited sequences in NCBI GenBank. *Bacillus paramycoides* and *Bacillus cereus* produced largest diameter of clearing zone compared to other biosurfactant producing bacteria on the oil spreading assay which indicated as the high quality of biosurfactants produced. Biosurfactant screening via emulsification index (E24) increased with presence of biosurfactants produced by *B. paramycoides* and *Bacillus cereus* due to the high interaction between water and oil which have industrial potential with greater ability in biodegrading the FOGs materials.

Keywords: Biosurfactant, FOG, bacteria, emulsification, biodegradation

ABSTRAK

Makanan yang mengandungi lemak, minyak dan gris (FOGs) biasanya dicuci ke dalam sinki dapur dan melekat pada dinding dalaman paip dapur, longkang dan paip pembetung serta membentuk penyumbatan yang menyebabkan penyekatan aliran air sisa. Kadangkala, bahan buangan FOG mengalir terus ke sungai atau laut dan membentuk lapisan minyak yang memberi ancaman kepada organisma akuatik dengan mengurangkan paras oksigen terlarut. Bakteria yang menghasilkan biosurfaktan bertindak sebagai agen biodegradasi untuk mengemulsikan bahan FOG daripada tersumbat dan menghalang penyumbatan di dalam longkang dan sistem kumbahan. Oleh itu, kajian ini bertujuan untuk mengasingkan dan mengenal pasti bakteria yang menghasilkan biosurfaktan. Ujian penyebaran minyak digunakan untuk menentukan kehadiran biosurfaktan dan Bacillus paramycoides and Bacillus cereus telah dikenal pasti melalui menggunakan teknik 16S rRNA dengan membandingkan urutan dalam NCBI GenBank. Bacillus paramycoides and Bacillus cereus telah membentuk zon jelas yang paling besar berbanding dengan bakteria-bakteria lain yang menghasilkan biosurfaktan pada ujian penyebaran minyak yang menunjukkan biosurfaktan yang dihasilkan mempunyai kualiti bermutu tinggi. Saringan untuk biosurfaktan melalui indeks pengemulsian (E24) telah meningkat dengan kehadiran biosurfaktan yang dihasilkan oleh Bacillus paramycoides and Bacillus cereus disebabkan oleh interaksi yang tinggi antara air dengan FOG yang mempunyai potensi dalam industri dengan keupayaan yang lebih besar untuk menguraikan bahan-bahan FOG.

Kata Kunci: *Biosurfaktan, FOG, bakteria, pengemulsian, biodegradasi*

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LIST OF ABBREVIATIONS

BLASTN	Basic Local Alignment Search Tool Nucleotide
BOD	Biological oxygen demand
Ca	Calcium
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
COD	Chemical oxygen demand
$CuSO_4.5H_2O$	Copper (II) sulphate pentahydrate
DAF	Dissolved air flotation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ET	Electrochemical
EtBr	Ethidium bromide
$FeSO_4.7H_2O$	Iron(II) sulphate heptahydrate
FFAs	Free fatty acids
FOG	Fat, oil and grease
HCl	Hydrochloric acid
HLB	Hydrophilic – lipophilic balance
H_3BO_3	Boric acid
K	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
KI	Potassium iodide
KOH	Potassium hydroxide
K_2SO_4	Potassium sulphate

MC	Mechanical coalescers
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$MnSO_4.4H_2O$	Manganese(II) sulphate tetrahydrate
MSM	Mineral salts medium
Na	Sodium
Na^+	Sodium ion
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium hydrogen phosphate
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
NH_4NO_3	Ammonium nitrate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PES	Polyethersulfone
rRNA	Ribosomal Ribonucleic acid
TAE	Tris-acetate-EDTA
TLC	Thin layer chromatography
TSA	Trypticase soy agar
TSB	Tryptic soy broth
VCP	Vitrified clay pipes
$ZnCl_2$	Zinc chloride

CHAPTER 1

INTRODUCTION

1.1 Research Background

Biosurfactants are surface-active metabolites, which are produced extracellularly or synthesized at the cell surface of microorganisms. The microorganisms which normally produce biosurfactants are bacteria, yeast, and fungi. One of the biosurfactant well known among scientists is rhamnolipids which is produced by *Pseudomonas aeruginosa*, an aerobic, and Gram-negative (Sohail & Jamil, 2020). Other examples of biosurfactant are sophorolipids produced by nonpathogenic yeasts, and lecithin can be obtained from the egg yolk. Biosurfactants are amphiphilic compounds with hydrophilic and hydrophobic. Biosurfactants are superior to chemical surfactants due to its ease biodegrade, low toxicity, high foaming ability, and perform high rate reaction at optimum pH, temperature, and salinity (Anandaraj & Thivakaran, 2010).

The first biosurfactants was known as surfactin, which was purified and characterized in 1968 by Kei Arima and his colleagues at Tokyo, Japan (Sekhon & Rahman, 2014). Arima and his colleagues used *Bacillus subtilis* to isolate a fair amount of surfactin and defined it as peptidelipid with carbon (59.6 %), hydrogen (9.0 %), and nitrogen (9.1 %) which have molecular weight about 1050 calculated using the vapor pressure method,. It was also found to consist of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, D-leucine (1:1:1:2:2) and unidentified fatty acids which are required for formation of fibrin clot by inhibiting the fibrin monomer from convert to fibrin polymer. In their research, they characterized the surfactin as an acidic compound which is soluble in alkaline water and other organic solvent, but does not soluble in water, and inorganic solvent. (Arima et al., 1968).

Biosurfactants such as glycolipids and lipopeptides are used in many industrial processes as the replacement for synthetic surfactants, such as greasing, wetting, soothing, fixing dyes, emulsification, stabilizing diffusion, preventing foaming, pharmaceutical industry, and food. Biosurfactants also used for bioremediation of organic aqueous wastes, and heavy metals as well as contaminated soil (Wang et al., 2018). Furthermore, biosurfactant also have an essential role in environmental protection, like oil spills control, biodegradation, petrochemical, and cosmetics industries (Patowary et al., 2017).

Based on Sumiardi et al. (2018), bacterial consortium which produce biosurfactants play important role in degrading the petroleum hydrocarbons compound mainly polycyclic aromatic hydrocarbon. In their research, the biosurfactant produce by bacteria was determined by growth curve at stationary phase after 12 hours and the produced biosurfactant have ability to reduce interfacial tension about 72 dyn . cm^{-1} to 48.7 dyn . cm^{-1}. Result showed positive for drop collapsing test, oil spreading test and hemolytic test which indicated presence and production of biosurfactant by bacterial consortium with 95.75 % of emulsification index (E24) (Sumiardi et al., 2018).

Fats, oils and greases (FOGs) like butter, excess cooked oil and other FOGs contained foods are often being washed down to the kitchen sink. This kitchen material sticks to the inner walls of kitchen pipes, drains and sewer pipes and forming clogs which cause the blockage that restrict the flow of wastewater. The blockage by FOGs can put wastewater pumping stations into unserviceable conditions and cause flood. However, sometimes waste FOGs material is flown directly to the mainstream like the river and forming an oil layer that prevents sufficient amount of sunlight and reduced the level of dissolved oxygen. This water pollution gives threats to the aquatic organisms.

By using oil spreading assay and polymerase chain reaction (PCR), it is believed that the formation of clearing zone allowed identification and characterization of the biosurfactant producing bacteria from FOGs.

1.2 Objectives

The objectives of this research were:

a) To isolate biosurfactant producing bacteria from fats, oils, and greases (FOGs) from Student Pavilion at UNIMAS.

b) To screen for biosurfactant producing bacteria using oil spreading assay and emulsification index (E24) test.

c) To identify the biosurfactants producing bacteria using 16S rRNA technique.

CHAPTER 2

LITERATURE REVIEW

2.1 Physical properties of FOG

FOG is characterized by an oily appearance and it normally exist in the solid form or liquid form. The FOG can be visualize as colourless, odorless, and tasteless in its pure state. FOG normally dissolve in organic solvent, such as alkane, ether, and trichloromethane, but, it insoluble in the water. FOG can float on the surface of water because it has less density than water. The presence of emulsifying agent, such as soap or detergent cause emulsions between FOG and aqueous media (Pérez-Díaz et al., 2017). The composition of fatty acid and double bonds structure will determine viscosity of FOG. FOG with low viscosity occurs due to the presence of the carbon chain with double bonds which cause structure of FOG to be loosely packed. The reason of FOG has a low pH value is because of the existence of huge amount of free fatty acids (FFAs) (Table 2.1). The formation of FFAs in FOG occur during extreme frying of food by hydrolysis and oxidation reactions (Husain et al., 2014).

Table 2.1. The common free fatty acids (FFAs) in FOG. (Adapted from Husain et al., 2014).

Name	Systematic name	Short-hand	Melting point (°C)
Butyric	Butanoic	4:0	-5.3
Capric	Decanoic	10:0	31.6
Lauric	Dodecanoic	12:0	44.8
Palmatic	Hexadecanoic	16:0	62.9
Stearic	Octadecanoic	18:0	70.1
Vaccenic	Octadecenoic	18:1	44.1
Linoleic	Octadecadienoic	18:2	-5.0
EPA	Eicosapentaenoic	20:5	-54.0
DHA	Dodosahexaenoic	22:6	-44.0

2.2 Chemical reactions related to FOG

The existence of sodium hydroxide (NaOH) and potassium hydroxide (KOH) cause the FFAs to readily undergoes saponification. The sodium (Na) and potassium (K) normally can be found in raw foods. During deep frying, the FFA in the frying oil will extract the sodium ion (Na^+) from foods to form sodium oleate which decrease the surface tension linking the narrow water layer on the surface of the fried food and cooking oil. This causes the amphiphilic lipids migrate to the food from the oil. Thus, the sodium oleate increase the oxidation in cooking oil by stimulating foaming (Husain et al., 2014).

The salts and food ingredients used at restaurants contain high concentration of sodium which cause arise of sodium in the wastewater. Common detergents and sanitizers cause saponification of FOG by hydrolysis reaction with the presence of the high amount of NaOH which is known as an alkaline catalyst. In Malaysia, vitrified clay pipes (VCP) were used and it was found that the presence of Na and K caused the deposition of FOG (Figure 2.1) (Husain et al., 2014). However, the hardness of water also cause deposition of FOG in sewer pipelines by associated with high concentration of calcium (Ca) in FOG samples (Otsuka et al., 2020).

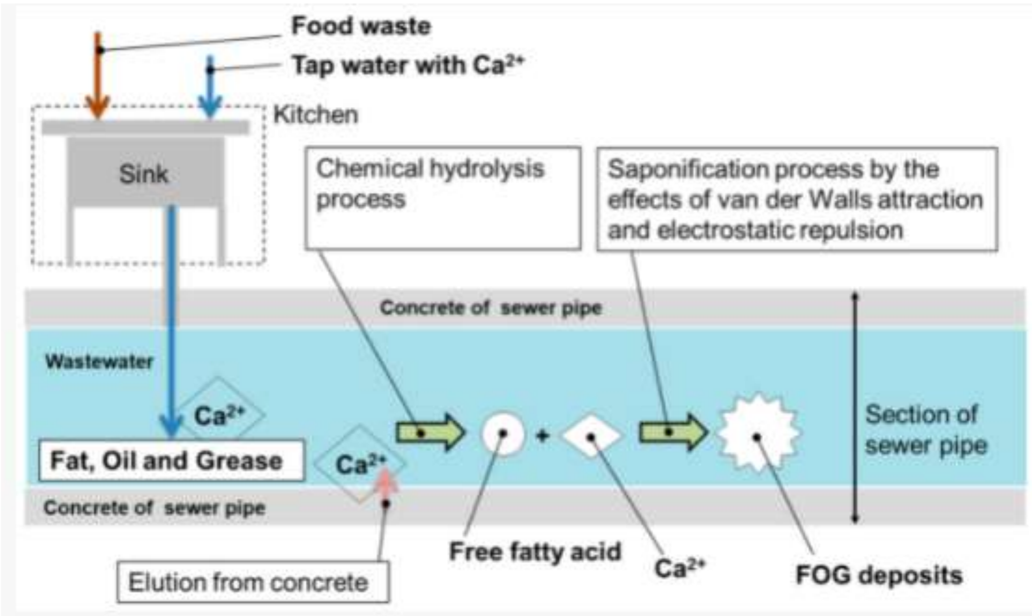

Figure 2.1. FOG deposition mechanism in sewage system. (Adapted from Otsuka et al., 2020).

2.3 FOG affect the biological oxygen demand (BOD), and chemical oxygen demand (COD).

According to Rimbach (2018), many restaurants and dairy production company have a problem in discharging waste products with FOG. This is because the waste products contain FOG which causes major problems in sewage system by causing blockage and contamination. BOD and COD measurements are often used to measure the concentration of contaminants in sewage system or wastewater. Normally, the value from the measurement of BOD and COD will be used to determine the amount of oxygen required to degrade the organic contaminants in sewage system. If the amount of oxygen required to degrade the contaminants is high, thus, the sewage system contained high level of organic waste. Microorganisms such as bacteria was used to degrade the sewage waste contaminants with FOG elements by producing certain enzyme which help to digest the contaminants in sewage system and release byproducts, such as carbon dioxide and water (Figure 2.2) (Kolev Slavov, 2017).

Figure 2.2. FOG deposition in sewer pipelines. (Adapted from Kolev Slavov, 2017).

2.4 FOG causes public health issue

The blockage of drains system and grease traps by FOG have high potential in causing damage in structure and facilities (Figure 2.3). If the blockage of drain and grease trap does not treat with the proper method, it can cause high risk in environmental health. This is because the blockage by FOG can form odours emitted it during decomposition of organic waste in the drain system. The emitted odours in the environment can attract insects, such as mosquitoes, cockroaches and flies, and vermin, such as rodents. These insects and vermin can cause public health problems and increases the risk of diseases such as malaria, typhoid fever, cholera, and leptospirosis (Martin, 2017).

Figure 2.3. Buildup of FOG in the grease trap. (Adapted from Martin, 2017).

To help avoid odours, there are few products used to help against the FOG deposition in the drain and grease traps. However, most of the products are threat for the environment and can be hazardous to the people who use them. Chemical reagents are also used to help against the built up of FOG in the drain and grease traps, however, most of the chemical cause adverse effect, such as inflammation of the eyes, nose or throat from fumes, or skin can become painful. Continues contact with chemical reagents lead to chronic illness (Martin, 2017).

2.5 Oily Wastewater treatment

There are few treatment methods used to manage the oily wastewater. The oily wastewater treatment can be classified into four methods, such as physical, chemical, mechanical and biological methods. In the physical method, dissolved air flotation (DAF) used to clean the oily wastewater. The DAF performed by introduce air at the bottom of the basin under extreme pressure which causes bubbles with pollutants arise to the top of the basin (Figure 2.4). Conventional DAF can generate microbubbles which have size smaller than 100 microns. The generated microbubbles adhere to the oil droplet and give rise to the tendency of droplets and cause float and move toward the surface of water. The important parameters need to monitor during performing DAF are compulsion and absorbance of air into wastewater. In conventional DAF, the compulsion should be equal to atmospheric pressure with high concentration of dissolved gas. DAF method produce high quality of treated water due to its ability to remove emulsified oil. However, the operation of each process are very expensive due to monitoring the pressure (Abuhasel et al., 2021).

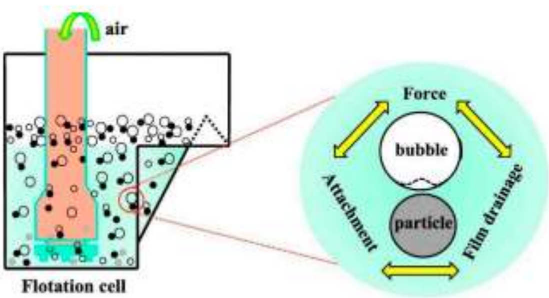

Figure 2.4. Process of dissolved air flotation (DAF) system. (Adapted from Abuhasel et al., 2021).

In chemical method, electrochemical (ET) technologies used to clean the oily wastewater. ET can be classified into three parts, such as electroflotation, electrocoagulation and electrofloculation. Combination of these three parts will produce very efficient oily wastewater treatment. In this system, the electrochemical cells involved by dipped the electrodes into oily wastewater. The ET system treat oily wastewater by using electrochemical oxidation (EO) with electro-oxidation (EIO) which form oxidizing agents

(Figure 2.5). The main focus of system is to oxidize the oil components and convert to carbon dioxide, water and biodegradable byproducts. However, this method not very efficient due to electrodes corrosion (Abuhasel et al., 2021).

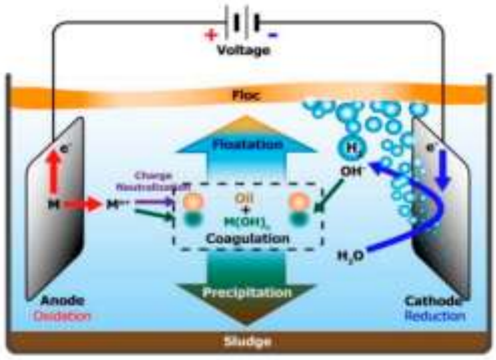

Figure 2.5. Oily wastewater treatment using Electrochemical (ET) system. (Adapted from Abuhasel et al., 2021).

Mechanical coalescers (MC) are used in the mechanical method. In the coalescers of MC, the small amount of oil droplets will collides and adhere with other components. This cause the formation of larger droplets and the density differences can separate these droplets by buoyancy. The MC will efficiently separate the oil droplet when the size of droplet is less than 10 µm. Due to space limitation, the MC used in the offshore platforms to treat the oily wastewater. The coalescers have high quality structures which service for long period, separate liquid to liquid phase efficiently, and only need small quantity of chemicals. The MC system have visualization through four steps, such as the attachment, the approach step, coalesce step and the release step (Figure 2.6). This method is only suitable for deep water and have complicate coalescer design (Abuhasel et al., 2021).

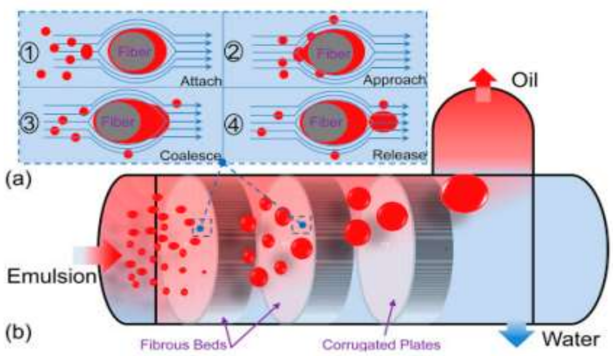

Figure 2.6. The coalescer design with four step of visualization. (Adapted from Abuhasel et al., 2021).

2.6 Biosurfactant producing bacteria as biological method

Biosurfactant is classified based on the type of microbes and their configuration of chemical. There are two kinds of biosurfactants that are classified according to weight of molecule, such as low molecular weight compounds, and high molecular weight compounds. The low molecular weight of biosurfactants, such as phospholipids act by lower the interfacial tension while the high molecular weight of biosurfactants are well known as polymers. Most of the biosurfactants are anionic and only a few biosurfactants are categorized as neutral charge. The composition of the fatty acid long chain represents the hydrophobic moiety while the composition of amino acid, phosphate group, carbohydrate part and a cyclic peptide represent the hydrophilic moiety in the biosurfactants (Figure 2.7) (Karlapudi et al., 2018).

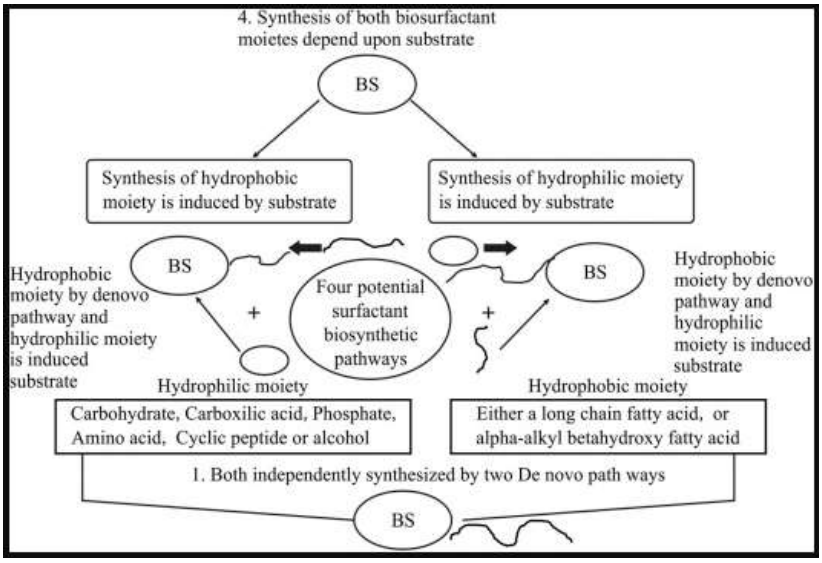

Figure 2.7. Synthesis of biosurfactant in bacteria. (Adapted from Karlapudi et al., 2018).

Biosurfactants are non-toxic agents and toxic friendly nature which is more suitable to use for biodegradation. Biosurfactants can stabilized or destabilized the emulsion by formation and breaking of bonds. The biosurfactants which act as emulsifiers are normally type of high molecular weight. Biosurfactants can degrade the oil components by rise surface area which cause absorbance of insoluble compound in water and then, form bonding with the heavy metals for removal (Karlapudi et al., 2018).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Cooking oil (fats, oils and greases)

Schott bottles

Sodium chloride (NaCl)

Potassium chloride (KCl)

Sodium hydrogen phosphate (Na_2HPO_4)

Potassium dihydrogen phosphate (KH_2PO_4)

Hydrochloric acid (HCl)

Sodium hydroxide (NaOH)

Distilled water

Sterile water

Potassium iodide (KI)

Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)

Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)

Copper(II) sulphate pentahydrate ($CuSO_4.5H_2O$)

Boric acid (H_3BO_3)

Manganese(II) sulphate tetrahydrate ($MnSO_4.4H_2O$)

Zinc chloride ($ZnCl_2$)

0.2 micron polyether sulfone (PES) membrane

Ammonium nitrate (NH_4NO_3)

Potassium sulphate (K_2S0_4)

Epsomite/ magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)

Calcium chloride anhydrous, $CaCl_2.H_2O$