Creation of Bacterial Culture System on Paper by Application of Inkjet Printing

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Creation of Bacterial Culture System on Paper by Application of Inkjet Printing

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Table 4.1 Printability, time required per sample and accuracy of SCS and MCS printers

Table 4.2 Survival rate of *E. coli* on PVA and CA hydrogel media after 24 h

Abbreviation

Abbreviation	**Description**
ADH	Adipic Acid Dihydrazide
CA	Calcium Alginate
CLSM	Confocal Laser Scanning Microscope
DOD	Drop On Demand
E. coli	*Escherichia coli*
GP	Glycoprotein
H-bonding	Hydrogen bonding
MCS	Multi Cartridge System
PEO-PAAc	Polyacrylic acid and polyethylene oxide
PS	Polystylene
PVA	Poly(vinyl alcohol)
PVAc	Poly(vinyl acetate)
SA	Sodium Alginate
SCS	Single Cartridge System
UV	Ultra Violet
XRD	X-ray diffraction

Chapter 1
General introduction

1.1 Bacterial culture system

Bacteria are present everywhere such as in natural soils, deep in rocks, in all water bodies, in the atmosphere, and also inside other living organisms. On the one hand bacteria can be harmful causing diseases in living organisms, while others are useful and commercially important in industries such as pharmaceutical and food industries [1,2]. Both make the research on bacteria one of the most active areas in biology and biotechnology, to extensively study and analyze bacteria species, it is indispensable to isolate pure bacteria cultures. In this work, we are am going to introduce a novel methodology for culturing bacteria.

1.1.1 Bacteria

Bacteria are single celled microbes which are found everywhere in nature. Bacteria can directly affect our health being the cause of many diseases (bacteria inflection) such as cholera, pneumonia, tetanus, leptospirosis, typhoid, appendicitis, gonorrhea, and etc. To effectively treat patients with bacterial infections, scientists have to classify the type and characteristic of the bacteria. The also play an important role in food and food production because bacteria are the main cause of rotten food (microbial spoilage) and food poisoning. Therefore, food preservation techniques are used to sterilize or control environmental condition to inhibit the growth of bacteria. On the other hand useful bacteria such as lactic acid bacteria are utilized for fermentation and waste water treatment.

Bacteria with 0.5-10 micron have different shapes as shown in Fig. 1.1. Bacteria are classified into 5 groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). They can exist as single cells, in pairs, chains or clusters [3].

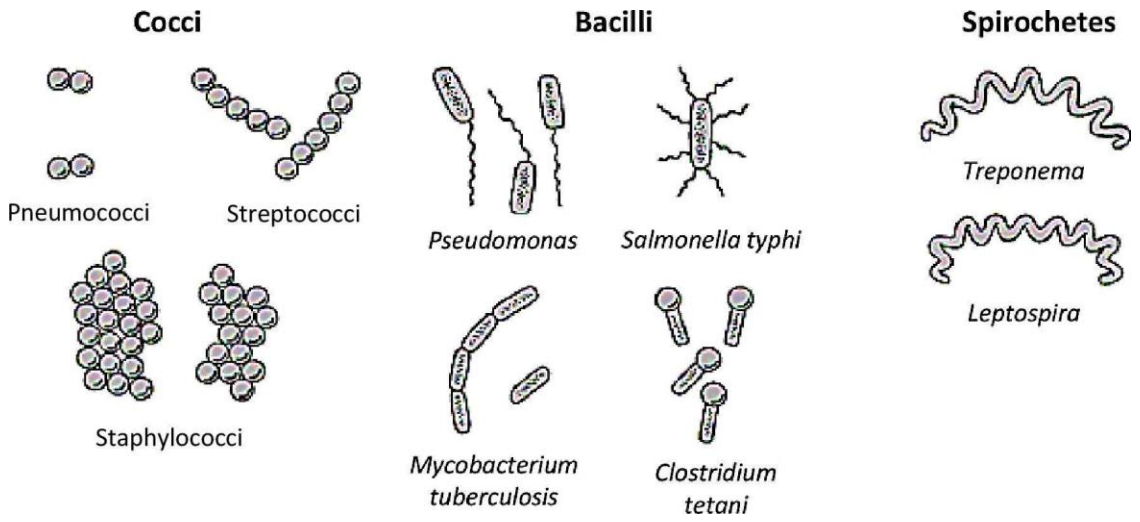

Fig. 1.1 Difference of bacteria shape [4].

Likewise, in case of bacterial growth on solid media as colonies, a colony is defined as a visible mass of microorganisms originating from a (single) mother cell, therefore a colony constitutes a clone of genetically identical bacteria. The number of cells within a colony can reach up to several billions. On a solid medium, shape, color, surface appearance, and size of colonies are often characteristic, and these visible features are used in the identification of particular bacterial strains as shown in Fig. 1.2 [5].

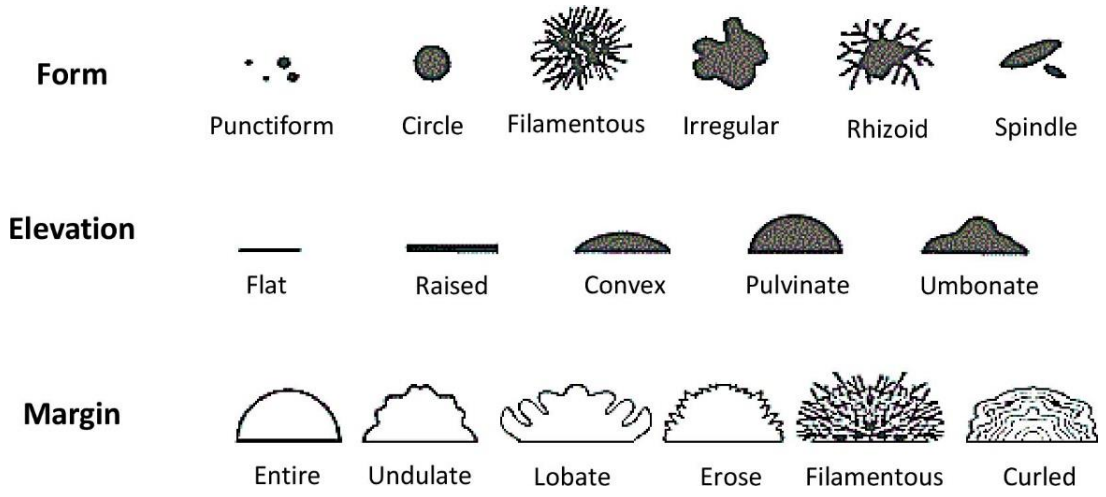

Fig. 1.2 Colony morphology of bacteria [6].

Bacteria reproduce by binary fission which is a division of one cell into two. The duration of cell division is called generation time. The generation time depends on the type of bacteria and the environment.

Bacteria are prokaryotes, lacking well-defined nuclei and membrane-bound organelles, and have chromosomes composed of a single closed DNA circle. Basically, all kinds of bacteria have the same general structure that consist of a cell wall, cell membrane, cytoplasm, single chromosome, and ribosomes. Some of them consist of capsules, glycocalyx, pili or fimbriae, mesosome, flagella, inclusion granule, and bacterial spore (Fig. 1.3).

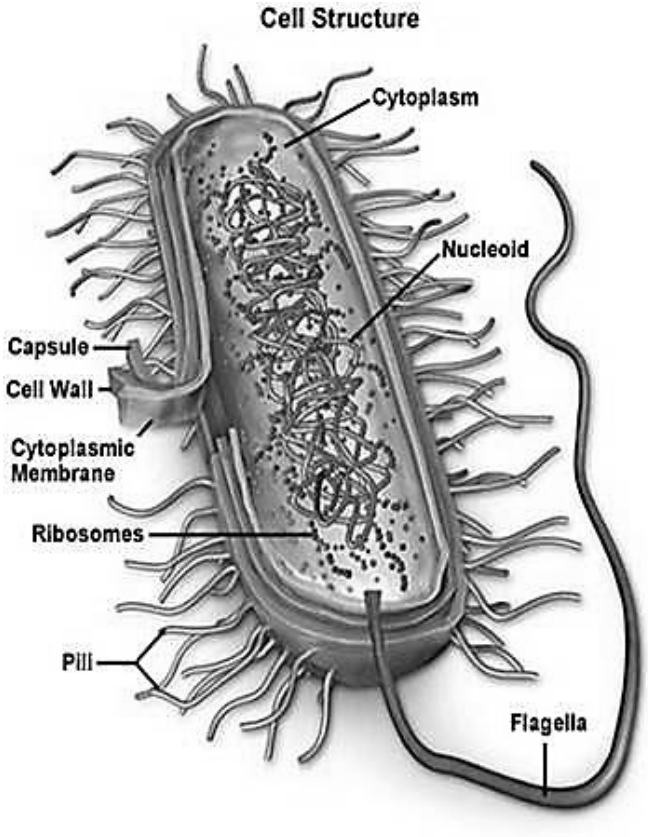

Fig. 1.3 Structure of bacteria [7].

There are a lot of factors that affect to the growth of bacteria such as nutrition:

1) carbon source such as carbohydrate,
2) electron source,

3) nitrogen source such as amino acids, peptides, proteins,
4) oxygen, sulfur and phosphorus source such as water, nucleic acid, nucleotide, phospholipids,
5) vitamin, and etc.,

oxygen: aerobic and anaerobic bacteria can be identified by growing them in test tubes of thioglycollate broth [8],

1) obligate aerobes need oxygen because they cannot ferment or respire anaerobically,
2) facultative anaerobes can grow with or without oxygen because they can metabolise energy aerobically or anaerobically,
3) aerotolerant organisms do not require oxygen as they metabolise energy anaerobically,
4) strict anaerobe or obligate anaerobes are poisoned by oxygen,
5) microaerophiles need oxygen because they cannot ferment or respire anaerobically (Fig. 1.4),

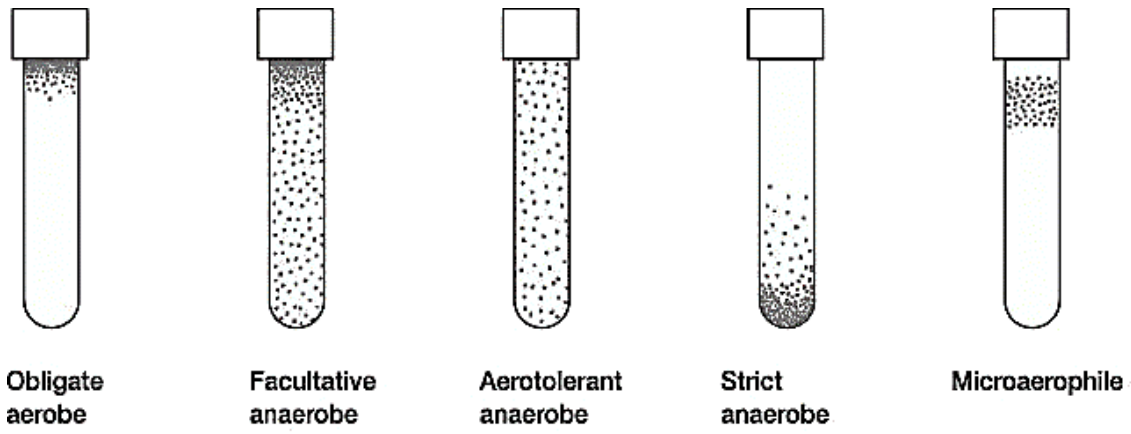

Fig. 1.4 Oxygen conditions of bacteria [9].

temperature: temperature control for bacteria growth also depend on their type,

1) thermophilic bacteria grow under high temperature,
2) mesophilic bacteria grow under medium high temperature,
3) psychrophilic bacteria grow under low temperature,

moisture content, and water activity.

1.1.2 Culture media

To culture bacteria, a nutrient media are required in a container, such as a Petri dish or test tube. There are several type of media such as solid or gel type, liquid or broth type. In this research, we applied the paper as the container and use gel type to culture the bacteria. There are three kinds of hydrogel that were used in the experiments: agarose gel, poly(vinyl alcohol) (PVA) hydrogel, and calcium alginate (CA) hydrogel.

Hydrogels are hydrophilic polymer networks that absorb from 10% to 20% up to thousands times of their dry weight in water [10] as shown in Fig 1.6. There are mainly two components that are interaction with water, namely 'hydrophilic group' such as -OH, -COOH, -$CONH_2$, -CONH-, and -SO_3H and hydrophobic group such as -CH_2-, and -CH_3 [11]. Hydrogels are formed by crosslinking polymer chains through physical, ionic, or covalent interactions [12].

Gelation refers to the linking of macromolecular chains together which initially leads to progressively larger branched yet soluble polymers depending on the structure and conformation of the starting material [13]. Continuation of the linking process results in increasing the size of the branched polymer with decreasing solubility. This is called the 'gel' or 'network'. The mechanical properties and viscoelasticity of hydrogels can be conveniently customized, making them highly suitable for a variety of biotechnological applications [14]. Because of the high water content of hydrogels [15], they are widely used for various pharmaceutical and biomedical applications. These include immunological assays [16], healing agents [17], and three-dimensional cell and tissue culture environments, in which hydrogels act as excellent mimics of the vivo state [13,18].

In this study, we used hydrogel which is formed by using crosslinking agent: physical crosslinking and chemical crosslinking. We expected that hydrogel can be a new choice for a culture media suitable for inkjet printer.

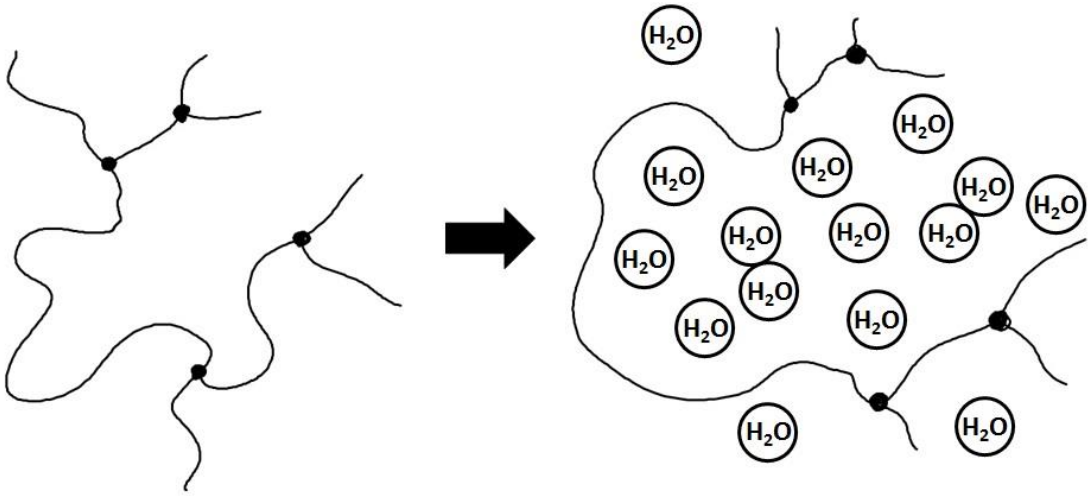

Fig. 1.6 Transition of polymer chains after molecules of water diffusion.

1.1.2.1 Heating/cooling a polymer solution

Gelation of agarose is a heating/cooling physically crosslinked gel type. Gels are formed when cooling hot solutions of gelatine or carrageenan [14]. Therefore, gelation of agarose depends on the temperature and there are 3 states of gelation as shown in Fig. 1.5. An agarose is a polysaccharide polymer material, generally extracted from seaweed. Agarose is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose [19].

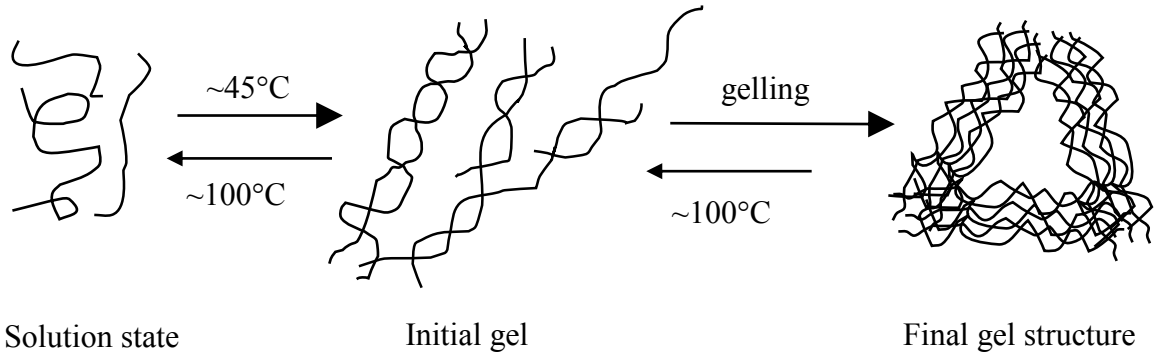

Fig. 1.5 State of the gelation of agarose.

1.1.2.2 H-bonding

Physical gels can be sub categorized as strong physical gels and weak gels. A strong physical gel has strong physical bonds between polymer chains and is effectively permanent at a given set of experimental conditions. Weak physical gels have reversible links formed from temporary associations between chains. These associations have finite lifetimes, breaking and reforming continuously. Figure 1.7 shows the physical bonds by hydrogen bonding (H-bonding) [14].

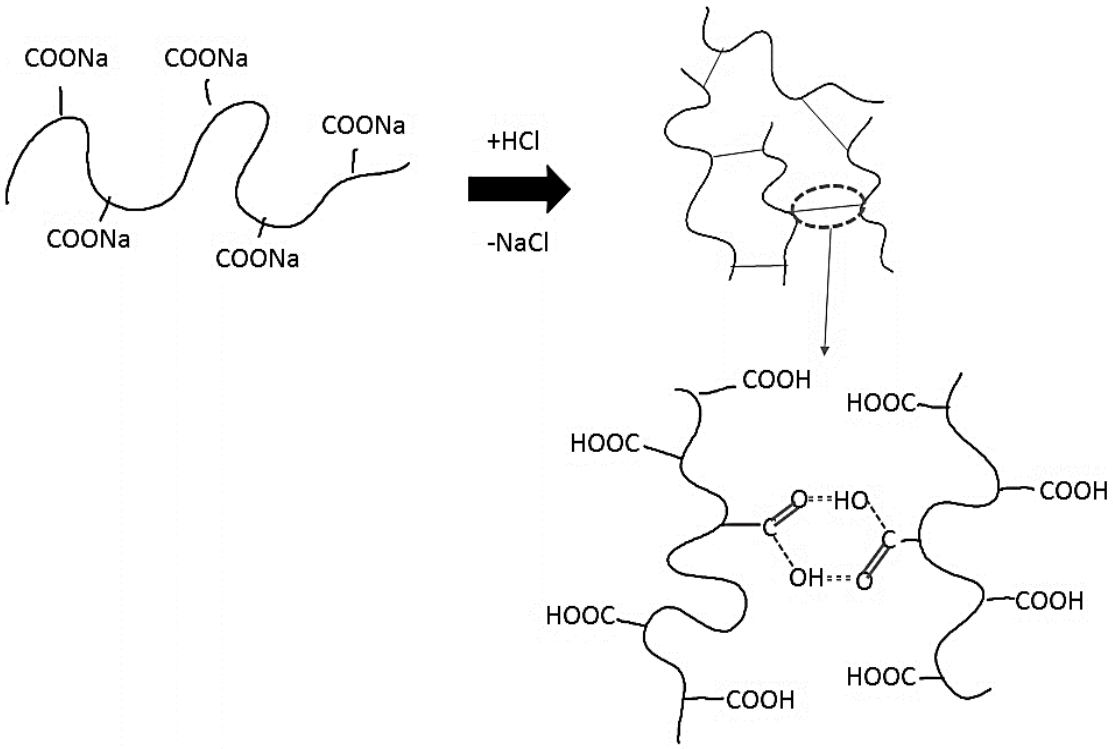

Fig. 1.7 Hydrogel network formation due to intermolecular H-bonding [14].

1.1.2.3 Chemical crosslinking

Chemical gelation is formed by covalent bonds and always results in a strong gel. Figure 1.8 shows the chemical crosslinking structure.

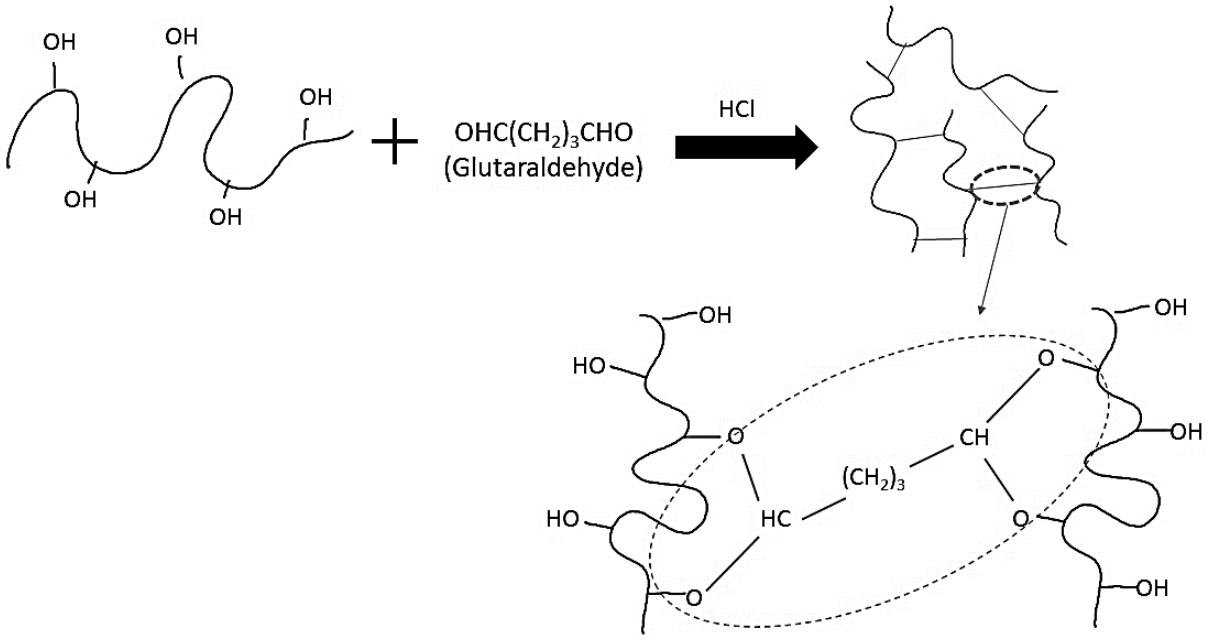

Fig. 1.8 Illustration of chemical cross-linked hydrogel network [14].

1.1.3 Cultivation method

To culture bacteria, a nutrient medium is required as well as a Petri dish or test tube as a container. In preparation for nutrient media, an autoclave is always required for sterilization at high temperature and pressure. Then, bacterial cells are transferred to a medium plate on a clean bench. In the same way, an autoclave was used for disposing bacteria.

We used broth media to prepare the suspension for bacterial cells printing and gel media to culture printed bacterial cells on paper. In order to obtain purified bacterial cells, isolation is required. To isolate bacteria there are several methods such as streak plate technique, serial dilution technique, heat shock technique, differential plating technique, and selective plating technique [20]. In this experiment, the streak plate technique was used to isolate bacteria and the serial dilute technique was used to prepare the suspension.

1.1.3.1 Streak plate technique

Streak plate technique is used to grow bacteria on a growth media surface such that individual bacterial colonies are isolated and sampled. Isolated colonies indicate a clone of cells, being derived from a single precursor cell. When the selected culture media is inoculated using a single isolated colony, the resulting culture grows from that selected single clone. To perform this procedure, the four ways or quadrant streak is mostly used [3,4] (Fig 1.9).

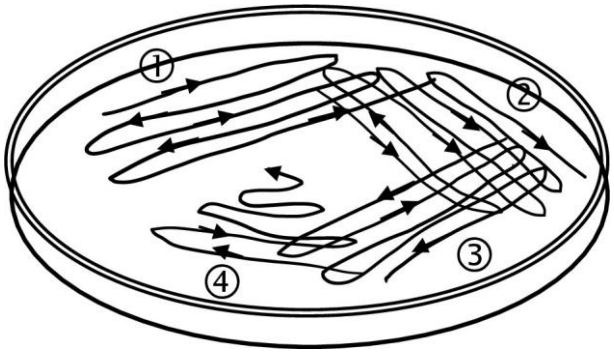

Fig. 1.9 Quadrant Streak Method [6].

The commonly used petri dishes are of hundred millimeter diameter. The gel surface of the plate should be dry without any moisture such as condensation drops. The source of inoculums can be clinical specimen, environmental swab, sedimented urine, broth or solid culture.

In the streaking procedure, a sterile loop or swab is used to obtain an uncontaminated microbial culture. The process is called "picking colonies" when it is done from an agar plate with isolated colonies and is transferred to a new agar or gelatin plate using a sterile loop or needle. The inoculating loop or needle is then streaked over an agar surface. On the initial region of the streak, many microorganisms are deposited resulting in confluent growth or the growth of culture over the entire surface of the streaked area. The loop is sterilized by heating the loop in the blue flame of the Bunsen burner, between streaking different sections, or zones and thus lesser microorganisms are deposited as the streaking progresses. The streaking process will dilute out the sample that was placed in the initial region of the agar surface [5]. Figure 1.10 shows the example of single colonies prepared by the streak plate technique.

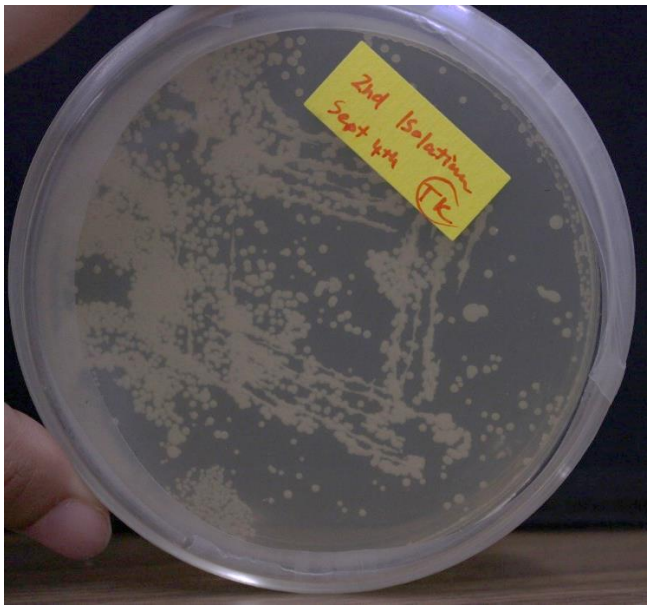

Fig. 1.10 The appearance of single colonies on agar plate by meaning of streak plate technique.

1.1.3.2 Serial dilution method

Serial dilution is a common technique used in many immunologic procedures. A small amount of serum or solute can be serially diluted by transferring aliquots to diluent. One of the most common series doubles the dilution factor with each transfer (1:2, 1:4, 1:8 …). These dilutions can be done in microliter plates or test tubes depending on the volumes of sample and diluent used [24]. The dilution is given by

CFU/g = [(number of colonies/quantity plated) x dilution factor]/gram of sample.

Figure 1.11 shows protocol of serial dilution technique. The original inoculum was transferred in 9 ml of saline. Then 1 ml of diluted inoculum was transferred into new 9 ml of saline. The number of dilution will be continued depending on how much dilution factor require. The 0.1 ml of each diluted inoculum from each test tube was poured in agarose plate and incubate. After the colonies appeared on agarose plate, the number of colonies were counted to calculate dilution factor (CFU/g).

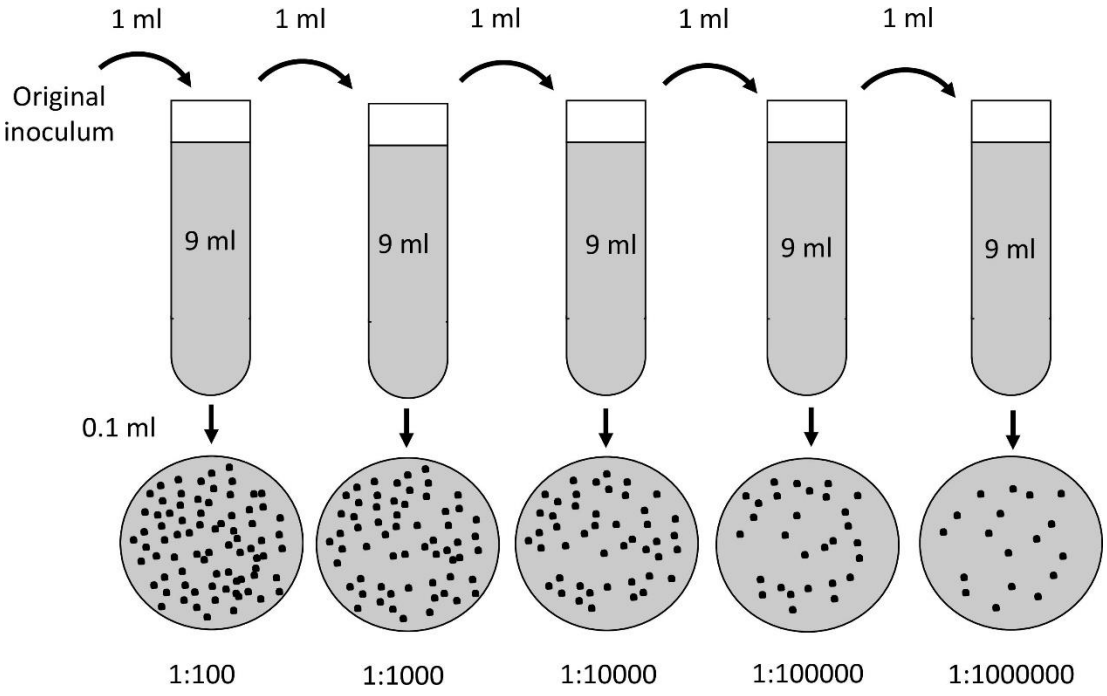

Fig. 1.11 Serial dilution method.

1.2 Inkjet technology

Inkjet printing is a type of computer printing that recreates a digital image by propelling droplets of ink onto paper, plastic, or other substrates. The rapid development of inkjet technology started in the late 1950s. Since then, many inkjet devices have been developed [25]. In this research, we used 2 types of inkjet printer: thermal inkjet, and piezoelectric.

1.2.1 Thermal inkjet

In a thermal inkjet printer ink droplets are ejected from an orifice by the explosive formation of a vapor bubble within the ink supply due to the application of a two part electrical pulse to a resistor within the ink supply [26]. The electrical pulse comprises a precursor pulse and a nucleation pulse; the precursor pulse preheats the ink in the vicinity of the resistor to a

temperature below the boiling temperature of the ink to preheat the ink while avoiding vapor bubble nucleation within the ink supply and the subsequently occurring nucleation pulse very quickly heats the resistor to near the superheat limit of the ink (Fig. 1.12).

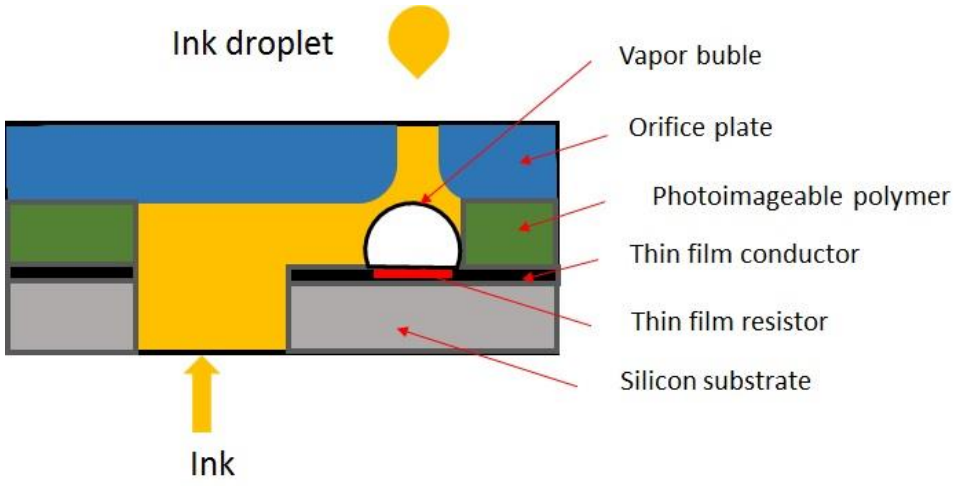

Fig. 1.12 Thermal inkjet system.

1.2.2 Piezoelectric

The driving force to eject a droplet with a piezo inkjet print head is generated by the actuator, which deforms the structure through the inverse piezo-electric effect (Fig 1.13).

The piezoelectric effect (electricity from an applied mechanical stress) was first discovered by Pierre and Jacques Curie in 1880. Their experimental demonstration consisted of a conclusive measurement of surface charges appearing on specially prepared crystals, which were subjected to mechanical stress. In 1881, Lippmann deduced mathematically the inverse piezoelectric effect (stress in response to an applied electric field) [27].

The piezoelectric actuator driven by the signal, optimized for the particular print head model, can precisely control the unrestrained ink meniscus vibration at the nozzle openings to

achieve multi-sized droplets ejection in a sequent manner as well as stabilized droplet ejection up to the extremely high ejection repetition frequency [28].

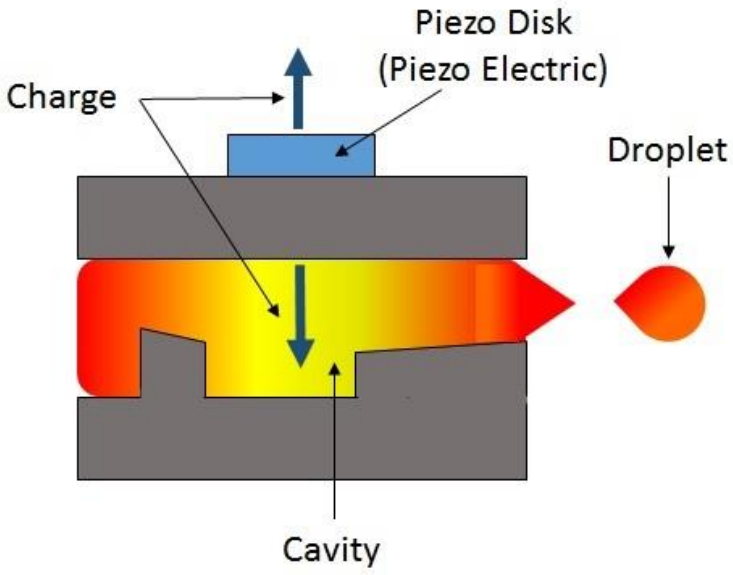

Fig. 1.13 Piezoelectric system.

1.2.3 Ink cartridge

Ink cartridge or inkjet cartridge is a component of an inkjet printer that contains the ink or fluid. Each ink cartridge contains one or more ink reservoirs. Also the number of head nozzle is different. Some of the inkjet printers are single cartridge system while others are multi-cartridge system.

1.2.3.1 Single cartridge system (SCS)

The single cartridge system printers were developed for highly accurate printing in specialize application such as printed electronics and bio-printing. Several kinds of fluid can be loaded in the cartridge and ejected by controllable head nozzles. The high accuracy of overprinting position was controlled by means of a CCD camera. In addition, the volume and direction of each droplet was controlled by jet waveform which can be designed by the user.

1.2.3.2 Multi-cartridges system (MCS)

The first commercial devices were introduced in 1951 by Siemens [29]. The MCS is well known as the third-party ink cartridges which can produce many color shades by mixing of the main colors (CMYK) depending on the percentage of each. Presently, more than 4 main colors were successfully installed in the inkjet printer to produce more color shades, to save ink and reduce the printing time. However, overprinting position control, small ink volumes, and individually head nozzle control are limited for the MCS printer used here.

In this research, the piezoelectric type SCS printer was used to dispense the suspension of bacteria while the thermal inkjet type MCS printer was used to dispense culture media.

1.3 Paper

Paper is a highly-engineered product that is part of almost every aspect of our daily life: books, magazines, catalogs, newspapers, notebooks, food packaging, and cleaning products. Paper is a material produced by pressing together moist fibers, cellulose pulp derived from wood, rags or grasses, and drying them into flexible sheets [30]. There are a lot of different types of paper developed for many different kinds of products and applications. The type of paper is classified by both its physical and chemical properties such as thickness, weight, surface, roughness, and wettability.

Although being already highly sophisticate for its classical fields of application, novel technologies are presently developed including new nanocellulose and hemicellulose based materials, improved manufacturing processes, and harness the possibilities inherent in the chemistry of cellulose [31].

In this research, we introduce paper as effective media for culturing bacteria. We modified the filter paper with hydrophobic/hydrophilic techniques to become the container of bacteria culture media.

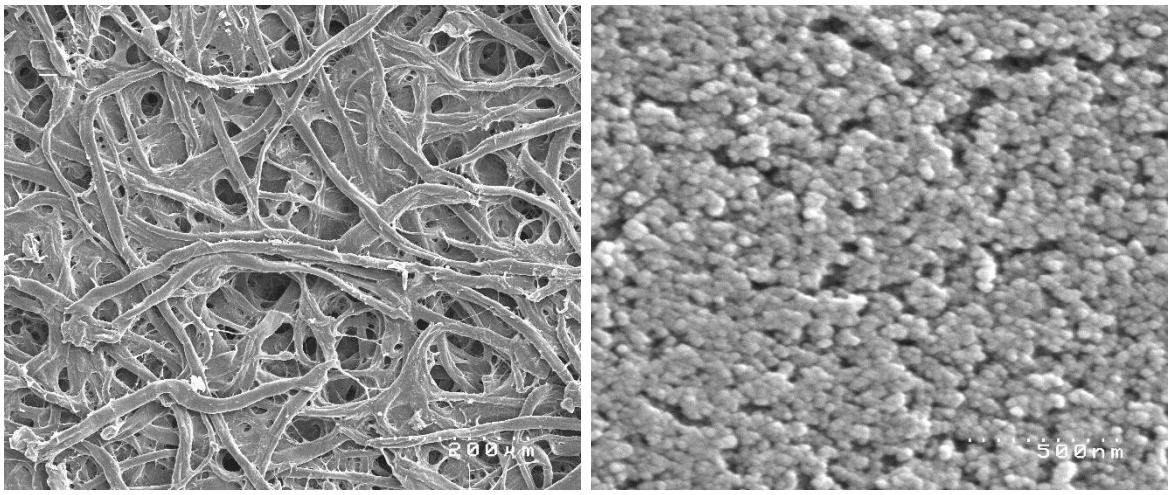

Fig. 1.14 Samples of paper surface: filter paper (left), pigment coated paper (right).

1.4 Application of paper and inkjet technology to bacterial culture system

Recently, various novel paper-based devices have been introduced. Paper is abundant available, inexpensive, and recyclable; it is easy to store, transport, manipulate, and dispose. Attempts have been made to fabricate paper-based medical sensors to analyze clinical body fluids and metabolic substances, such as blood [32], urea [33], and glucose [34]. A multiplex detection system for *Escherichia coli* was developed using an enzyme-based lab-on-paper test strip [32]. Furthermore, fabrication methods and design techniques of paper-based microfluidic channels using wax for portable bioassay were discussed [35].

Paper can be divided into multi-channels for liquid-transporting sensor tests by using a patterning technique to separate hydrophobic/hydrophilic areas. We would apply this useful technique to bacterial cultures under various conditions. Hydrophilic areas were built on a small

sheet of hydrophobized filter paper in order to accommodate dispensed media and bacteria. In addition, paper is a combustible substrate; therefore grown bacteria could be simply disposed by combustion. Thus, no autoclave is required in the disposal process.

There is another factor of inconvenience caused by manual operation. Bacterial cells are commonly transferred by manually in culture tests. The transferred volume cannot be constant or controllable. To solve the instability problem due to manual procedure, we proposed an inkjet as a dispensing method. To observed under the microscope, the growth of a rod-shaped cell like *E. coli* appears quite simple [36]. A freshly divided cell elongates with little or no increase in girth. Eventually a transverse wall is laid down near the center of the cell; when the cell reaches approximately twice its original size; it separates into two cells nearly equal in size. Therefore, *E. coli* cells transfer was tested as a model of bacterial cells by using inkjet technology. In particular, inkjet printing is often used as one of the measures for manufacturing electronics because of its advantages such as resource savings and non-contact processes attained by the drop on demand (DOD) system [37]. That is why inkjet technology is no longer only an office printing technology [38]; it has gradually become a versatile tool in various fields for accurately dispensing very small quantities of fluids on substrates, such as human organs or cells [39-43].

Therefore, inkjet technology is a promising technique for transferring bacterial cells in a small with stable and controlled volumes. In addition, ink-jet ejection is easily controlled using standard software that can print any designed patterns. Moreover a charge-coupled device camera, which is usually equipped with high-performance inkjet printers, can be used to examine the pattern dispensed on a substrate. Processing, such as hydrophilic/hydrophobic patterning, with an ink-jet printer is a time-saving, low-cost, and controllable technique providing a powerful alternative to manual operation.

Through this technology, one can minimize the hazard of accidental exposure to harmful or pathogenic bacteria while handling them. As shown in Fig 1.15 we proposed in reference [44] to create a bioassay system by applying an inkjet printer and paper.

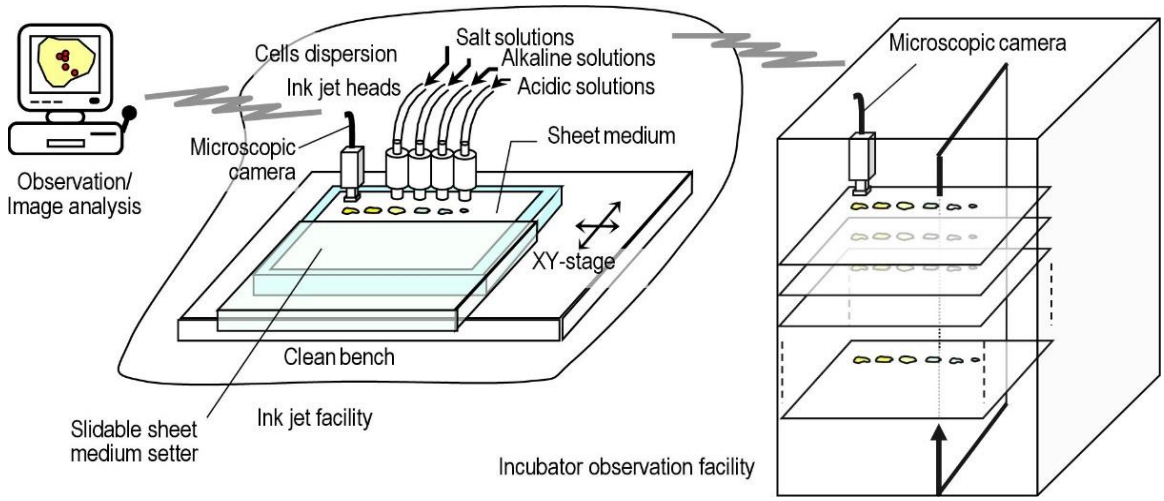

Fig. 1.15 Bioassay system using inkjet printing and paper substrate [44].

1.4 Objective of this study

In this research, I attempt to present a new method to create a well-ordered and reproducible system for dispensing bacteria on a microscopic scale by using advantages of paper and inkjet technology. Also, to find the easy-to-use medium which can be printed by using inkjet printer.

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Chapter 2

Development of a bacterial culture system using a paper platform to accommodate media and an inkjet printer to dispense bacteria

2.1 Abstract Generally, bacterial culture is performed manually and is subject to error. Here, we created a novel, well-ordered, and reliable system for dispensing bacteria microscopically by using paper and an inkjet printer for controlled patterning. For paper to accommodate a culture medium, hydrophobic/hydrophilic patterns were incorporated onto the paper by immersing paper in a toluene solution of polystyrene and drying for complete hydrophobization, followed by etching discrete, small areas of hydrophilicity by inkjet printing with toluene. Agar was hydrolyzed with sulfuric acid for appropriate viscosity and dispensed with an inkjet printer. In a separate experiment, bacterial cells were sequentially printed on a medium, and colonies were observed microscopically. The results of this experiment ensured the successful dispensing of bacteria using inkjet printing. An almost constant number of particles per droplet were ejected using polystyrene latex as a model of bacterial dispersion. Consequently, we expect this technology to be adapted for the development of a paper-based bioassay system.

2.2 Introduction

Bacteria are present everywhere: in soils, deep in rocks, in all water bodies, and in the atmosphere, including on and inside other living organisms [1]. Several kinds of bacteria are commercially important in industries such as pharmaceutical and food industries [2]. However, some bacteria are harmful. To extensively research bacteria species, microbiologists isolate pure cultures and further analyze them. To culture bacteria, a nutrient medium is required in a

container, such as a Petri dish or test tube. If growth under different conditions is needed, several sets of containers are required. To culture bacteria, a nutrient medium is required as well as a Petri dish or test tube as a container. In preparation for nutrient media, an autoclave is always required for sterilization at so high a temperature and pressure that high electric power is consumed. Then, bacterial cells are transferred to a medium plate in a clean bench. In the same way, an autoclave was used again for disposing bacteria. In case of observation under different conditions, several sets of containers are additionally required. Therefore, we came with an idea to use paper that is easy to handle as a container instead of a Petri dish. The paper container would use ethylene oxide gas for sterilization and combustion for disposal.

Recently, various novel paper-based devices have been presented. Paper is ubiquitous, inexpensive, and recyclable; it is easy to store, transport, manipulate, and dispose. Attempts have been made to fabricate paper-based medical sensors to analyze clinical body fluids and metabolic substances, such as blood, urea [3, 4], and glucose [5]. A multiplex detection system of *Escherichia coli* was developed using an enzyme-based lab-on-paper test strip [3]. Furthermore, fabrication methods and design techniques of paper-based microfluidic channels using wax for portable bioassay were discussed [6]. Paper can be divided into multi-channels for liquid-transporting sensor tests by using a patterning technique to separate hydrophobic/hydrophilic areas. We considered that we would apply this useful technique to bacterial cultures under various conditions. Hydrophilic areas were tried to be built on a small sheet of hydrophobized filter paper in order to accommodate dispensed agar and then bacteria. In addition, paper is a combustible substrate, therefore grown bacteria could be simply disposed with the paper substrate after combustion. Thus, no autoclave is required in the disposal process.

There is another factor for inconvenience caused by manual operation. Bacterial cells are commonly transferred by manually in culture tests. The transferred volume cannot be constant or controllable. To solve the instability problem due to manual procedure, we proposed an inkjet dispensing method. However, [7] observed under the microscope, the growth of a rod-shaped cell like *E. coli* appears quite simple. A freshly divided cell elongates with little or no increase in girth. Eventually a transverse wall is laid down near the center of the cell; when the cell reaches approximately twice its original size; it separates into two cells nearly equal in size. Therefore, *E. coli* cells transfer was tested as a model of bacterial cells by using inkjet technology. Because

inkjet technology is no longer only an office printing technology [4]; it has gradually become a versatile tool in various fields for accurately dispensing very small quantities of fluids on substrates, such as human organs or cells [8-11]. Therefore, inkjet technology is a promising technique for transferring bacterial cells in a small, stable and controlled volume. In addition, inkjet ejection is controlled using software that can print any designed patterns, and a charge-coupled device camera, which is usually equipped with high-performance inkjet printers to examine the pattern dispensed on a substrate. Processing, such as hydrophilic/hydrophobic patterning, with an inkjet printer is a time-saving, low-cost, and controllable technique that is an alternative to manual operation. Through this technology, researchers can avoid harmful or pathogenic bacteria from directly touching them as a concomitant effect.

In this chapter, we present a new method to create a well-ordered and reproducible system for dispensing bacteria on a microscopic scale by using advantages of paper and inkjet technology. As shown in Fig. 2.1, we used paper as a media platform for bacterial culture and inkjet printing technology to dispense small amounts and for manageable patterning of bacterial suspension. Bacterial growth on a nutrient medium contained in a Petri dish is sometimes evaluated visually by positive/negative judgment followed by a statistical analysis [12], counting the number of colonies [13], color of colonies [14], and more semi-quantitatively, scores based on the number of quadrants in which growth was observed [15]. The system we are creating aims to quantify bacterial growth with a microscope and image analysis as the future work, which will be useful to routine laboratories using agar plates.

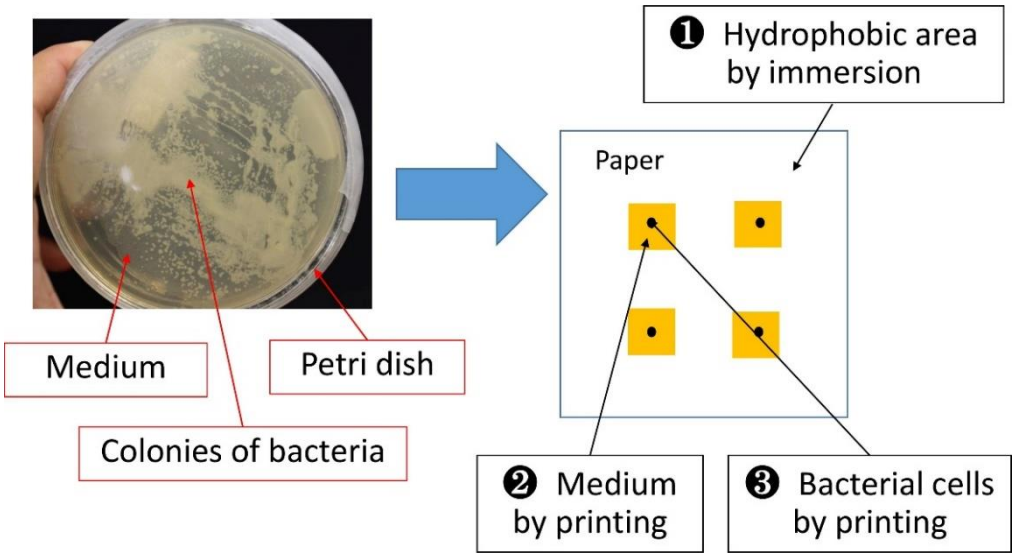


Fig. 2.1 Image of bacterial culture system using a paper platform.

2.3 Materials and methods

2.3.1 Preparation of the Hydrophilic/Hydrophobic Pattern

We selected paper as a potential new substrate to facilitate efficient observation of bacterial growth under various conditions. Previous research [4] presented a method to modify paper to form hydrophobic and hydrophilic areas in the same substrate by using polystyrene (PS) and toluene. In the present study, we used PS to serve as a hydrophobic barrier to block spreading of the aqueous medium that was printed on hydrophilic areas. In addition, filter paper was chosen as a substrate to settle the medium because of its high absorbency. First, the filter paper was soaked in a 3.0 wt% toluene solution of PS for 1 h (Fig 2.2) and then allowed to dry at room temperature for 15 min, to allow the entire filter paper to become hydrophobic. Squares (10 mm × 10 mm) were etched to make hydrophilic areas by printing toluene with Solvent Blue 35 at 0.015 wt% (blue toluene) for visualization. All patterns were listed in table 2.1.

We found that with 3 head nozzles and overprinting the area twice for the best organized patterns with distinctive borders. Finally, hydrophilicity was tested by dropping 2 μL of water on the etched areas, and the degree of penetration was observed and compared (Fig 2.3).

Table 2.1 Print conditions of hydrophilic area

Pattern	Number of Head nozzle	Drop spacing (μm)	Overprinting (time)
1	3	70,50,20,10,5	1,2
2	5	70,50,20,10,5	1,2
3	9	70,50,20,10,5	1,2
4	10	70,50,20,10,5	1,2
5	16	70,50,20,10,5	1,2

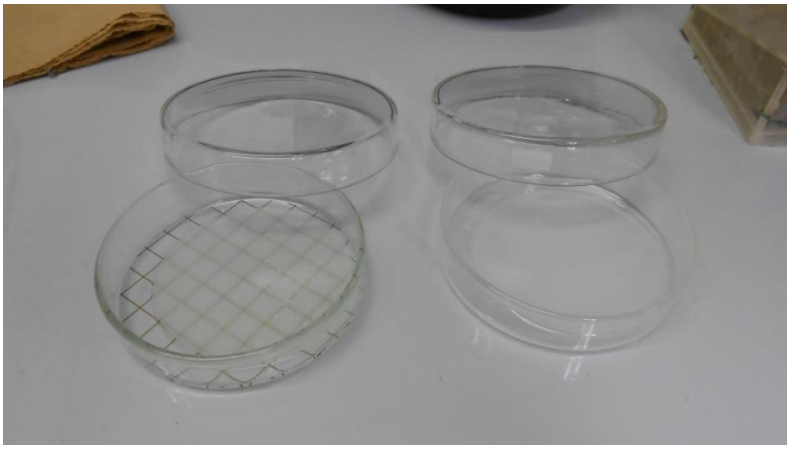


Fig. 2.2 Filter paper soaked in toluene solution of PS.

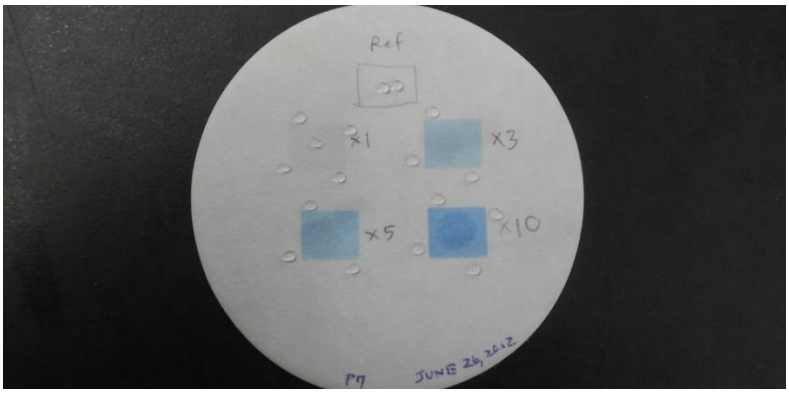


Fig. 2.3 Hydrophilicity test by dropping 2 μL of water on etched areas.

2.3.2 Modification of Agar

A common culture medium for bacteria is an agar medium that contains necessary nutrients. We attempted to print a nutrient agar medium by using the inkjet printer for dispensing small quantities of the medium on hydrophilic areas of the filter paper. However, the medium quickly solidified at room temperature, which is not conducive to printing. Therefore, we modified the molecular structure of agar by hydrolysis. Agar contains agarose as a major component, which is a linear polymer made up of the repeating unit of agarobiose—a disaccharide made up of D-galactose and 3, 6-anhydro-L-galactopyranose. Glycosidic bonds between each agarobiose are easily hydrolyzed by acids, such as sulfuric acid (H_2SO_4), that shorten polysaccharide chains. The optimum hydrolysis of agar was sought to generate agar polymers conducive to printing. First, 0.5 g of agar powder was dissolved in 20 mL of 0.05%, 0.1%, and 0.02% H_2SO_4 aqueous solutions, and then the solutions were heated at 100°C for 10, 15, 20, 25, 30, 40, 45, and 50 min. To stop the acid hydrolysis, the agar solutions were cooled on ice water for 5 min. Sodium hydroxide (NaOH) was added to neutralize the solutions for bacterial culture. Sodium sulfate (Na_2SO_4) produced during neutralization was removed by electrodialysis (Microacilyzer S1 with a membrane cartridge AC110-10, ASTOM, Japan). Viscosity of each hydrolyzed agar solution was measured by using a Cannon-Fenske Routine Viscometer. Finally, the hydrolyzed agar was mixed with yeast extract, Bacto tryptone, and

sodium chloride (NaCl). The agar mixture was loaded into an ink cartridge wrapped with a film-type heater (Fig. 2.4) and was printed on rectangular hydrophilic areas (5 mm x 5 mm). The drying speed of printed hydrolyzed agar was recorded to evaluate water retention.

Fig. 2.4 Ink cartridge wrapped with film-type heater.

2.3.3 Bacterial Culture on Paper

Bacterial growth was examined by transferring a suspension of bacteria and comparing the growth on a normal agar plate and on hydrolyzed agar medium on paper. First, bacteria floating in the air were taken randomly (Fig 2.5) from stationary phase and isolated for pure culture by the streak technique. At least 3 repetitions of this technique were performed to confirm the purity of the bacteria and classify the morphology of the bacterial colonies. Serial dilution [18] was applied to reduce the concentration of the bacterial suspension (Fig 2.6). Finally, a bacterial suspension of 2.7×10^7 CFU/mL was transferred onto hydrolyzed agar to observe the bacterial growth.

Fig. 2.5 Culture of air-floating bacteria on agarose medium in Petri dish.

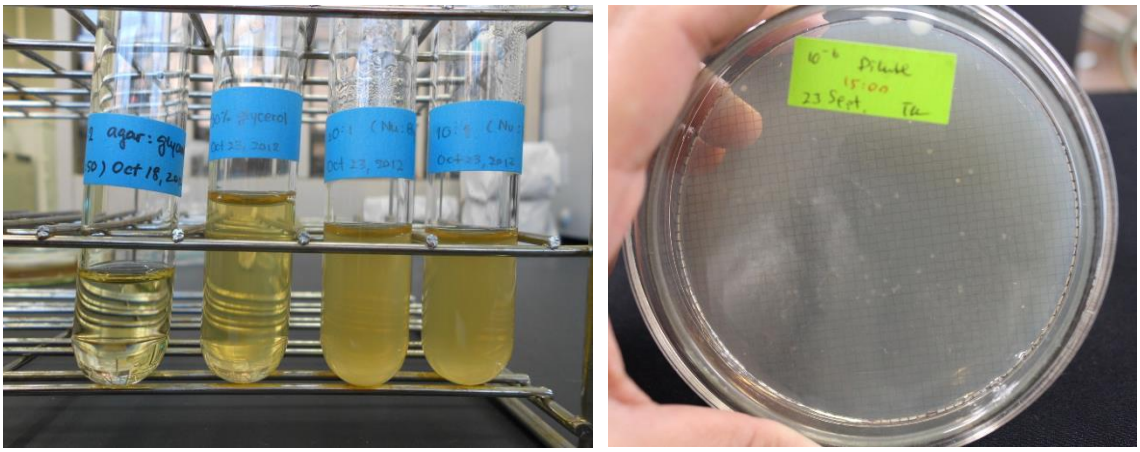


Fig. 2.6 Serial dilution method (left) and diluted colonies on agarose plate (right).

2.3.4 Ejection of *Escherichia coli* on Medium

E. coli cells were printed with a testing inkjet printer (Dimatix DMP-2831, Fujifilm, Japan) as shown in Fig. 2.7, with a piezoelectric actuator, on a standard culture medium to confirm bacterial cell applicability and viability. First, a 5-mm-thick square sheet of culture

medium was prepared and placed on the stage of the inkjet printer. Next, *E. coli* cells were loaded into a cartridge and printed following patterns designed using a specialized application. The pattern was considered to be a rectangle of 5 × 10 dots arranged on a sheet at intervals of 5 mm. The printed *E. coli* cells were incubated at 37°C for 24 h, and the resultant colonies were examined.

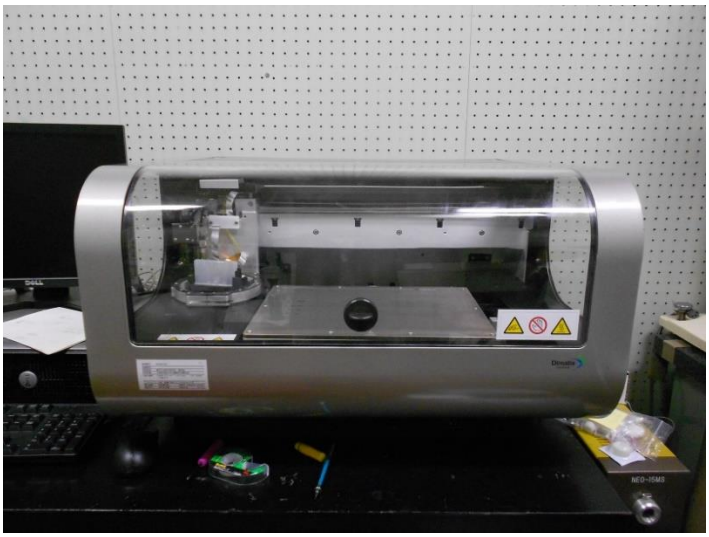


Fig. 2.7 Dimatix DMP-2831.

2.3.5 Preparation of Emulsified Particulates

Polystyrene acrylate hollow latex particles with a diameter of about 1 μm (latex particles; PAT8125; ZEON Corporation, Japan) were selected as a bacterial cell model to ensure the ability to print a constant number of particles. The stock latex emulsion, consisting of 26.5% solids, was diluted to 0.001% and then mixed with a small amount of glycerol to obtain an appropriate viscosity for inkjet printing (Fig. 2.8). Latex particles were subsequently printed on a sheet of photo-grade inkjet printing paper (Super photo grade; Kassai, Fujifilm, Japan) using an application to manage printing conditions such as printing frequency and number of droplets in 1 dot on the paper. A square pattern of 11 × 11 dots was printed only once or overprinted 3 or 5 times on the paper (Fig. 2.9), and photographs of the printed dots were taken by a scanning electron microscope (S-4200, Hitachi, Japan) to count the number of latex particles.

Fig. 2.8 Latex emulsions mixed with glycerol.

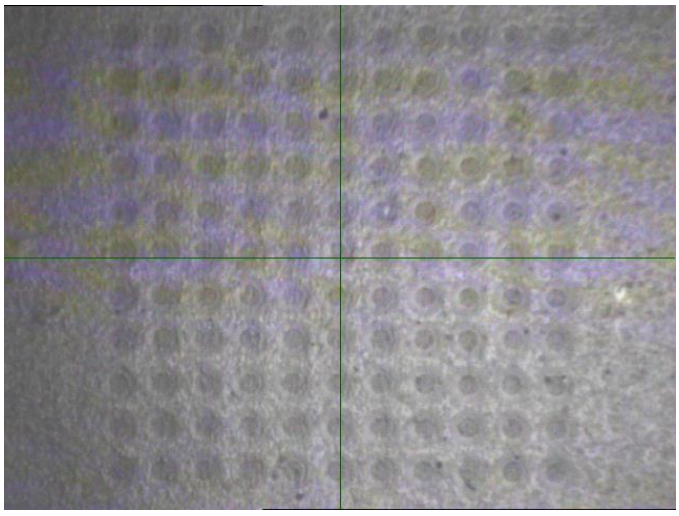


Fig. 2.9 One square pattern of 11 × 11 dots.

2.3.6 Confocal Laser Scanning Microscopy

Automatic and selective monitoring of bacterial growth by a confocal laser scanning microscope (CLSM 700; AxioObserver, Carl Zeiss Microscopy) was performed to develop this bioassay system. A dried piece of filter paper with a bacterial colony on the agar medium was subjected to fluorescence microscopic observation with an excitation wavelength (ex) of 555 nm (green) and an emission wavelength (em) ranging from 500–630 nm (blue to red) for the agar and ex of 639 nm (red) and em > 640 nm (red) for the hydrophilic area. CLSM was used to obtain a regular light transmission image showing the fiber network, and the composite image was created from the 3 images.

2.4 Results and discussion

2.4.1 Hydrophilic/Hydrophobic Patterning

After the filter paper was soaked in a toluene solution of PS and dried, hydrophobicity of the filter paper surface was tested by dropping 2 μL of water on the whole surface, as shown in Fig. 2.10.

Hydrophobicity of areas etched by printing blue toluene on the hydrophobic filter paper was checked by dropping water again. Darker blue regions, with overprinting of blue toluene, demonstrated a higher hydrophilic property of faster water penetration. For optimum hydrophobic/hydrophilic patterning, a 40 min printing method including 3 head nozzles and overprinting twice was chosen (Fig. 2.11).

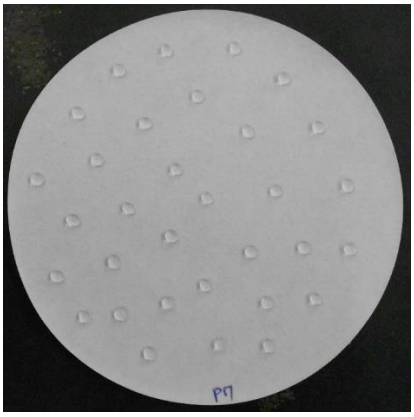


Fig. 2.10 Hydrophobicity test of filter paper surface by dropping 2 μL of water.

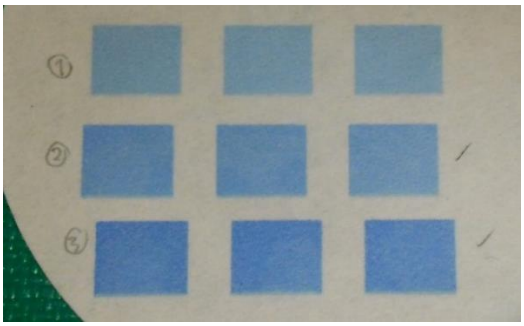
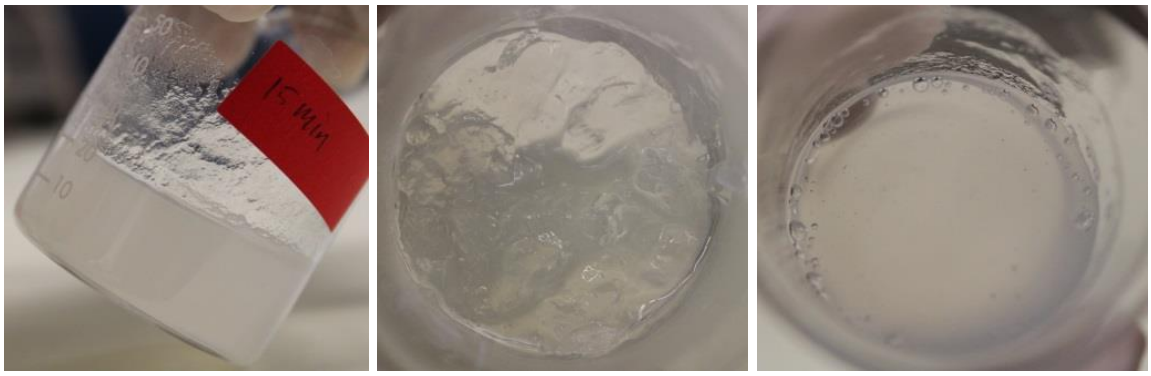


Fig. 2.11 Blue toluene patterns overprinted twice with 3 head nozzles.

2.4.2 Optimized Modification of Agar for Inkjet Printing

As shown in Fig. 2.12, hydrolyzed agars were classified into 3 groups, depending on the state of the material: gel, paste, and aqueous states. Agar powder dissolved in 0.05% H_2SO_4 aqueous solution and heated for 30 min attained an optimum hydrolysis level, which was a viscosity conducive to inkjet printing (Fig. 2.13). Additionally, a film-type wrapping heater was used to change the hydrolyzed agar from the paste to aqueous state. As a result, heating guaranteed smooth ejection of the hydrolyzed agar medium by maintaining the aqueous state and avoided clogging the nozzle heads. However, 50 μL aliquots of the hydrolyzed agar medium dried so quickly that the moisture content of the agar was maintained only for 16 h.



(a) (b) (c)

Fig. 2.12 Three phases of modified agar; (a) solid (gel), (b) paste, and (c) liquid.

To confirm the survival of bacterial cells on paper, the isolate originally from air-floating bacteria were transferred on the hydrolyzed agar medium manually. Bacterial colonies were observed after 10 h on a normal agar medium. However, colonies were not observed clearly on the hydrolyzed agar medium on plates or paper. Colonies that grew on the wet hydrolyzed agar medium on the plate were observed after 3 days, although colonies on paper had dried completely. Because we developed a culture system for bioassays that will be processed within a few hours, the drying time of 16 h is sufficient to maintain a suitably moist environment.

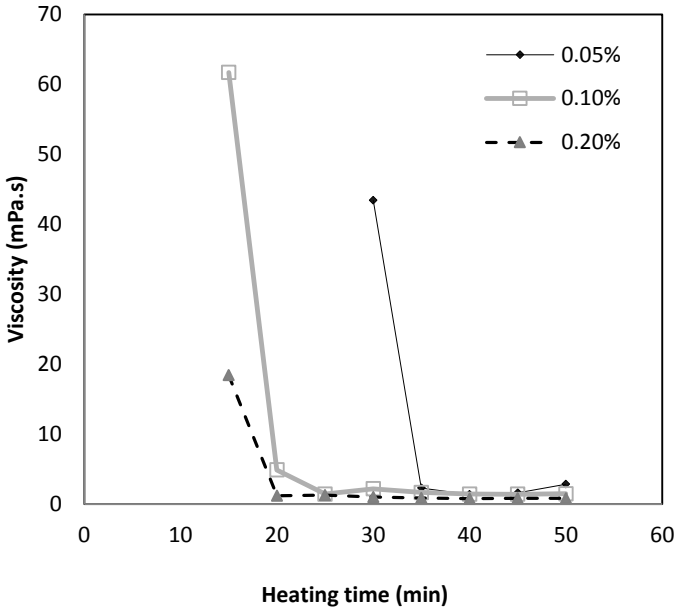


Fig. 2.13 Viscosity of agar hydrolyzed at three different concentrations of H_2SO_4.

To compare bacterial growth on normal and hydrolyzed agar media, we observed that each colony on normal agar medium grew separately and laterally, allowing clear observation (Fig. 2.14). However, on hydrolyzed agar medium, many overlapping colonies grew, and it was difficult to discern individual colonies. Overlapping likely occurred, because droplets of the hydrolyzed agar were printed on top of others, during overprinting, and in the paste state over and over again and the surface tends to be rough. Even if bacteria can grow and are observable on the hydrolyzed agar, quantitative evaluation cannot be expected.

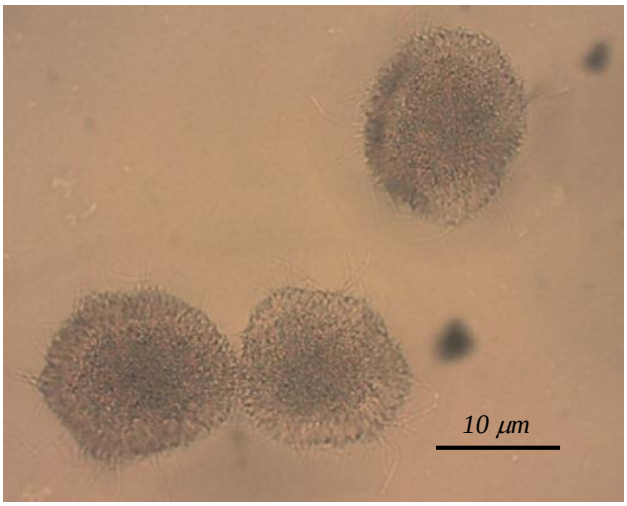


Fig. 2.14 Colonies of bacteria growing on modified agar medium.

2.4.3 Ejection of *E. coli* on agar Medium

After a suspension of *E. coli* cells was printed, the normal agar medium sheet was immediately observed microscopically. Individual *E. coli* cells were not visible; however, mature *E. coli* colonies appeared as white spots after culture for approximately 30 h and were photographed (Fig. 2.15) and microscopic colony of *E. coli* (Fig. 2.16).

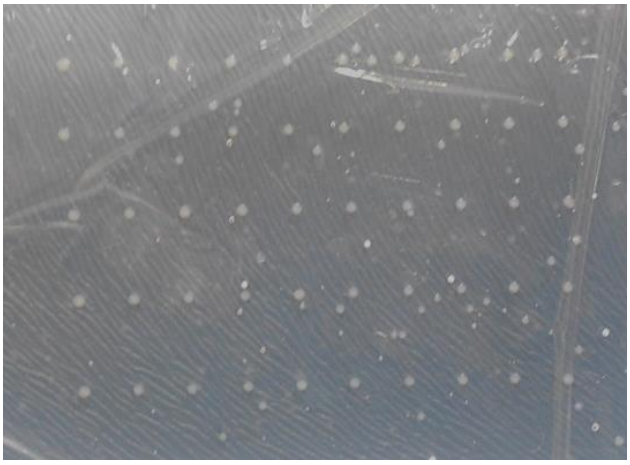


Fig. 2.15 Arrangement of cultured *E. coli* colonies after printing on a sheet of agar medium.

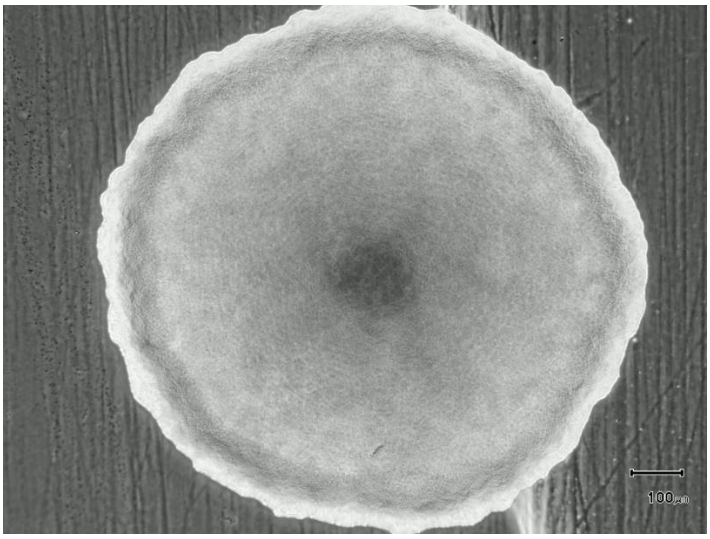


Fig. 2.16 The colony of *E. coli* was observed by microscope.

These results suggest that *E. coli* cells that were ejected through narrow nozzles were still viable, although they were subject to high shear stress in the nozzles. Previous reports discuss problems of low survival rates in such kinds of inkjet bio-dispensing systems [2,16]. However, the survival rate obtained using this system was satisfactory, considering that bacterial growth was observed for all printed dots. These results also suggest that this inkjet printing method is capable of dispensing fairly regular arrangements of *E. coli* cells, although some satellite colonies are visible next to the primary colonies in the photograph (Fig. 2.15). This deviation in the regular arrangement was due to droplet split, which occurred immediately as the droplets were released from the nozzle; deviation was solved by increasing viscosity and decrease surface tension by adding a small amount of glycerol to the bacterial solution.

2.4.4 Stability in Number of Ejected Particles

After we confirmed the ability to dispense viable bacterial cells by inkjet, we verified that the inkjet printer could dispense regular arrangements of non-ink liquid dots and eject consistent numbers per droplet of latex particles, a model for bacterial cells. The inkjet printer is designed

to eject droplets with volumes of 1-10 pL. Although, the average diameter of the latex particles is approximately 1 μm, and latex particles were expected to print smoothly, we sought to avoid clogging of the head nozzles or aggregation between particles. A small amount of glycerol was added to latex particles to increase the viscosity and decrease the surface tension [17]. Figure 2.9 shows printed dots of latex particles at certain intervals on photo-grade inkjet paper, which resulted in the formation of well-dispersed particles for easy counting and a potentially desirable inoculation level of bacterial cells. For the measurements, the number of primary particles with a diameter of approximately 1 μm was counted, and coexisting particles much smaller in size were excluded from counting. We randomly selected 10 out of 121 dots for counting. The statistical data are listed in Table 2.2. Consequently, the average number of latex particles per droplet was consistently approximately 15, irrespective of the number of droplets per dot. This finding suggests that liquids containing bacterial cells can be dispensed evenly and regularly onto a culture medium. In addition, the circular shape of 1 dot evenly formed from 5 droplets (Fig. 2.17(c)), indicating that overprinting several times can be applied to adjust the number of cells per dot.

Table 2.2 Number of latex particles observed in printed dots by SEM.

Number of droplets per dot	Number of particles			
	Mean number per dot	*Mean number per droplet*	*Standard deviation*	*Confidence interval (95%)*
1	14.4	14.4	2.67	1.65
3	44.0	14.7	5.14	3.18
5	79.2	15.8	5.24	3.25

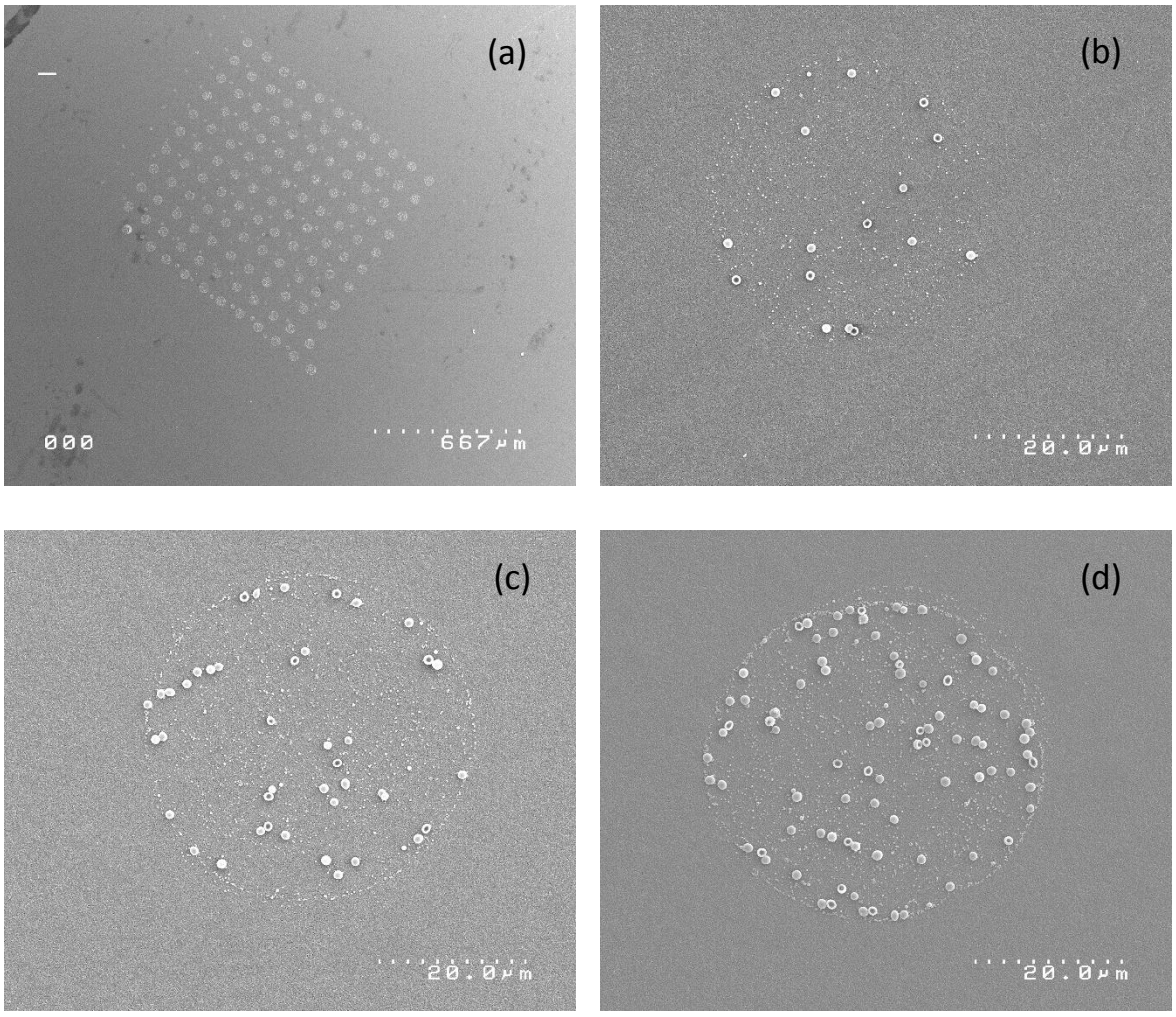


Fig. 2.17 Latex particles in 1 dot observed by scanning electron microscope; (a) A pattern of 11 × 11 dots, (b) 1 time overprinting, (c) 3 times overprinting, and (d) 5 times overprinting.

2.4.5 Fluorescence Monitoring of *E. coli G*rowth and Agar Location

Light transmission and fluorescence optical micrographs were captured by CLSM. The sample of colonies of bacteria which grew on agar media on paper (Fig. 2.18) was examined by CLSM. Figure 2.19 shows an overall paper structure consisting of fibers of the filter paper with a dark area. Figures 2.20 show fluorescence images of agar medium and toluene blue adsorbed by

fibers, respectively. It should be noted that the false-colors appearing in the images are not the same as those visible to the human eye. The combined image (Fig. 2.21) clearly indicates that the agar medium spread and settled within the hydrophilic area, and the dark area in Fig. 2.21 corresponds to the blue dye. Unfortunately, the bacterial colonies could not be distinguished by CLSM, because the auto-fluorescence of the bacteria was similar to that of the medium. However, CLSM is a promising technique to distinguish mixed components in the paper. This technique would be incorporated in the bioassay system as an automatic evaluation of bacterial growth.

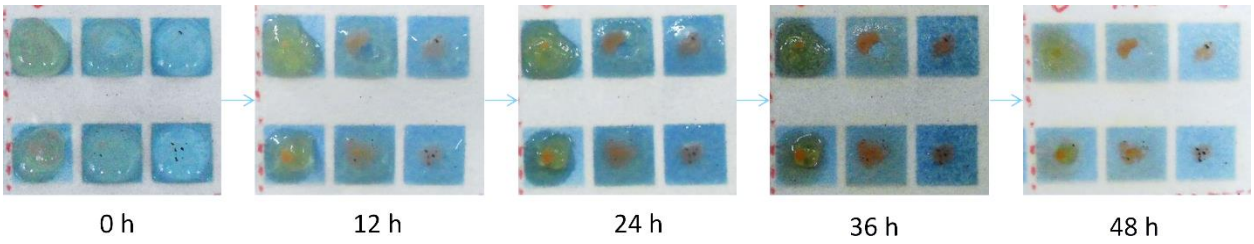


Fig. 2.18 The sample of colonies of bacteria grew on agar media on paper.

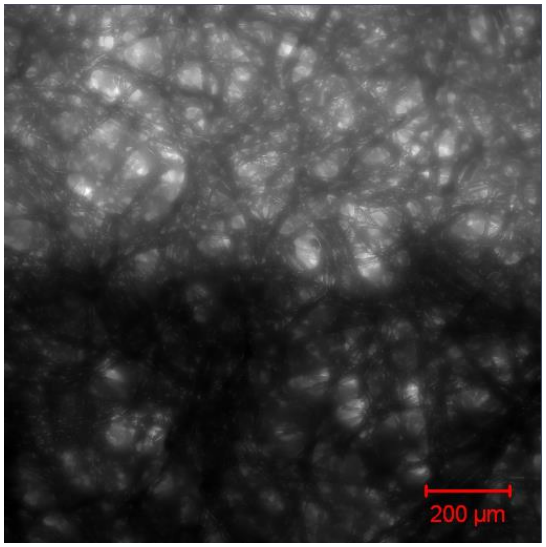


Fig. 2.19 The overall paper structure consisting of fibers of the filter paper.

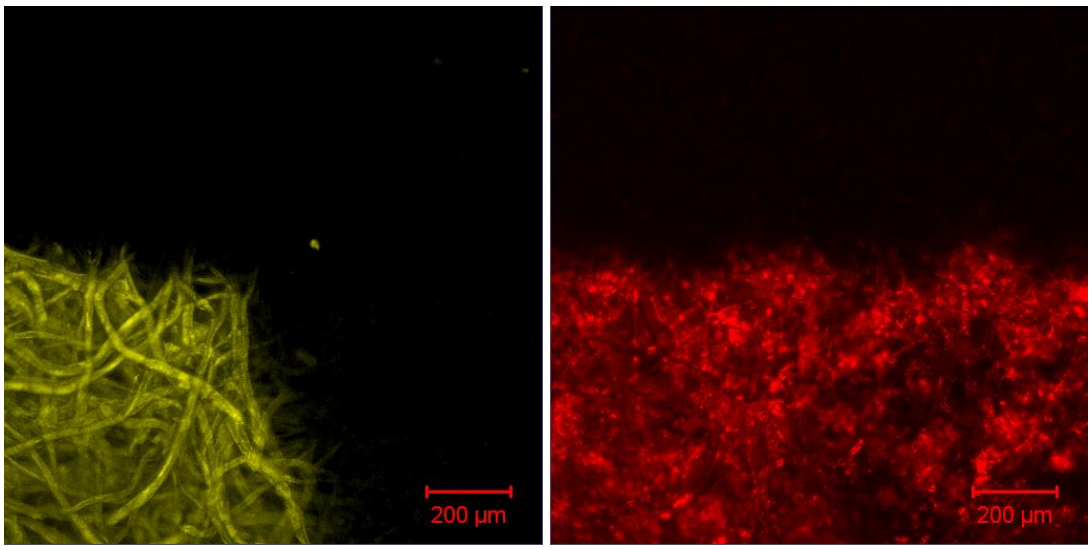


Fig. 2.20 The fluorescence images of agar medium (left) and toluene blue (right).

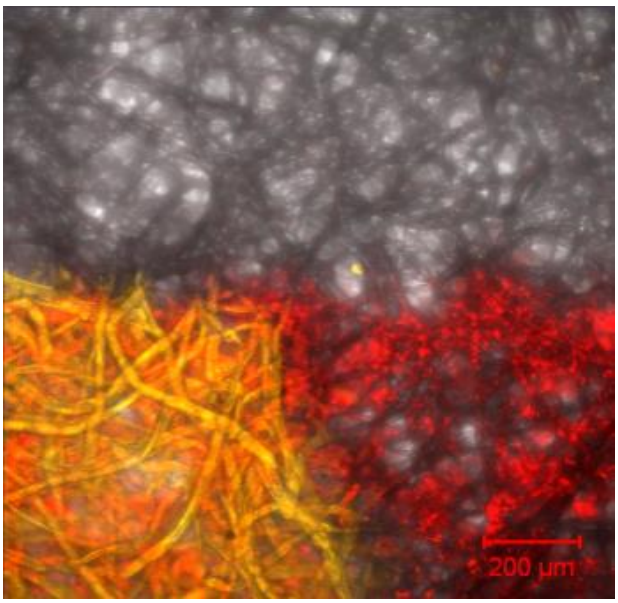


Fig. 2.21 Combined image of agar medium on hydrophilic areas of filter paper as observed by CLSM.

2.5 Conclusions

The inkjet printer-assisted bioassay system we are currently developing in combination with a paper substrate is a promising technique to more efficiently evaluate bacterial growth rates. The hydrophilic/hydrophobic patterning provided a paper substrate suitable for accommodating an inkjet printed culture medium. Agar was hydrolyzed to adjust the viscosity, making it conducive to printing on paper and bacterial growth. CLSM was performed to detect the locations of the agar, blue dye, and fibers. The discrete locations on the paper substrate were discernible in the fluorescence images. In a separate experiment, *E. coli* cells were printed on a sheet of agar medium, and the growth of colonies with high survival rates were confirmed. We confirmed the regularity of the number of ejected cells in 1 droplet with latex particles as a cell suspension model. Finally, we developed inkjet assisted paper-based substrate as a part of a bioassay system.

2.6 References

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Chapter 3

Application of inkjet-printed poly(vinyl alcohol) (PVA) hydrogel to bacterial culture media

3.1 Abstract Inkjet printing and paper were applied for respectively dispensing and containing culture media and bacteria to create a comprehensive bacterial culture system. Poly(vinyl alcohol) (PVA) hydrogel was then applied as culture media because of its advantageous long storage life, ability to control the gelation time, and ability to expel via the inkjet head nozzles without clogging. PVA hydrogel was formed by physical crosslinking from a mixture of PVA and adipic acid dihydrazide (ADH) as a crosslinking agent. The gelling conditions depended on the concentrations of PVA and ADH, and the mixture ratio. We determined an optimum mixture ratio of 4.5:0.5:95 (PVA:ADH:H_2O) for suitability of inkjet printing. A solution of a mixture PVA/ADH was successfully printed and allowed to gel by itself on paper. Finally, bacterial growth was confirmed on the hydrogel medium.

3.2 Introduction

Inkjet printing systems, which are capable of outputting variable information from sheet to sheet, are currently applied for personal use and advanced commercial printing [1]. In particular, inkjet printing is often used as a means for manufacturing electronics because it provides advantages such as resource savings and noncontact processes carried out via the drop on demand (DOD) system [2]. Advanced printing applications are not limited to the field of electronics. Indeed, inkjet and laser printing technologies [3] have gradually become a versatile tool in a variety of other fields and have been used to accurately dispense very small quantities of fluids on substrates, such as human organs [4,5], cells [6], or bacteria [7].

The hazards caused by careless handling of pathogenic bacteria can be minimized by inkjet printing, as it is possible to dispense the minimum required amount of a bacterial suspension. In this study, SCS and MCS inkjet printer were used as a dispensing device in a

comprehensive bacterial culture system. Thus, the system that we introduce here provides a low-cost, easy-to-use, efficient means for bacterial culture. In chapter 2, an inkjet printer was used to function as a dispensing device and clean bench. In addition, paper was used as a container for the culture media.

Conventionally, bacterial culture systems utilize agarose gels containing nutrients as the medium. Agarose is a galactan formed by linking approximately 500 agarobioses consisting of d-galactose and 3,6-anhydro-l-galactopyranose linked by α-(1→3) and β-(1→4) glycosidic bonds [8]. In chapter 2, I utilized an inkjet printer to dispense both agarose medium and a bacterial suspension on a paper substrate, with the goal of creating stable patterns and dispensing accurate amounts of bacterial cells. However, agarose contains long chains of polysaccharides, which makes it unsuitable for smooth ejection from the head nozzles of the inkjet printer. Therefore, agarose was hydrolyzed by using sulfuric acid to reduce the degree of polymerization. After hydrolysis, the agarose became acidic and was thought to inhibit bacterial growth. To avoid this problem, the pH was adjusted to neutral using sodium hydroxide, and the hydrolyzed agarose was successfully ejected by the inkjet printer. However, the hydrolysis processes took a long time, and the viscosity adjustment required precise control. Therefore, here we introduce poly(vinyl alcohol) (PVA) hydrogel as a suitable alternative for agarose. PVA combines the advantages of agarose with a higher compatibility for inkjet printing.

Hydrogels are formed by crosslinking polymer chains through physical, ionic, or covalent interactions and are well known for their ability to absorb water [9]. Moreover, the mechanical properties and viscoelasticity of hydrogels can be conveniently customized, making them highly suitable for a variety of biotechnological applications [10]. Because of the high water content of hydrogels [11], they are widely used for various pharmaceutical and biomedical applications. These include immunological assays [12], healing agents [13], and three-dimensional cell and tissue culture environments, in which hydrogels act as excellent mimics of the vivo state [14,15].

PVA is a polymer that can be used to form hydrogels. Moreover, PVA is capable of simulating natural tissue and is readily accepted by the human body [13]. PVA is generally made by dissolving another polymer, poly(vinyl acetate) (PVAc), in an alcohol such as methanol and treating it with an alkaline catalyst, such as sodium hydroxide (NaOH). The resulting hydrolysis reaction removes the acetate groups from the PVAc molecules without

disrupting their long chain structure [16]. However, the incomplete removal of the acetate group yields resins that are less soluble in water and more soluble in certain organic liquids. Therefore, PVA can be synthesized to have varying interactions with water.

Additionally, PVA can be cross-linked with many types of cross-linking agents. In this study, adipic acid dihydrazide (ADH) was mixed with PVA as a cross-linking agent to produce PVA hydrogels. The PVA hydrogel is classified as a reversible or physical gel. PVA hydrogel networks are held together by hydrogen bonding as shown in Fig. 3.1.

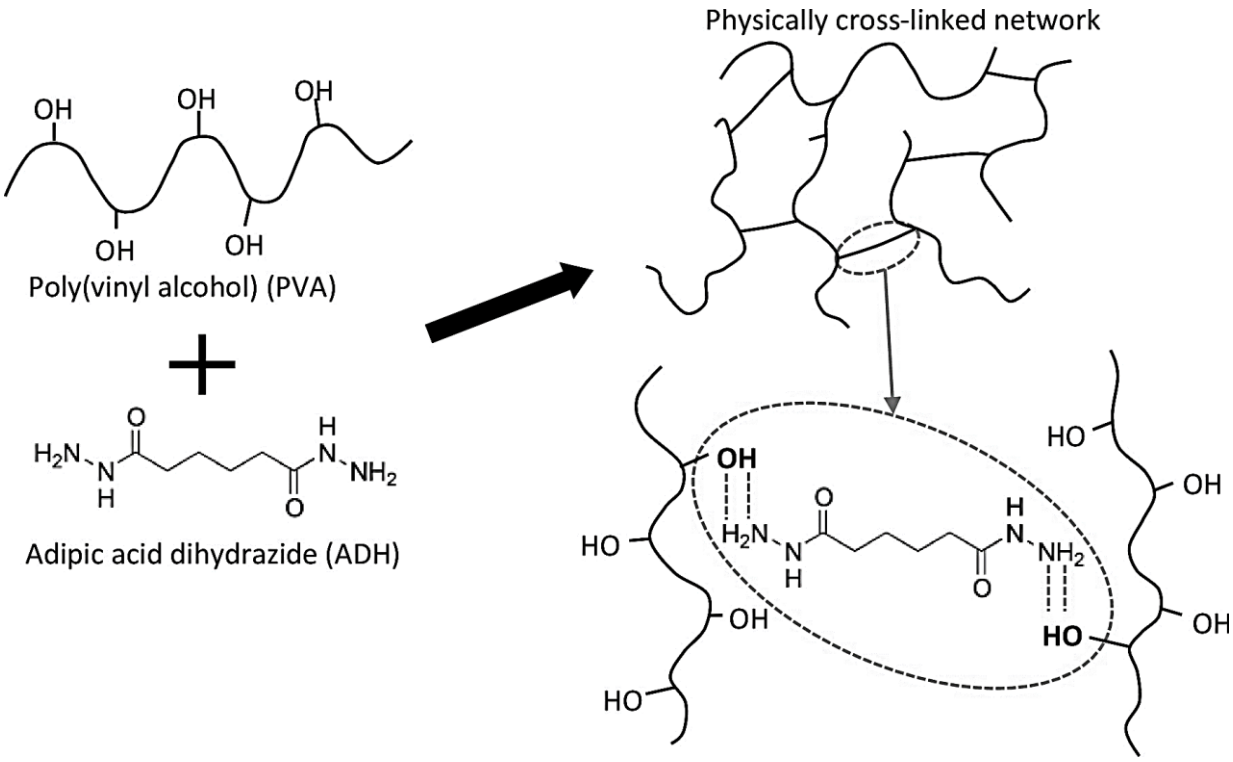

Fig. 3.1 Formation of a PVA hydrogel network based on intermolecular hydrogen bonding.

In physically cross-linked gels, dissolution is prevented by physical interactions between different polymer chains [17]. All such physical interactions are reversible and can be disrupted by changes in physical conditions or by application of stress [18]. We observed that PVA hydrogel reverted to a PVA/ADH mixture solution after several days under ambient conditions. The gelation behavior of a PVA hydrogel depends on the concentration of PVA, ADH and the ratio of PVA and ADH in the mixture, and the mixing intensity; all of

these parameters are closely related to the ejection from an inkjet printer. PVA hydrogel is one opportunity of biomaterials which could be a promising new medium for developing a bacterial culture system coupled with an inkjet technology.

3.3 Materials and methods

3.3.1 Preparation of PVA hydrogel ink

First, PVA hydrogels were prepared by mixing PVA (Gohzenol Z-100, Nippon Gohsei, Japan) and ADH (Adipic acid dihydrazide, Nippon Kasei Chemical, Co., Ltd, Japan) solutions at several concentrations and a variety of different mixture ratios. The viscosities of the PVA solutions were then examined with a glass capillary viscometer (Cannon-Fenske type) in a water bath (Kinematic Viscosity Bath TV-5s, Japan) to evaluate the suitability of the hydrogel for inkjet printing. In practical printing, an ink cartridge is filled with a mixture of PVA and ADH before gelling. Next, an ADH solution was poured into a PVA solution, and the mixture was stirred for 5 min.

Because PVA hydrogel was formed by physical crosslinking that the gelation can be sub categorized as strong gels and weak gels [15]. The strong gels cannot be reversible or irreversible and weak gels can be reversible. We, therefore, defined the gelation of PVA hydrogel by gel strength (rigidity modulus). The gel strength was measured using a rheometer (CR-500DX, Sun Scientific Co., Ltd, Japan) under the following conditions: 60 mm/min intrusion velocity with a plunger having a pressure-sensing head of 10 mm in diameter and 10 mm in full depth (Fig 3.2). The force sensed by the sensing head was recorded over the full depth of 10 mm and the result was reported as the final force in N at 10 mm in depth and plotted on a ternary phase diagram. The rheometer was also used to characterize the gelation time [17] by measuring the gel strength every 5 min starting from the initial preparation of the mixture until a finite gel strength was observed. After determination of the mixture ratio suitable for gel formation (in terms of gel strength, as evaluated using the ternary phase diagram), the gelation times of the PVA/ADH mixtures were measured.

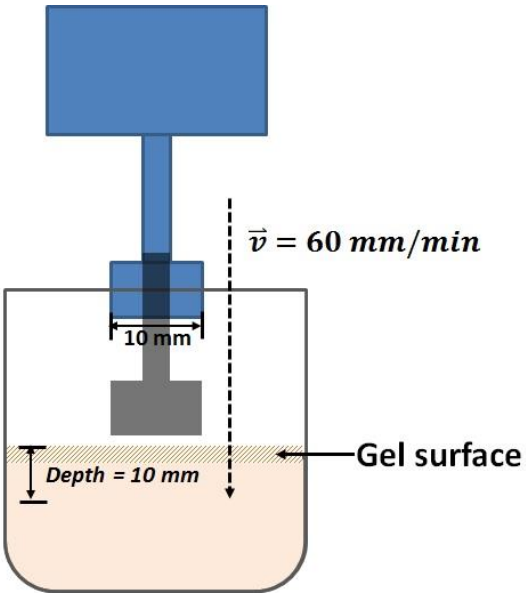

Fig. 3.2 Rheometer method.

3.3.2 Ultraviolet (UV) absorption spectra of PVA hydrogel

The PVA/ADH mixture in a ratio of 4.5:0.5:95 (PVA:ADH:H_2O) was investigated by ultraviolet spectroscopy. The investigated wavelength range was from 200 nm to 300 nm with a step size of 20 nm using an ultraviolet spectrophotometer (UV-3100PC, Shimadzu Scientific Instruments, Japan) (Fig. 3.3.). The PVA/ADH mixture was measured immediately, 1 min, 10 min, 15 min, and 20 min after mixing to monitor the transitional condition of PVA hydrogel formation with time. And also the amounts of remaining free ADH in the PVA hydrogels was observed because ADH has been reported to inhibit the growth of microorganisms [9]. For comparison, we also measured the UV absorption spectrum of PVA solution and ADH solution (both at 0.05%).

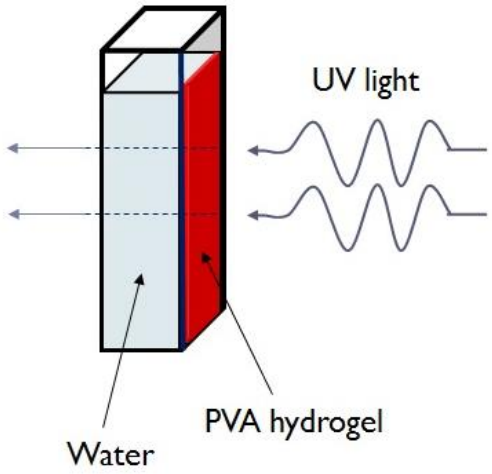

Fig. 3.3 UV absorbance measurement method.

3.3.3 X-ray diffraction of PVA hydrogel

In additional, in order to observe the crystallization of PVA hydrogel, X-ray diffraction patterns of PVA/ADH mixtures were measured by an X-ray diffratometer (D8 ADVANCE, Bruker Corporation) each over a 2θ angle range from 10 to 50 degrees for 207 s. The X-ray generator sourced from the Cu-Kα emission with a wavelength λ of 0.15408 nm was operated at 40 kV and 40 mA. A mixture of 9 mL of 10% PVA and 1 mL of 5% ADH were stirred with a glass rod for 30 s and 200 μL of the mixture was dispensed in a cavity 25 mm in diameter and 0.5 mm in depth of a silicon specimen holder (Fig. 3.4). When 3 min passed since the mixture, the first X-ray diffraction pattern started to be measured. Subsequent measurements were made in 8, 13, 18, 23, 28, and 33 min after the mixture. It was confirmed that the mixture completely became a gel in approximately 15 min after the mixture considering that the mixture was observed to completely lose fluidity and stick to the bottom without any deformation even when the small beaker containing the rest of the mixture was turned upside down. For comparison, pure water was also subjected to X-ray diffraction measurement.

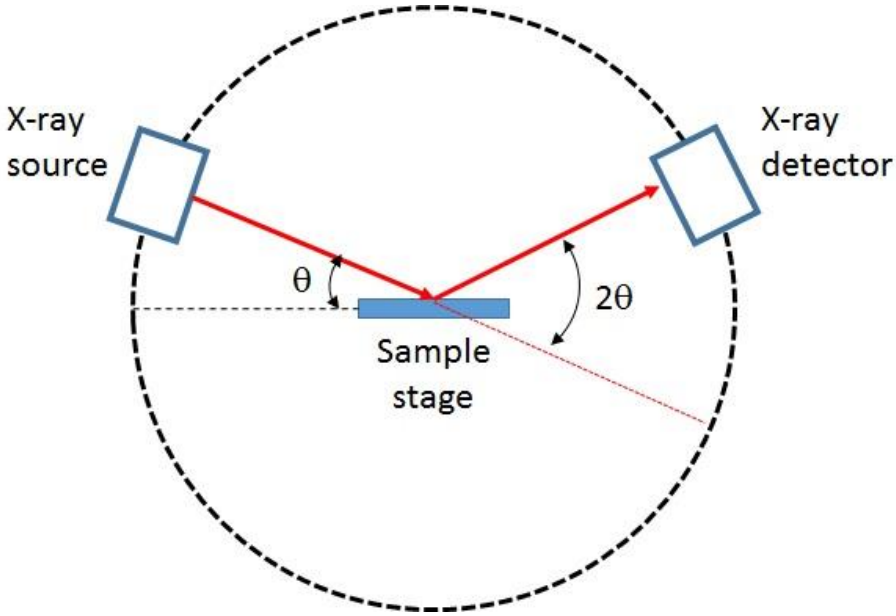

Fig. 3.4 XRD measurement method.

3.3.4 Preparation of paper substrates

Because conventional filter paper is too water absorbent to hold a PVA/ADH mixture solution firmly, it was necessary to choose a more hydrophobic paper substrate. Recently, we introduced a method to modify paper to form hydrophobic and hydrophilic areas in the same substrate by using polystyrene (PS) and toluene [8]. In this experiment, the same procedure (see Chapter 2, 2.3.1) was used but the hydrophilic level of etched area was decreased by decreasing the overprinting time of blue toluene. The condition of printing was changed from twice to once over printing time.

3.3.5 Modification of inkjet printer

In chapter 2, we used SCS inkjet printer with heat wrap to control the temperature of modified agarose in the cartridge. However, we found that the gelation of PVA/ADH mixture did not depend on the temperature. Therefore, the SCS inkjet printer was used without heat wrap. In addition, the MCS inkjet printer was used also to compare the efficiency as dispensing method. Because the cartridge of SCS inkjet printer can provide only a small

amount of media so this is time consuming. We expected that the MCS inkjet printer which can provide more amount of fluid will be better method as culture media dispenser.

A modified inkjet printer (iP2000, Canon, Japan) was used in this experiment for printing PVA/ADH mixture solution. Continuous printing was required in order to obtain a culture medium with sufficient thickness. However, reloading a paper sample with the printed culture medium was not successful because the printed media was smeared by feeding through the rollers of the printer. Therefore, for continuous printing without reloading, the printer was modified by perforation. After both the upper and lower parts of the printer stage were perforated, a paper sample was set on the far left side of the printing area, and dummy paper was loaded onto the paper tray (Fig. 3.5). Then, the sponge in the ink cartridge was removed (Fig. 3.6), and the cartridge was washed with distilled water. To sterilize the cartridge for further use, the cartridge was sprayed with aqueous ethanol (70%).

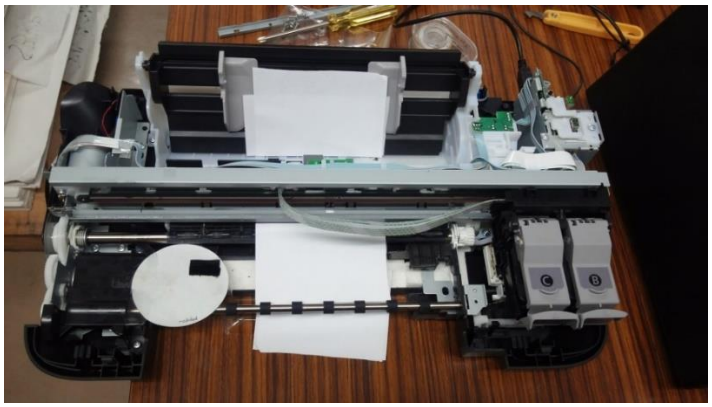

Fig. 3.5 Modified inkjet printer.

Fig. 3.6 Cartridge with a sponge removed.

3.3.6 Culture media printing

We used 2 different kinds of inkjet printers as dispensing tools, namely a SCS (Dimatix DMP-2831, Fujifilm, Japan) and MCS (iP2000, Canon, Japan) Subsequently, the efficiency of the 2 systems was compared in terms of the compatibility with the hydrogel culture media and length of time required for one complete printing operation.

3.3.6.1 SCS printer

As the SCS printer allows to set only one cartridge, all solutions: PVA/ADH mixture and nutrient for bacterial culture (see Chapter 2, 2.3.2) were loaded into separate cartridges and printed one by one respectively. The methods for the SCS printer are shown in Fig. 3.7. The printing condition was 40 V in ejection voltage, 16 head nozzles in use, 50 μm in drop spacing, and at least 10 times overprinting.

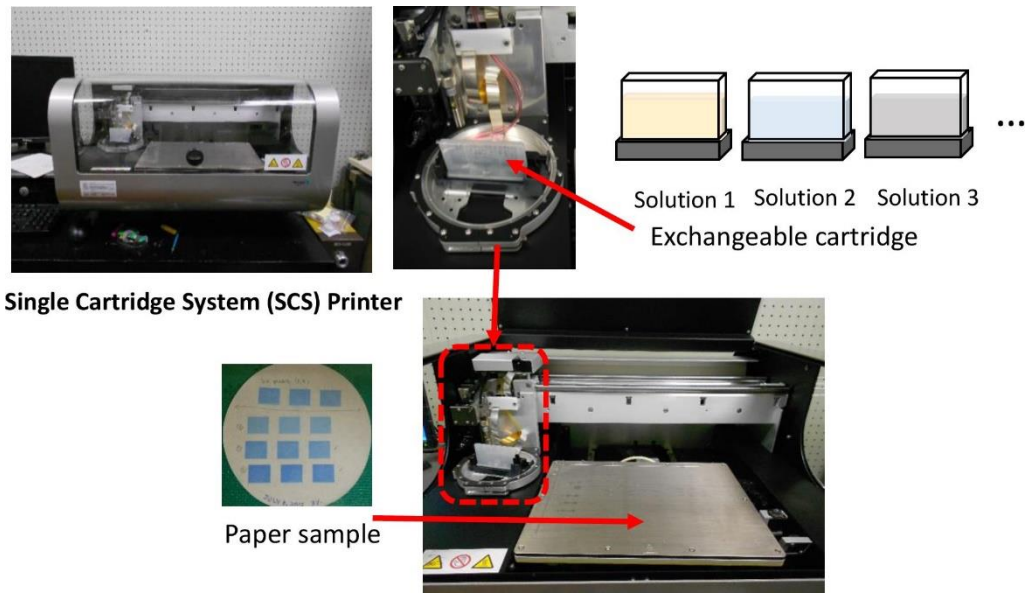

Fig. 3.7 Medium printing by SCS printer sequence.

3.3.6.2 MCS printer

The modified inkjet printer was used (see Chapter 3, 3.4.5). To load hydrogel medium materials, the sponge in the ink cartridge was removed and the cartridge

was washed with distilled water. To sterilize the cartridge for subsequent bacterial culture tests, the cartridge was sprayed with aqueous ethanol (70%).

The MCS printer for color printing can be equipped with up to 4 different cartridges loaded with 4 different kinds of inks so that all solutions composing a PVA/ADH mixture could be printed at the same time. In addition, the ejected amount of each solution was controlled by specifying CMYK percentages as shown in Fig. 3.8. The photographs of the modified MCS printer are shown in Fig. 3.9.

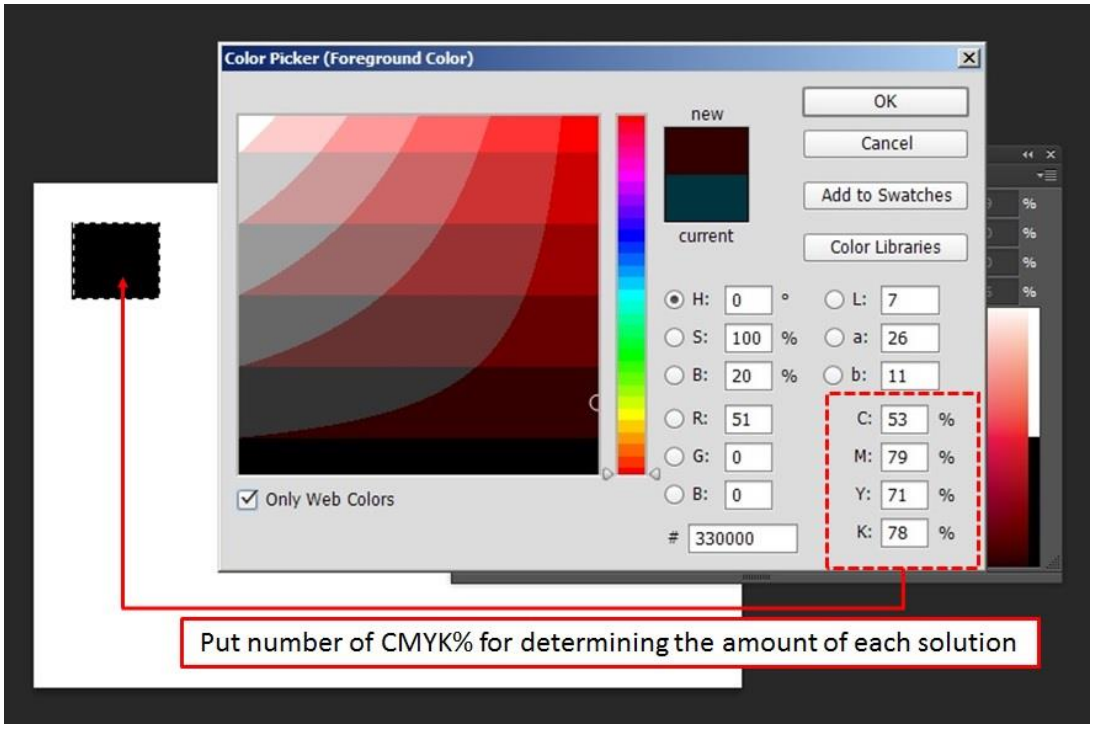

Fig. 3.8 Amounts of each solution determined by percentages of CMYK; Cyan = NaCl, Magenta = Yeast extract, Yellow = Bacto trypton, Black(K) = PVA/ADH mixture.

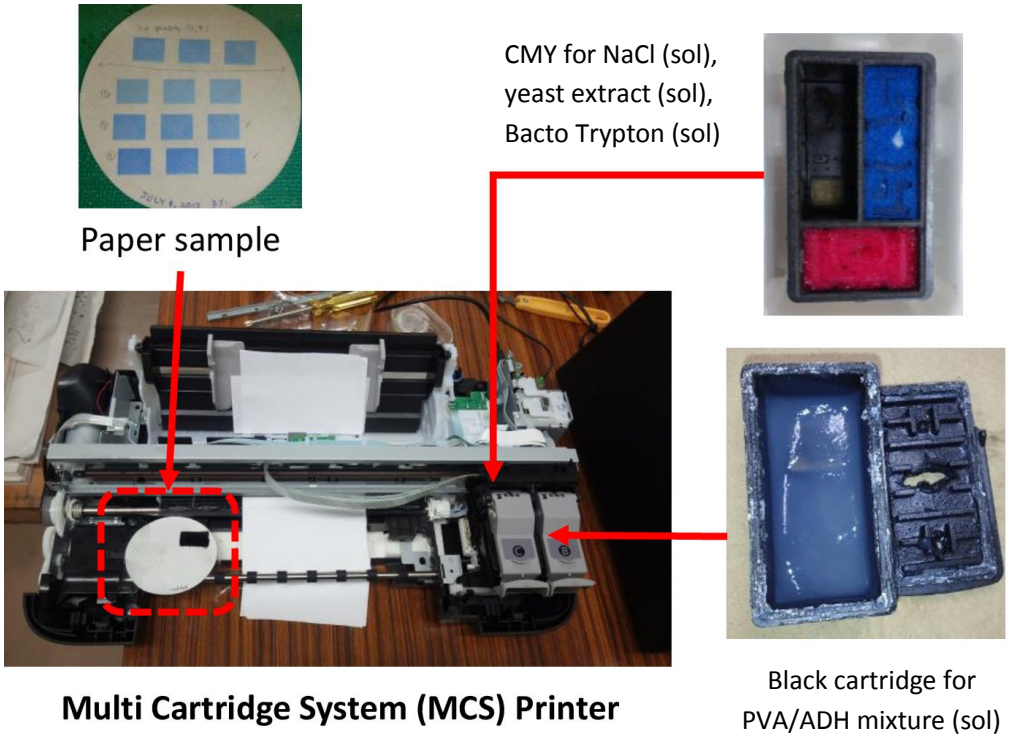

Fig. 3.9 Processing of MCS printer and procedure of medium printing.

3.3.7 Bacterial culture

In Chapter 2, *Escherichia coli* (*E. coli*) was tested as an example of bacteria for the printability on a SCS printer (See Chapter 2, 2.4.3). It could be printed successfully and a high survival ratio was obtained. Therefore, in this work, firstly, bacteria were taken randomly from a stationery surface and isolated for pure culture by the streak plate technique. Secondly, the morphology of the bacterial colonies was classified (see Chapter 2, 2.3.3). The concentration of the bacterial suspension was diluted to 2.7×10^7 colony-forming units (CFU)/mL using the serial dilution method. Then, 0.5 mL of the diluted bacteria was mixed with 1 mL of glycerol and loaded into a cartridge of the SCS printer. First, we printed 16 areas of PVA hydrogel on a sheet of hydrophobic/hydrophilic paper. Next, the bacterial suspension was printed on the top of the PVA hydrogel media. In total, we prepared 5 sheets containing 16 areas of bacteria. After 24 h, the average number of grown colonies per area was determined by optical microscopy. The survival rate is defined as the proportion of the number of bacterial growth areas to the total number of areas. In the same way, the contamination rate was defined as the proportion of the number of contaminated areas to the total number of areas.

3.4 Results and discussion

3.4.1 Characteristics and optimization of PVA hydrogel

Figure 3.10 shows 2 examples of the force curves used to determine the gel strengths of the hydrogels. The sensed force attained a maximum value at a depth of 10 mm for every curve. The gel strengths (maximum force values) are plotted in the ternary phase diagram as shown in Fig. 3.11 as a function of dry mass percentages of PVA and ADH, with water content in percentage of the mixture. The ratios of the three components comprising the gel were distributed as shown in the shaded area in Fig. 3.11. Some PVA/ADH mixtures gelled by themselves and had various gel strengths. No force (practically less than 1 N) was detected for the others (no hydrogel formation identified). The gel strength depended mainly on the dry mass percentages of PVA. However, when the concentration of ADH exceeds 0.75% dry mass, gelation was prevented for any ratio of PVA/ADH/H_2O mixture. Also the percentage of PVA was reduced to less than 4.50%, hydrogel could not form. In contrast, 25% or more PVA produced hydrogel without the addition of a cross-linking agent and cannot be handled as an ink.

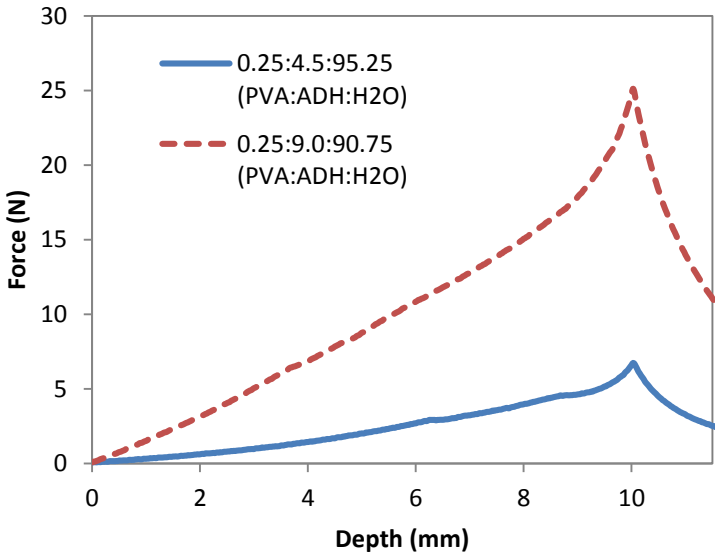

Fig 3.10 Force curves of the cylindrical plunger sensor in intruding PVA hydrogel over time down to 10 mm in depth.

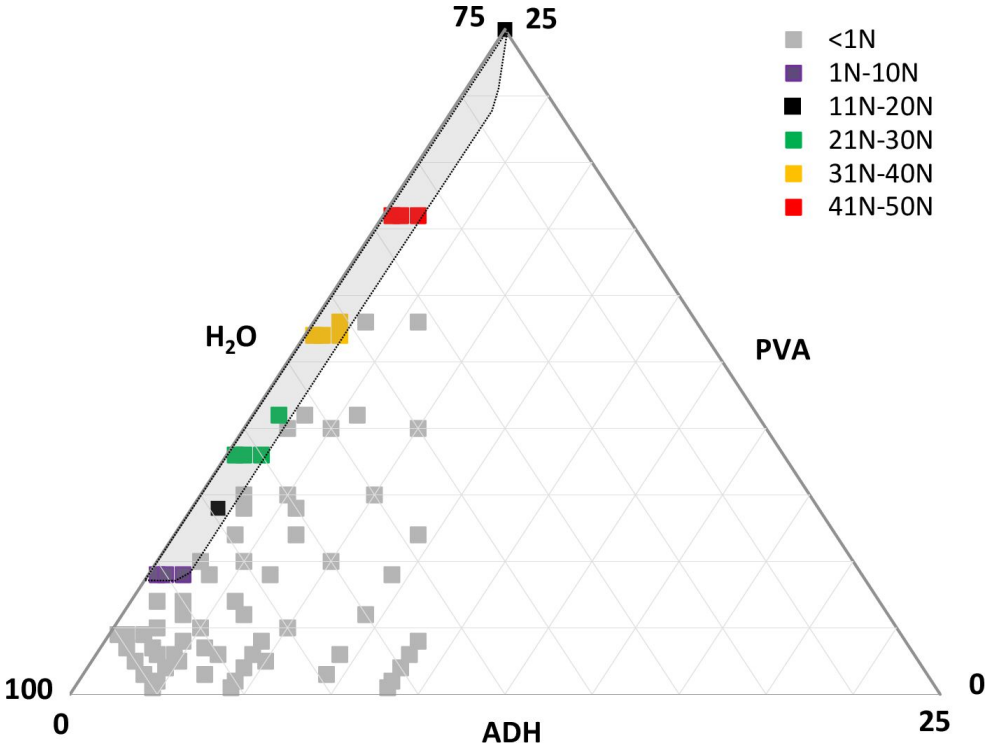

Fig. 3.11 Ternary phase diagram of gel strength as a function of the ratios of the 3 components composing PVA hydrogel and ratios of gelation was observed in the shaded area.

Moreover, increasing the dry mass percentages of PVA also affected the gelation time, as shown in Fig. 3.12.

Practically, strong gel is more suitable for cultivation than weak gel. We found that the gel strength of PVA hydrogel in period of 31N-40N and 41N-50N were strong gel or irreversible gel. However, to identify the optimal concentrations of PVA and ADH suitable for inkjet printing, we examined all PVA/ADH mixtures in the shaded area in the ternary phase diagram with respect to their printing capabilities.

The mixture has to be chosen such that it gels as fast as possible on paper while also remaining stable in solution long enough to be stored in the printer cartridge during the preparation process. Consequently, we identified 4.5% dry mass PVA and 0.5% ADH as the best combination with respect to printability and quality of the printed patterns. Even if this condition provided the weak gel but it allowed to keep gel for at least 2 days before which

was long enough for the rapid bacterial culture system. It has to be noted that even at PVA dry mass percentages higher than 13.5%, the mixture gelled with an appropriate gelation time; however, the viscosity was too high for inkjet printing.

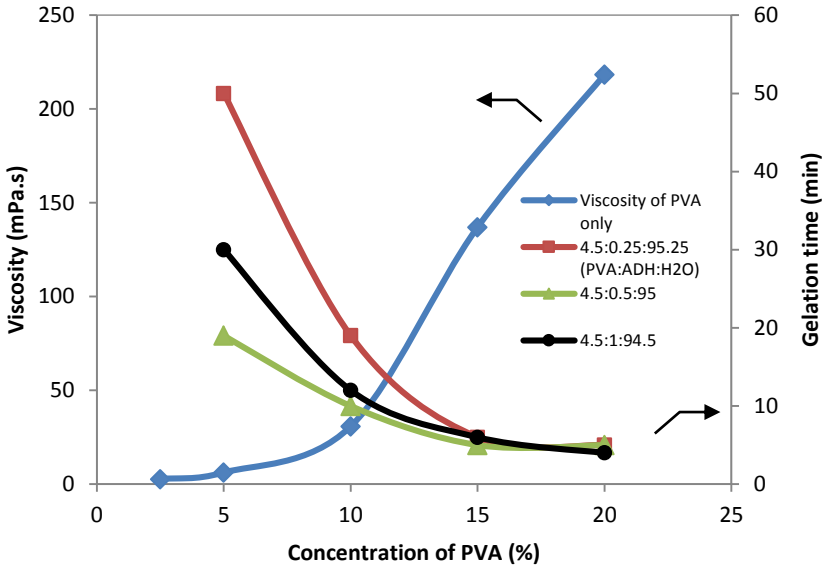

Fig. 3.12 Viscosity of PVA solutions and gelation time of the PVA hydrogel at three concentrations (PVA:ADH:H_2O).

3.4.2 UV absorption spectra and X-ray diffraction pattern of PVA hydrogel

Figure 3.13 shows the UV absorption spectra of the PVA solution, ADH solution, and PVA/ADH mixtures. Four spectra of the mixtures were measured at each stage of the transition from solution to full gelation for the identical sample. Some studies have shown that hydrogen bonding can affect UV absorption [19, 20]. In the UV spectra, a shoulder peak appeared for the PVA/ADH mixture around 240 nm and could be assigned to the hydrogel structure. The shoulder peak height continued to increase with time, suggesting continuous gelation. Between 15 min and 20 min in elapsed time, the difference in the spectra was slight. This behavior is likely to correspond to the approach to the complete gelation. Although the curve of the ADH solution exhibited much higher UV absorption in the shorter wavelength band than PVA at the same concentration, there was no clear peak for quantifying ADH independent of the hydrogel.

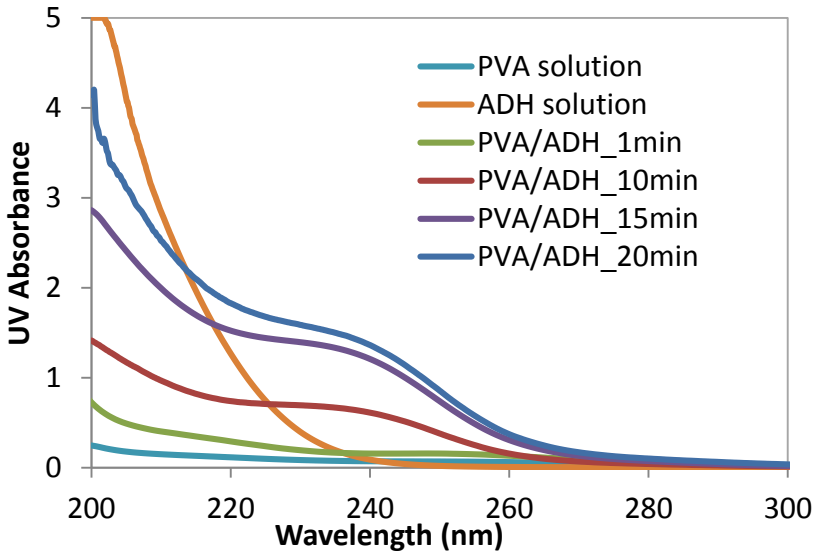

Fig. 3.13 UV absorbance of ADH, PVA, and the PVA/ADH mixtures with increasing degrees of gelation at 1, 10, 15, and 20 min (full gelation).

The change of the X-ray diffraction pattern of a PVA and ADH mixture with time is shown in Fig 3.14. All of the patterns were the same with that of pure water irrespective of elapsed time. It means that the PVA hydrogel does not have a specific crystal structure.

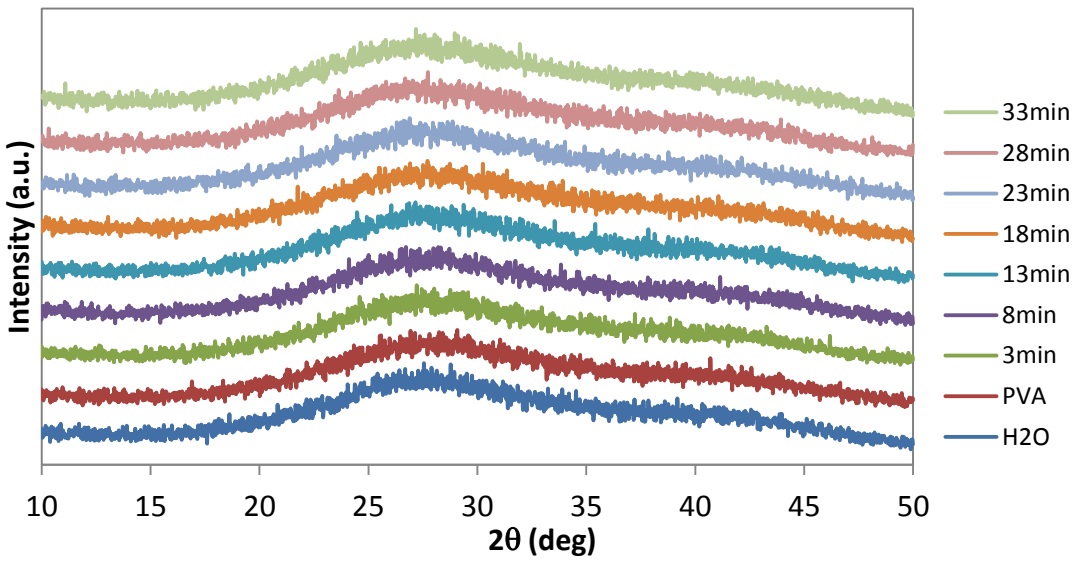

Fig. 3.14 Change of X-ray diffraction patterns of PVA/ADH mixture with time that elapsed since mixture.

3.4.3 Culture media printing

Media printing was tested by printing all solutions using both the SCS and MCS printers to evaluate the compatibility between the printers and solutions. The result in Table 3.1 shows that the maximum concentration which could be printed by the SCS printer is only 2.25% dry mass of PVA in PVA/ADH mixture. For the multi-cartridge MCS printer the range of printable mixture ratios is much wider. By means of the MCS printer we could successfully print 2.25%, 4.5%, and 9% dry mass of PVA in PVA/ADH mixtures. We found that the SCS printer was not suitable for PVA/ADH mixture printing. As described in the previous topic (see Chapter 3, 3.4.1), 4.5% dry mass PVA and 0.5% ADH with the MCS printer was identified as the best combination. It combines adequately low viscosity which ensures continuous ejection with no clogging and a relatively large amount of PVA per droplet for rapid gelling. Additionally, this mixture maintained stable in the solution state long enough to be stored in an ink cartridge.

Table 3.1 Printability and accuracy of SCS and MCS printers

Printability of solutions	**SCS**	**MCS**
PVA/ADH/H_2O mixture		
2.25:0.5:97.25	✓	✓
4.5:0.5:95	✕	✓
9:0.5:90.5	✕	✓
13.5:0.5:86	✕	✕
18:0.5:81.5	✕	✕
NaCl 0.05 g/L	✓	✓
Bacto trypton 0.1 g/L	✓	✓
Yeast extract 0.05 g/L	✓	✓
Accuracy error (%)	0.00	0.44

✓: successfully printed ✕: unable to eject

Using a camera installed near the head nozzles we found that the SCS printer has a high accuracy in positioning of the head nozzles which is important for overprinting the solution on the paper. However, the MCS printer is less time-consuming because all solutions

are printed at the same time and, among others, the composition of all inks can be altered very easily by changing CMYK percentages from area to area on the same paper substrate.

3.4.4 Confirmation of bacterial growth on PVA hydrogel medium

For PVA hydrogel medium the *E. coli* growth on the paper was confirmed 24 h after inoculation. The counted number of colonies is listed in Table 3.2. The low deviation suggests that liquids containing *E. coli* cells could be dispensed evenly and regularly onto a culture medium. Figure 3.15 shows growth of colonies 6 h after inoculation.

Table 3.2 Survival rate of *E. coli* on PVA hydrogel media after 24 h.

	PVA hydrogel
Number of areas measured	80
Mean number of colonies per area	6.78
95% Confidence intervals of number of colonies per area	0.27
Survival rate (%)	67.1
Contamination rate (%)	26.3

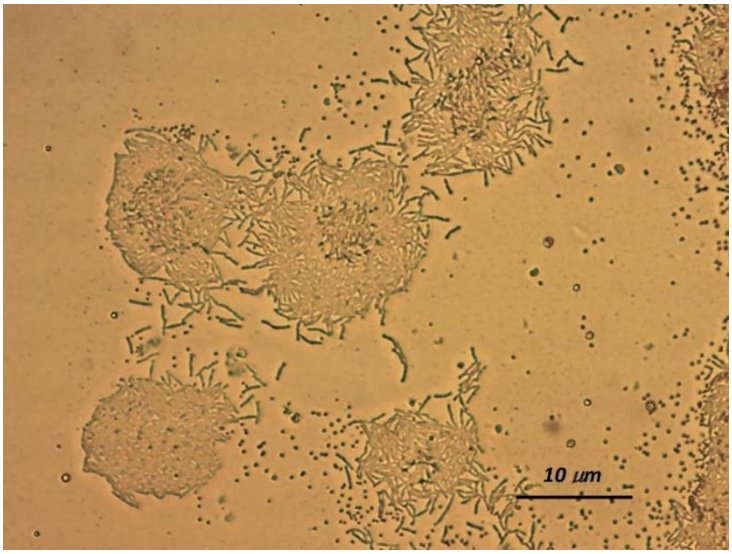

Fig. 3.15 *E. coli* colonies growing on PVA hydrogel medium after 6 h.

The results showed that PVA could be printed by using SCS and MCS printers on paper. In addition, living *E. coli* cells could be printed and survived the printing process.

Both printing systems have their own advantages and can be used complementary depending on the desired application. The results showed that the SCS printer has higher accuracy in positioning of the printing location and dispensing stable amounts of inks. On the other hand, the MCS printer is less time-consuming and it is easier to achieve of various compositions of medium components by means of simple CMYK color adjustment. Summarizing, the system we developed in the present work provides a low-cost, easy-to-use, efficient means for bacterial culture. Essentially we modified an inkjet printer to function as a dispensing device and clean bench and paper was used as a container for the culture media.

3.5 Conclusions

In this study, we combined inkjet printing technology and paper to create an automated, simple bacterial culture system. PVA hydrogel, formed with physical crosslinking by mixing PVA and ADH aqueous solutions, was applied as a culture medium. This mixture exhibited a viscosity that was low enough for successful ejection by inkjet printing in the solution state. The mixture gradually gelled over time and became a rigid gel in about 20 min after mixing when the mixture ratio was 4.5:0.5:95 (PVA:ADH:H_2O). This gelation time was appropriate for handling during the protocol for inkjet printing. Measurement of the UV spectrum of the PVA hydrogel showed a peak at approximately 240 nm that was not observed in that of PVA or ADH; this peak was therefore ascribed to the hydrogel structure. A PVA/ADH mixture in the solution state was loaded in an inkjet cartridge and successfully printed and gelled on paper. Finally, bacterial growth was confirmed on the PVA hydrogel medium. In summary, we showed that PVA hydrogels were an easy-to-use medium for inkjet printing.

3.6. References

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Chapter 4

Application of inkjet-printed Calcium alginate (CA) hydrogel to bacterial culture media

4.1 Abstract we attempted to create a stable system for dispensing bacteria microscopically by using inkjet technology on paper to improve the conventional bacterial culture system conducted by uncontrollable human operation. To use paper as a container supporting a culture medium, a hydrophobic/hydrophilic pattern was built by immersing it in a toluene solution of polystyrene for hydrophobic of the whole sheet and printing toluene for etching hydrophilic areas on the paper. Moreover, sodium alginate and calcium chloride solution were printed to form hydrogel as a culture media. Then, bacterial cells were subsequently printed on media on hydrophilic areas, and microscopically observed. The cells were observed to be stably arranged, and the subsequent growth of them was successfully achieved.

4.2 Introduction

Presently, inkjet technology is promises and challenges for applying to biology field because of its advantages such as resource savings and non-contact processes attained by the drop on demand (DOD) system [1]. Various kinds of research have been presented about printing technology that has gradually become a versatile tool for accurately dispensing very small quantities of fluids on substrates, such as bacterial communities [2], heterogeneous tissue construction [3], protein and cell printing [4], organ 3D printing design [5-7], tissue engineering development [8-9], cell molecular self-assembly [10], DNA [11-13], hybrid cell [14], even or membranes [15]. Also, in this research, inkjet printer was used as a dispensing machine for developing bacterial culture system.

Conventionally bacterial culture systems have done by using agarose gel as a container of nutrients. Agarose consists of many polysaccharide chains. In Chapter 2, an inkjet printer was applied to dispensing both of agarose media and bacterial suspensions on paper substrates aiming at stable patterns and accurate amounts of bacterial cells. In case of pathogenic bacteria, excess bacteria are considered as human-caused hazards but it probably was decreasing because inkjet printers could dispense tiny amounts of bacterial suspensions. However, agar solidifies on cooling; therefore, the temperature must be maintained at approximately 37°C in preparation for printing. This requisite was too severe for inkjet printing practices. Therefore, agarose was hydrolyzed by using sulfuric acid to reduce the degree of polymerization. After hydrolysis, the agarose became acidic and was considered to inhibit bacterial growth. To avoid this problem, the pH was adjusted to neutral using sodium hydroxide, and the hydrolyzed agarose was successfully ejected by the inkjet printer. However, the hydrolysis processes took a long time, and the viscosity adjustment required precise control. In Chapter 3, we applied PVA hydrogel as a culture medium and the result showed that PVA hydrogel could be used for bacterial culture and suit to inkjet printer. Here, we present another type of hydrogel called Calcium alginate (CA) hydrogel.

Polysaccharide-based hydrogels are useful for numerous applications, from food and cosmetic processing to drug delivery and tissue engineering. The formation of hydrogels from polyelectrolyte solutions is complex, involving a variety of molecular interactions. The physical gelation of polysaccharides can be achieved by balancing solvophobic and solvophilic interactions. Polymer chain reorganization can be obtained by solvent exchange, one of the processing routes forming a simple hydrogel assembly [16].

Alginic acid or alginate, which is an anionic polysaccharide consisting of homopolymeric blocks of β-D-mannuronate and α-L-guluronate residues derived from cell walls of brown algae, forms a gel in the presence of calcium and dissolves to a solution upon addition of a calcium chelating agent [17].

Sodium alginate also is an anionic polysaccharide distributed widely in the cell walls of brown algae which is one of nature polyelectrolytes. Sodium alginate is used to gel in presence of calcium, as shear-thinning thickener in absence of calcium, to stabilize emulsions or foams and to form films [18].

Calcium ions as a crosslinking agent diffuse from a bulk solution into flocculated sodium alginate (SA) [17]. The hydrogel forms by chemical reaction, the calcium ions displace sodium ions from the alginate, holding the long alginate molecules together such that a gel formed (Fig. 4.1). Unlike agarose, the PVA/ADH mixture and the SA/Calcium chloride ($CaCl_2$) components remain under ambient condition long enough in a solution state to be processed by printing. Several studies pointed out the benefits of sodium alginate to bacterial cells and tissue culture, such as increasing the survival of bacteria [19], enhancing the bacterial yield [20], and proliferation [21].

In this research, we used sodium alginate solution and calcium chloride ($CaCl_2$) solution to produce gel by using inkjet printer for bacterial culturing on paper. Especially, a wide range of choice of culture medium would make this bacterial culture system more flexible and versatile.

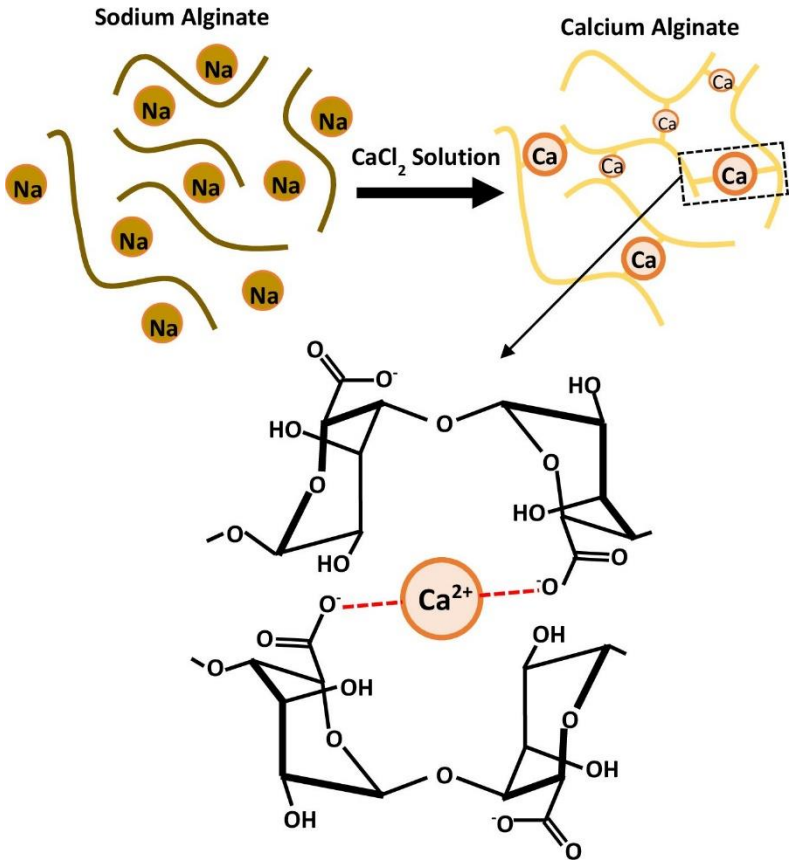

Fig. 4.1 Reaction of SA and $CaCl_2$.

4.3 Materials and methods

4.3.1 Preparation of paper substrates

The process of hydrophobic/hydrophilic paper fabrication was mentioned in Chapter 3, 3.3.4. Figure 4.2 shows the picture of water droplet on hydrophobic paper. The 2 μL of water was dropped on hydrophobic paper to examine hydrophobic level.

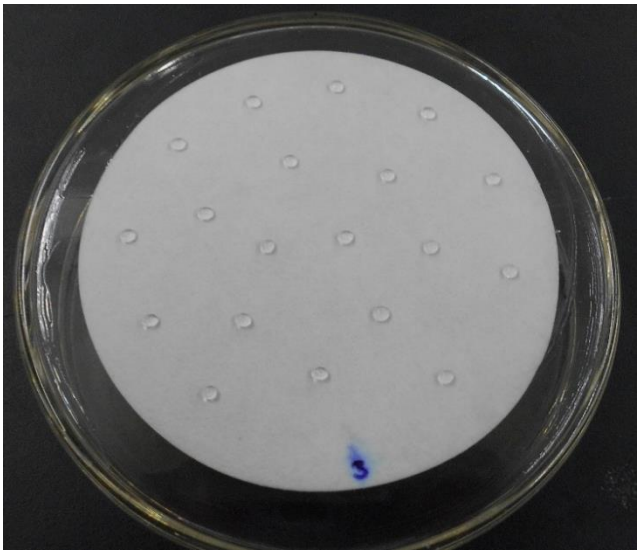

Fig. 4.2 Drops of water on hydrophobic filter paper.

4.3.2 Preparation of CA hydrogel ink

Usually, media are prepared by mixing a SA solution, 0.05% (w/w) of $CaCl_2$ solution, 0.05 g/L of yeast extract solution and 0.1 g/L of bacto tryptone solution in required ratios, where the unit g/L means a solute in g dissolved in 1 L of an aqueous solution. The ratio was controlled based on the amount ejected from each cartridge. However, the inkjet ejection performance is mainly determined by the ink viscosity [16]. In particular, SA solutions have higher viscosities than the other solutions and the viscosity dependents on the concentration. Therefore, the SA solutions at several concentrations (0.01%, 0.025%, 0.05%, and 0.1 % (w/w)) were prepared and their viscosities were measured with a glass capillary viscometer. The gelation of CA hydrogel was formed by an irreversible reaction. However, in order to provide CA hydrogel without

remaining initial substances, the ratio of SA and $CaCl_2$ solutions was examined by measuring pH values. In the experiment, the pH values of SA and $CaCl_2$ solutions were 8.0 and 7.0, respectively. After CA hydrogel was formed, the pH value of a remaining solution was examined (Fig 4.3). Then the ratio without remaining initial substances was adjusted.

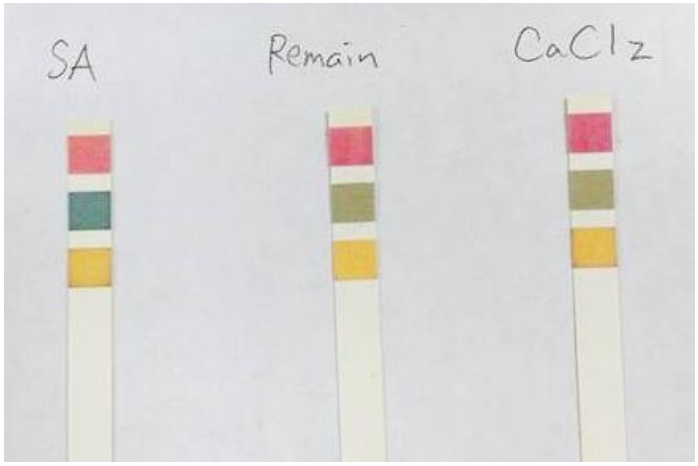

Fig. 4.3 The example of pH value of a remaining solution test.

4.3.3 Culture media printing

As mentioned in Chapter 3, We used 2 different kinds of inkjet printers as dispensing tools, namely a SCS (Dimatix DMP-2831, Fujifilm, Japan) and MCS (iP2000, Canon, Japan) Subsequently, the efficiency of the 2 systems was compared in terms of the compatibility with the hydrogel culture media and length of time required for one complete printing operation.

4.3.3.1 SCS printer

Usually, the medium was prepared from mixture of SA solution, $CaCl_2$ solution, yeast extract solution and bacto tryptone solution. However, the viscosity of sodium alginate solution was dependent on the concentration. Thus, SA solutions at several concentrations (0.1%, 0.25%, 0.5%, and 1.0 % (w/w)) were prepared for examining the optimum concentration for inkjet printer. Because the SCS printer allows setting only one cartridge, all solutions were loaded into each cartridge and printed one by one respectively. The methods for

the SCS printer are shown in Fig. 3.7 (see Chapter 3, 3.4.6.1). The printing condition was 40 V in ejection voltage, 16 head nozzles, 50 μm in drop spacing, and at least 10 times overprinting.

4.3.3.2 MCS printer

SA solutions at several concentrations were prepared in the same way as for SCS printer. The modified MCS inkjet printer (see Chapter 3, 3.4.5) was used to print all solutions. Also the amount of each solution could be controlled by CMYK (Cyan = $CaCl_2$, Magenta = Yeast extract, Yellow = Bacto trypton, Black(K) = SA solution) percentage.

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4.3.4 Bacterial culture

Preparation of bacterial suspension was mentioned in Chapter 2, 2.3.3. After 0.5 mL of the diluted bacteria was mixed with 1 mL of glycerol and loaded into a cartridge of the SCS printer, we printed 16 areas of CA hydrogel on a sheet of hydrophobic/hydrophilic paper. Next, the bacterial suspension was printed on the top of the CA hydrogel media. In total, we prepared 5 sheets containing 16 areas of bacteria. After 24 h, the average number of grown colonies per area was determined by optical microscopy. The survival rate is defined as the proportion of the number of bacterial growth areas to the total number of areas. In the same way, the contamination rate was defined as the proportion of the number of contaminated areas to the total number of areas. In this chapter we compared the survival rate and contamination rate with PVA hydrogel.

4.4 Results and discussion

4.4.1 Hydrophilic/Hydrophobic Patterning

After hydrophobic/hydrophilic pattern was checked by dropping water, the results show that 20 min printing method including 3 head nozzles and overprinting once was chosen for optimum hydrophobic/hydrophilic patterning (Fig. 4.4).

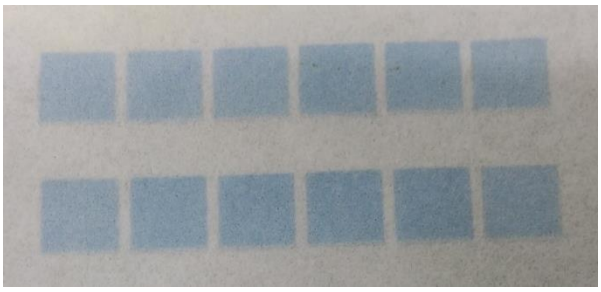

Fig. 4.4 Hydrophobic/hydrophilic patterning.

4.4.2 CA hydrogel medium

The viscosity of the sodium alginate solutions was examined for an optimum viscosity level for the printers. The determined dependence of the viscosity on the concentration is shown in Fig. 4.5.

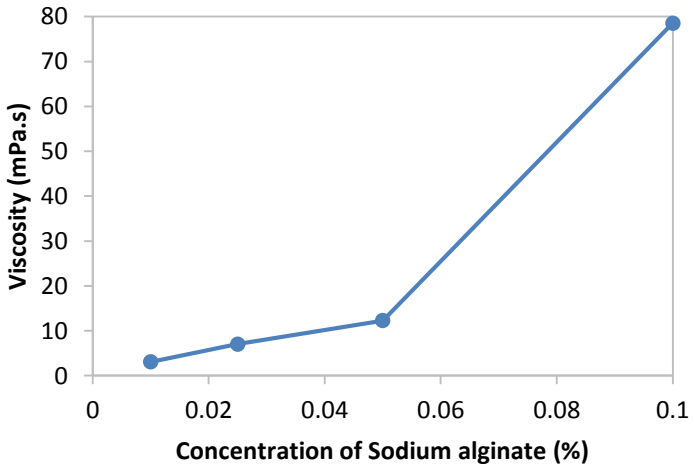

Fig. 4.5 Viscosity of SA solutions at various concentrations.

4.4.3 Culture media printing

Media printing was tested by printing all solutions using both the SCS and MCS printers to evaluate the compatibility between the printers and solutions. The result in table 4.1 shows that all components could be printed by SCS and MCS printers. High concentrations of SA solutions could not be printed by SCS. Considering the viscosities of PVA and SA solutions, SCS is likely to allow to print inks with viscosities of lower than 10 mPa·s. A 0.025% (w/v) of SA solution was chosen as the ink for loading. Other solutions for making CA hydrogel were found to be all printable. To adjust the mixture ratio of all the components to follow the standard CA medium composition such as a suitable mass ratio of 5:6 (SA:$CaCl_2$), the ejection percentage of each color cartridge called CMYK for MCS was decided to be 50% of SA, 30% of $CaCl_2$, 15% of Bacto trypton, and 5% of Yeast extract. The CA hydrogel media printed on a filter paper substrate is shown in Fig. 4.6.

Table 4.1 Printability, time required per sample and accuracy of SCS and MCS printers

Printability of solutions		SCS	MCS
	SA 0.1%	✓	✓
	SA 0.25%	✓	✓
	SA 0.5%	✕	✓
	SA 1.0%	✕	✓
	$CaCl_2$ 0.5%	✓	✓
	Bacto trypton	✓	✓
	Yeast extract	✓	✓
Time required per sample		SCS	MCS
	Average (min)	120	40
	Accuracy error (%)	0.00	0.44

✓: successfully printed ✕: unable to eject

Using a camera installed near the head nozzles we found that the SCS printer has a high accuracy in positioning of the head nozzles which is important for overprinting the solution on

the paper. However, the MCS printer is less time-consuming because all solutions are printed at the same time and, among others, the composition of all inks can be altered very easily by changing CMYK percentages from area to area on the same paper substrate.

Fig. 4.6. CA hydrogel on hydrophilic areas on hydrophilic/hydrophobic patterned paper.

4.4.4 Bacterial growth on the PVA hydrogel and CA hydrogel media

For both PVA hydrogel medium and CA hydrogel medium the *E. coli* growth on the paper was confirmed 24 h after inoculation. The counted number of colonies is listed in Table 4.2. The average number (95% confidence intervals) of colonies per hydrophilic area was found to be comparable for both hydrogels, with a slightly higher number for PVA hydrogel. This low deviation suggests that liquids containing *E. coli* cells could be dispensed evenly and regularly onto a culture medium. *E. coli* cells on CA hydrogel media are shown in Fig. 4.7.

Table 4.2. Survival rate of *E. coli* on PVA and CA hydrogel media after 24 h

	PVA hydrogel	**CA hydrogel**
Number of areas measured	80	80
Mean number of colonies per area	6.78	5.73
95% Confidence intervals of number of colonies per area	0.27	0.22
Survival rate (%)	67.1	90.4
Contamination rate (%)	26.3	7. 7

The results showed that PVA and CA hydrogels could be printed by using SCS and MCS printers on paper. In addition, living *E. coli* cells could be printed and survived the printing process. SA is a polysaccharide which is indigestible for most organisms so that microbial growth does not affect the gel used, meaning that CA hydrogel media maintain the stable quality [19]. The survival rate of the CA hydrogel was higher than that of the PVA hydrogel which exhibits an acidic pH [22,23]. In our experiment, the pH of the PVA hydrogel was 6.0 and for the CA hydrogel the pH was 7.0.

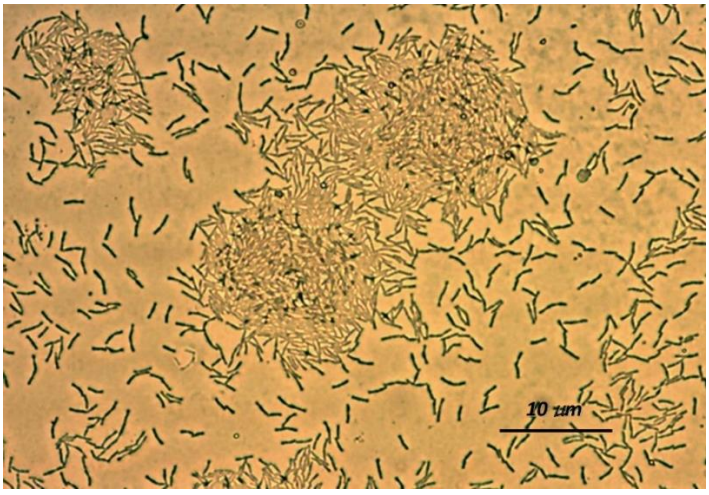

Fig. 4.7 *E. coli* colonies growing on CA medium after 6 h.

Both printing systems have their own advantages and can be used complementary depending on the desired application. The results showed that the SCS printer has higher accuracy in positioning of the printing location and dispensing stable amounts of inks. On the other hand, the MCS printer is less time-consuming and it is easier to achieve of various compositions of medium components by means of simple CMYK color adjustment. Summarizing, the system we developed in the present work provides a low-cost, easy-to-use, efficient means for bacterial culture. Essentially we modified an inkjet printer to function as a dispensing device and clean bench and paper was used as a container for the culture media. An actual productive unit based on the techniques developed can be realized as sketched in Fig. 1.6 (see Chapter 1, 1.4) which shows the possible schematics of a future system for efficient bioassay.

4.5 Conclusions

In this chapter, we combined inkjet printing and paper technologies for creating a prototype of an automated and simple to operate bacterial culture system. CA hydrogel, formed by chemical reaction between SA solution and $CaCl_2$ solution. We used two different kind of printers, namely a single cartridge SCS printer and a multi-cartridge MCS printer to dispense the culture media and bacterial suspension. The SCS printer showed on the expense of processing speed a high accuracy in positioning of the printing location and provided an accurate overprinting. On the other hand, the MCS printer had a larger margin of accuracy error in the time sequence; however, it achieved fast printing at low cost simply by outputting CMYK values each based on the demanded amounts of a SA solution, $CaCl_2$ solution, and nutrients. In addition, the SCS printer allows a very accurate inoculation of the bacteria onto the medium using accurate overprinting. The CA hydrogel medium had a higher survival rate of bacteria and lower contamination rate compared to PVA hydrogel medium. In summary, we demonstrated that PVA hydrogel and CA hydrogel were an easy-to-use medium for inkjet printing.

4.6 References

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Chapter 5

Overall Conclusion

In this study, we present a new method to create a well-ordered and reproducible system for dispensing bacteria on a microscopic scale by using advantages of paper and inkjet technology. We used paper as a media platform for bacterial culture and inkjet printing technology to dispense small amounts and for manageable patterning of bacterial suspension.

In Chapter 2, we simulated bacterial culture system on hydrophilic/hydrophobic paper by using inkjet printer as a dispensing method.

The results are as follows:

1) The hydrophilic/hydrophobic patterning provided a paper substrate suitable for accommodating an inkjet-printed culture medium separately.
2) Hydrolysis was applied to agarose to adjust the viscosity, making it conducive to printing on paper.
3) Bacterial growth on hydrolyzed agarose was confirmed.
4) In a separate experiment, *E. coli* cells were printed on a sheet of agarose medium and the growth of colonies with high survival rates were confirmed.
5) We confirmed the regularity of the number of ejected cells in 1 droplet with latex particles as a cell suspension model.

In Chapter 3, because the hydrolysis processes of agarose took a long time, and the viscosity adjustment required precise control. Therefore, here we introduce poly(vinyl alcohol) (PVA) hydrogel as a suitable alternative for agarose. PVA combines the advantages of agarose with a higher compatibility for inkjet printing.

The results are as follows:

1) The PVA hydrogel was applied to be a container of nutrient for bacterial culture.
2) PVA hydrogel, formed by physical crosslinking.
3) The PVA/ADH mixture exhibited a viscosity that was low enough for successful ejection by inkjet printing in the solution state.
4) The mixture gradually gelled over time and became a rigid gel in about 20 min after mixing when the mixture ratio was 4.5:0.5:95 (PVA:ADH:H_2O).
5) The gelation time about 20 min was appropriate for handling during the protocol for inkjet printing.
6) Measurement of the UV spectrum of the PVA hydrogel showed a peak at approximately 240 nm that was not observed in that of PVA or ADH; this peak was therefore ascribed to the hydrogel structure.
7) Measurement of the XRD of PVA hydrogel showed that the PVA hydrogel does not have a specific crystal structure.
8) A PVA/ADH mixture in the solution state was successfully printed and gelled on paper.
9) Bacterial growth was confirmed on the PVA hydrogel medium.

In Chapter 4, we used sodium alginate (SA) solution and calcium chloride ($CaCl_2$) solution to produce Calcium alginate hydrogel and applied to bacterial culture media. SA solution and $CaCl_2$ solution was successfully printed on paper. Especially, a wide range of choice of culture media would make this bacterial culture system more flexible and versatile.

The results are as follows:

1) SA and $CaCl_2$ solutions were used to be the initial substances to produce CA hydrogel which is formed by chemical crosslinking.
2) To adjust the mixture ratio of all the components to follow the standard CA medium composition such as a suitable mass ratio of 5:6 (SA:$CaCl_2$), the ejection percentage

of each color cartridge called CMYK for MCS was decided to be 50% of SA, 30% of $CaCl_2$, 15% of Bacto trypton, and 5% of Yeast extract.

3) SA and $CaCl_2$ solutions could be printed efficiently by inkjet printer.
4) Consequently, bacterial growth on alginate gel was confirmed.
5) Compared to the other two hydrogel systems, alginate gel was considered to be a promising choice for media printing because of an optimum adequacy in handling and printability.

The reported experiments in this thesis are the first step to develop an efficient bacterial culture system with stability and accuracy. For practical and industrial application, instrumentation with suitable inkjet head nozzles and a sterilization system, and laboratory testing with various bacteria would be required and create the novel bacterial culture system.

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