

Bacterial Bioprinting on a Flexible Substrate for Fabrication of a Colorimetric Temperature Indicator by Using a Commercial Inkjet Printer

Abstract

Background: Bacterial sensors are recommended for medical sciences, pharmaceutical industries, food industries, and environmental monitoring due to low cost, high sensitivity, and appropriate response time. There are some advantages of using bacterial spores instead of bacteria in vegetative forms as spores remain alive without any nutrient for a long time and change to vegetative form when a suitable environment is provided for them. **Methods:** For biosensor fabrication, it is important to define how the bacterial spores are delivered to the substrate media. The main purpose of this paper is an investigation of transferring bacterial spores on a flexible substrate media using a commercial inkjet printer (HP Deskjet 1510). It should be noted that in the previous researches, the special printers were used to transfer bacteria on rigid films. **Results:** These printed bacterial spores are used as a colorimetric temperature indicator. The custom-made bio-inks are prepared by bacterial spores along with a gelling agent and pH indicator. **Conclusions:** Finally, transformation of bacterial spores into vegetative bacteria is occurred by changing of temperature. A color change in the bio-prints is demonstrated because the bacterial transformation and growth change the environmental pH to an acidic level.

Keywords: Bacterial spores, biosensor, colorimetric indicator, flexible substrate, printer

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Introduction

Printing techniques enable easy deposition of different kinds of active and functional substances in patterns with a specific size and shape. Properties such as accessibility and dissolution rate of these bioactive patterns can be modified by changing these factors. Inkjet printing has been seen as one of the most promising methods of transferring bioactive materials to carrier substrates such as paper. It has several advantages, such as the possibility to exactly control the amount of transferred material, and it is a noncontact printing method that the printing equipment does not come into contact with the printing substrate. Therefore, inkjet printing does not make any harsh demands on the quality of the printing substrate and all kinds of substrates can be used, ranging from smooth to very rough and even 3D surfaces^[1,2] In 2010, Cho *et al.* developed patterning methods based on the inkjet printing technique that employed bacteria and generated two-dimensional bacterial cell arrays on the agar media using a custom-made commercially available partially reformed

thermal inkjet printer. In that study, they aimed to apply the inkjet-printed bacterial cell array to biosensors.^[3] In 2015, Srimongkon *et al.* created an automated bioassay system based on inkjet printing. They printed polyvinyl alcohol and calcium alginate (CA) hydrogels on paper by using inkjet printer as the culture media and compared them to each other.^[4] Temperature markers are simple devices attached to the label of foods, which check and record the temperature history of the product. This system allows manufacturers, distributors, retailers, and consumers to check, at a glance, whether perishable foods have been correctly transported and stored, or experienced excessive temperature and time exposure, usually by the colorimetric method.^[5] During the past 30 years, numerous systems have been proposed, of which only a few reached the prototype, and even less have reached the market, usually due to cost and accuracy issues.^[6]

This project aims were to print bacterial spores and eventually produce microbial temperature indicator and propose this concept for different applications, especially in the medical and food industries. Here, a thermal inkjet printing is used as bacterial

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deposition system. Until recently, inkjet printing was used exclusively for printing documents. This technology can be used to print bio-inks-containing living cells using a few minor changes, such as removal of the ink sponge, washing the cartridge, and replacing its ink with bio-ink. Solution constituents should not harm the bacterial spores during the printing process.^[7] This printing method improves the quality as well as the processing speed compared to conventional deposition methods for bacteria. When the bacterial spores are exposed to the suitable environment, they react and start to grow and alter the acidity and consequently the color of the printed bio-ink that shows changes in ambient temperature. Finally, this method is a new diagnostic concept in the medical, pharmaceutical, and food industry that examines product spoilage by monitoring the temperature effect on bacterial growth.

Materials and Methods

Printer

A two-dimensional commercial inkjet printer (HP Deskjet 1510, USA) was applied for this study. This inkjet printer is designed for home and office applications that can easily be controlled and managed with a reasonable price. This device has two separate black-color (K) cartridges and tri-color inkjet cartridges including cyan (C), magenta (M), and yellow (Y) with the ability to refill the cartridges [Figure 1].

The printers use CMYK color system for printing. This system enables us to simply supply the amount of each solution and the cartridge activity by CMYK values in a user-friendly software.^[4]

The device is a thermal inkjet printer that a current pulse through the heating element leads to a temperature elevation and rapid vaporization of the ink in the chamber and formation a bubble, which causes a large pressure increase and propelling a droplet of ink onto the paper.^[8]

Preparation of the bio-ink

For each chamber of the ink cartridges, separate solutions of bacterial spores, culture medium, guar gum, and borax was prepared at room temperature as bio-inks. After this preparation, the CMYK cartridges were refilled with the bio-inks to enable the printer for bio printing, and the percentage of each of the solutions was controlled using Photoshop Software.

1. The bacterial spores – Two types of the bacteria spores, *Geobacillus stearothermophilus* (GBS) (PTCC 1713) and *Bacillus atrophaeus* (BA) (PTCC 1722), are obtained from Roshan Rai Sepahan Company and used in the bio-ink at a concentration of 10^7 CFU. These bacteria are Gram-positive and rod-shaped with a thick inner cell membrane and are highly resistant to heat and chemicals. This thermophilic bacterium that is a major cause of food spoilage can widely be found in

the soil, hot springs, and ocean sediments. The GBS and BA grow at 56°, and 37°, respectively, and these temperatures were used during the test to evaluate the growth and change in color of the printed samples.^[9] Polyvinylpyrrolidone solution (1% in deionized water) was used to prevent rapid bacterial deposition in the cartridges

2. Culture medium – tryptic soy broth (TSB) (Merck Millipore, USA) is used in microbiology laboratories as a broth medium to grow aerobic bacteria. The main components of TSB are the casein and tryptone soya that are dissolved under gentle heat and then was autoclaved for 15 min at 121°C. Bromocresol purple (Merck Millipore, USA) and bromothymol blue (Merck Millipore, USA) are two pH agents that were used in culture mediums for GBS and BA, respectively
3. Guar gum (Sigma Chemical Company, St. Louis, MO) – guar gum is an additive that is frequently used in the food, pharmaceutical, and makeup industries. One of the main tasks of guar gum is to give the higher viscosity to the solution. However, overall this material is used as an active ingredient in creating cohesion, consolidation, and parser in pharmaceuticals, foods, and cosmetics.^[10] Here, this is useful for trapping bacteria and culture medium in a higher thickness on the substrate. Without this, the substrate will have less bio-ink on its surface and therefore will evaporate rapidly. To increase culture medium content, the guar gumdrop on the printed bio-inks could manually added
4. Borax (Sigma, St. Louis, MO, USA) – Borax is used as a source of borate which uses the property complex of borate with other agents in water to form complex ions with various materials and generally is used as a gelling agent.^[11]

Photoshop software (Adobe Systems, USA)

This graphical software is used to draw printing pattern circles. Printing resolution was adjusted to 200 points per inch (PPI). In addition, CMYK color capability was applied to determine the amount of each bio-ink solution [Figure 2a and b].

Methods

The inkjet printer was applied to print bacteria on a flexible substrate such as polyvinyl chloride (PVC) film. For this purpose, as it is revealed in Figures 1 and 2, the cap of both cartridges was removed, and the cartridges were washed well with distilled water in an ultrasonic bath to completely drain the inside ink. Here, the custom-made bio-ink was used for printing instead of the printer inks. Instead of the cyan, magenta, yellow and black inks, the cartridge chambers were filled with the water-based bio-inks made of guar gum solutions (0.25%), borax (1%), culture medium, and bacterial spore solution, respectively. Amounts of each solution in printing is determined by percentages of CMYK using Photoshop Software. To ensure visibility of the

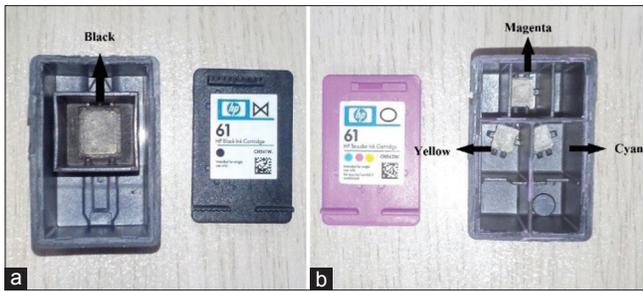


Figure 1: (a) Empty black cartridge after absorber sponge removal. (b) Empty tri-color cartridge with 3 chambers

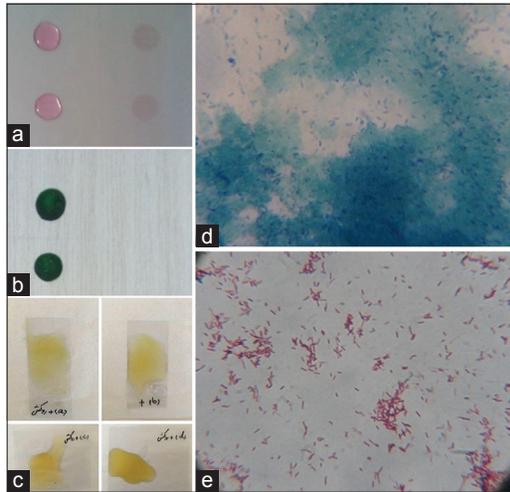


Figure 3: (a) Printed spores of *geobacillus stearothermophilus* and samples that covered with guar gum solution. (b) Printed spores of *Bacillus atrophaeus* and samples that covered with green gel. (c) Printed *Bacillus atrophaeus* on a flexible substrate after incubation in a suitable environment demonstrate a color change from green to yellow. (d) Light micrograph of a malachite green-stained smear of *Bacillus atrophaeus* cells showing greenish-blue spores. (e) Growth of printed *geobacillus stearothermophilus* is demonstrated by staining with Safranin e showing reddish vegetative bacteria

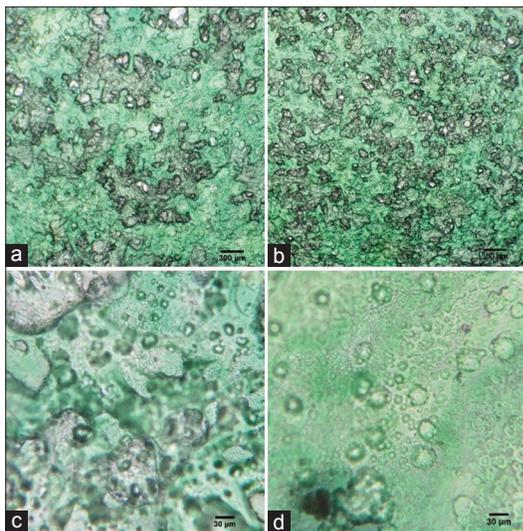


Figure 5: Images of coated *Bacillus atrophaeus* that printed on the polymeric substrate; (a) 100% borax (4x) (b) 0% borax (4x), (c) 100% borax (40x) (d) 0% borax (40x)

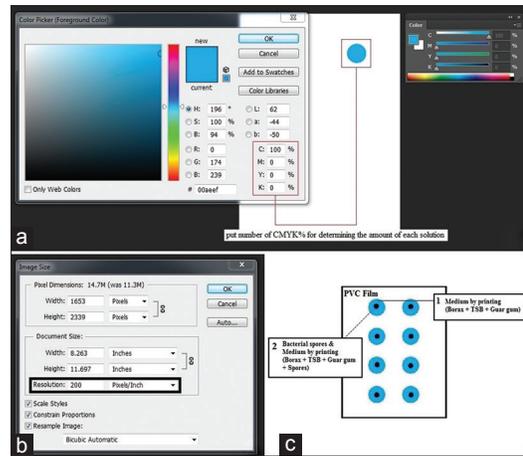


Figure 2: (a) Amounts of each solution determined by percentages of CMYK; Cyan: Guar gum 0.2%, Magenta: Borax 1%, Yellow: TSB, Black (K): Bacterial spores. (b) Adjust the print resolution to 200 PPI for printing circles on the substrate. (c) Schematic of two-step methods; PPI: Points per inch

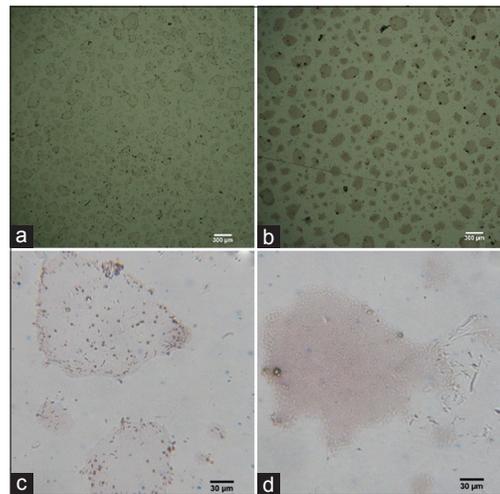


Figure 4: Images of *Bacillus atrophaeus* printed on the polymeric substrate; (a) 100% borax (4x) (b) 0% borax (4x), (c) 100% borax (40x) (d) 0% borax (40x)

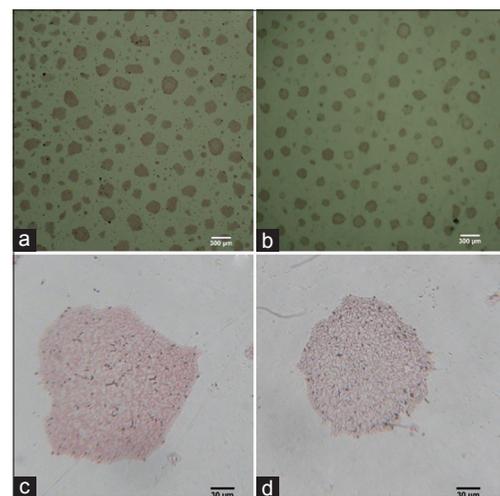


Figure 6: Images of printed *geobacillus stearothermophilus* on the polymeric substrate with 4x, and 40x magnifications; (a) 100% borax (4x) (b) 0% borax (4x), (c) 100% borax (40x) (d) 0% borax (40x)

printing, each solution was mixed with an edible food dye. The guar gum solution dyed with edible inks to the cyan, borax to magenta, culture media to yellow and bacterial spores to black. For guar gum and TSB printing, the maximum percentage in the software (100%) was chosen to confirm enough gelling agent and nutrient for bacterial growth, respectively.

The intended patterns were formed on the paper first, and after proving the feasibility of the process, the patterns were printed onto the PVC substrate. The circular pattern dimension was 50 × 50 pixels and had a resolution of 200 PPI [Figure 2c].^[12] Each pattern was printed in a 2-step process. First, a circle comprising of different concentrations of borax was printed. Then in the second step, different combinations of the compounds mentioned above in addition to spore was printed on the top of the underlying pattern [Table 1]. The top printed layer was providing and maintaining moisture that is necessary for bacterial growth and in addition since it is gelatinous, it can enhance the bacterial immobilization on the PVC film [Figure 3a and b].

Spore staining (Schaeffer–Fulton or Wirtz–Conklin)

To stain endospores, special stains and procedures should be used. Strongly resistant endospores to decolorization are the basis of the Schaeffer–Fulton or Wirtz–Conklin method. This method also differentiates the spores from sporangia and vegetative cells.

The cells and spores were obtained from the PVC film (printed BA and GBS on a flexible substrate). The samples were smeared with a drop of tap water on the clean glass slide and allowed to air-dry. Vegetative bacterial cell or the endospore was fixed onto the slide by passing slides through the Bunsen Burner flame 4–5 times. The slide was placed on the staining rack and overlaid with 5% aqueous malachite green (Fisher Scientific Co. Fair Lawn, NJ, USA) (5 g of malachite green oxalate was dissolved in 50 mL of deionized water, and stirred gently to prevent foaming, and filtered if necessary) and kept saturated with dye. The slide was heated with a Bunsen flame for 5 min (the process was steaming and was not baking). Malachite green from both sides of the microscope slide was washed with tap water for about 30 s. The smear, while still wet, was then counterstained with 0.5 percent aqueous Safranin O (0.5-g Safranin O was dissolved into 10 ml of 95% ethyl alcohol, then this solution was diluted with deionized water to 100 ml.) for 60–90 s. Finally, both sides of the slide were rinsed to remove the secondary stain and

then dried with bibulous paper. Eventually, vegetative cells, endospores-containing cells and the free endospores were observed with light microscope under ×100 (oil immersion) total magnification. The vegetative cells and the spores (both endospores and free spores) were appeared red and green, respectively.^[13,14]

Results

As a result of the borax reduction, the number of printed dots and spores of BA increased and spore distribution became denser [Figure 4].

The crystals of BA printing became much more regular and finer, as a result of the reduction of borax printing from 100% to 0% [Figure 5].

There was a negligible effect on the number of dots and spores of GBS that were printed [Figure 6].

Reduction of borax made the bacteria loosen on the printed substrate and made them susceptible to detachment. The borax content can increase hydrogel volume of guar gum, therefore, could increase cell attachment on the substrate. The low gel content of the printed bacteria causes less growth susceptibility of printed samples. For this reason, a drop of a mixture of guar gum (1% in culture medium) had been added by using a pipette. Printed samples were incubated in a suitable environment (for GBS at 56° and BA at 37°), and after 24h, the color change from green to yellow was visible [Figure 3c]. Thus, it can be used as an indicator of the temperature.

Optical images of stained bacteria with malachite green shows that the bacterial spore shapes after growing had been changed from oval/ spherical to rod shape. There was more bacterial growth in samples coated with guar gum (1% in deionized water) with culture medium comparing to noncoated ones [Figure 3d and e]. Although, there was a risk for bacterial apoptosis due to high-temperature pulses exerted by the printer cartridge head. However, in a separate study, the number of viable bacteria was counted before and after printing and no significant reduction in the number of viable bacteria was observed (data not shown). This can be due to the following: (a) application of bacterial spores as they are more resistant to harsh environments, (b) application of thermophilic bacterium in this study, and (c) due to short intervals of heat exposure during the printing process.

Conclusion

The commercial inkjet printers have the capability to be applied for transferring biological solutions. In this study, considering optimum solutions for each chamber of cartridge we had deposited spores on the PVC films with an acceptable attachment to the film. This attachment was obtained by addition of borax as gelling agent to guar gum during the printing. To prepare enough culture

Table 1: Amount of borax and spore solutions in 2-steps of four printing methods

	Methods							
	First		Second		Third		Fourth	
	Step 1	Step 2	Step 1	Step 2	Step 1	Step 2	Step 1	Step 2
Borax	100	100	50	50	25	25	0	0
Spore	0	100	0	100	0	100	0	100

medium for bacterial growth a drop of guar gum solution was poured on each sample while a color change indicator was included. As it was visualized, the color change shows bacterial growth and reproduction.

Reduction of borax in the printing process generally had no effect on the number and density of spores printed on the substrate, but this reduction altered the crystals of the coating that were used for BA printed spores. As a result, crystals were smaller and more regular due to a reduction in gelling agent. In general, in this study, only the borax percentage and its effects on the hydrogel volume of guar gum and cell bacterial attachment on the substrate were evaluated.

Uncoated samples showed bacterial growth, but they did not show the color change. The growth of bacteria after staining them can be seen, and it was more noticeable in coated samples. Due to the lack of color reagent, guar gum coated samples could not show color changes, but samples covered with pH indicator could be used as temperature indicator. The greater thickness of printing and coating could provide better growing conditions for bacterial spores, and this issue should be considered in the future studies. In addition, monitoring the color change of the printed bio-ink during a period of heat exposure could lead to the production of biological time-temperature indicator.

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Conflicts of interest

There are no conflicts of interest.

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