รายงานวิจัยฉบับสมบูรณ์

โครงการพลศาสตร์ของหน้าคลื่นชีวภาพที่เกิดขึ้นในโคโลนีแบคทีเรีย

โดย ผศ.ดร.อรอุมา เขียวหวาน

มีนาคม 2552

สัญญาเลขที่ RMU4880018

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ในงานวิจัยนี้พลศาสตร์และภาวะไม่คงตัวในการเคลื่อนที่ของหน้าคลื่นของโคโลนีบาซิลลัส ซับทิลิส TISTR 008 ได้ถูกศึกษา โดยพบว่าโคโลนีของแบคทีเรียชนิดนี้สามารถแสดงลวดลาย เช่น ลวดลายที่ขอบของโคโลนีกลมเรียบหรือลวดลายแบบที่ขอบของโคโลนีมีรอยหยักคล้ายรูปนิ้วมือ ในงานนี้ภาวะไม่คงตัวในการเคลื่อนที่ของหน้าคลื่นของโคโลนีนั้นสอดคล้องกับความหยักของขอบของโคโลนี ซึ่งสามารถหาค่าในเชิงปริมาณได้โดยการใช้มิติแฟร็กทัล (fractal dimension) จากผลการทดลองพบว่า ภาวะไม่คงตัวในการเคลื่อนที่ของหน้าคลื่นของโคโลนีเพิ่มขึ้นแบบเป็นเชิงเส้น เมื่อความเข้มข้นของอาหารลดลง และ/หรือ เมื่อความแข็งของวุ้นเลี้ยงเชื้อเพิ่มขึ้น ผลการทดลองในระดับที่เห็นด้วยกล้องจุลทรรศน์เผยให้ทราบว่า การเพิ่มจำนวนประชากรในพื้นที่ที่จำกัดซึ่งส่งผลให้เกิดการซ้อนพับของมัดของสายโซ่ของเซลล์แบคทีเรียนั้นเป็นสาเหตุของการแพร่แบบไม่เป็นเชิงเส้นและเกิดภาวะไม่คงตัวในการเคลื่อนที่ของหน้าคลื่นในที่สุด

การศึกษานี้ได้เสนอแบบจำลองของปฏิกิริยาคู่ควบกับการแพร่เพื่ออธิบายการเกิดลวดลายที่พบในการทดลอง ในแบบจำลองนี้เรามีสมมุติฐานว่าเซลล์แบคทีเรียมี 2 แบบ คือ เซลล์แบบที่มีการเคลื่อนที่และมีการแบ่งตัว และอีกแบบคือเซลล์ที่ไม่มีการเคลื่อนที่และไม่มีการแบ่งตัว และเนื่องจากการเคลื่อนที่ของเซลล์นั้นขึ้นอยู่กับความหนาแน่นของเซลล์แบบที่มีการเคลื่อนที่และมีการแบ่งตัว ซึ่งมีลักษณะไม่สม่ำเสมอจากการที่มีการซ้อนพับของมัดของสายโซ่ของเซลล์ ดังนั้นในแบบจำลองนี้เราได้นำเสนอการแพร่ของเซลล์แบบไม่เป็นเชิงเส้น เราพบว่าแบบจำลองที่นำเสนอสามารถจำลองการเกิดลวดลายของโคโลนีภายใต้การเปลี่ยนแปลงค่าความเข้มข้นของวุ้นเลี้ยงเชื้อและความเข้มข้นของอาหาร รวมทั้งการเกิดภาวะไม่คงตัวในการเคลื่อนที่ของหน้าคลื่นของโคโลนี ได้เป็นอย่างดี

คำหลัก : ภาวะไม่คงตัว ; บาซิลลัส ซับทิลิส ; ระบบแบบปฏิกิริยาคู่ควบกับการแพร่ ;
การเกิดลวดลาย

Abstract

Project Code : RMU4880018

Project Title : Dynamics of biological fronts formed by bacterial colony

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In this research the dynamics and the instabilities in propagating colonial front formed by *Bacillus subtilis* TISTR 008 was studied. Depending on environmental conditions, colonies of this bacterial species grown on the surface of agar mediums show patterns such as a smooth circular pattern or a fingering pattern. In this work the instability of colonial fronts corresponds to the roughness of the boundary of the bacterial colony. In order to quantitatively determine the instabilities in colonial fronts, the fractal dimension is used as a characteristic parameter to indicate the amount of the roughness or instabilities of colonial fronts. Experimental results show that instabilities of propagating colonial fronts are linearly increased with decrease of peptone concentration and/or increase of agar concentration. Microscopic observations reveal that cell reproduction in a limited space resulting in the formation of bundles of chains of bacterial cells is a cause of instability in propagating fronts.

The study proposes a reaction-diffusion model to describe the colonial patterns obtained from the experimental results. The essential assumption is that there exist two types of cells; active cells that move actively, grow and perform cell division, and inactive ones that do nothing at all. Since the movement of cells is dependent on the density of active cells, a nonlinear diffusion is proposed. The model is able to reproduce the experimental results of variation of agar concentration and peptone concentration, including instabilities of the propagating fronts.

Keywords : Instability ; *Bacillus subtilis* ; Reaction-diffusion system ; Pattern formation

1. INTRODUCTION

Recently, much attention has been paid to pattern formation, which is fascinating phenomena in nonlinear systems. Pattern formation was found in various fields such as, chemistry, physics, and biology [1]. Examples of patterns observed in chemical and biological systems are shown in Fig. 1.1. Spiral pattern in the Belousov-Zhabotinsky (BZ) reaction (Fig. 1.1(a)) is a famous example in chemical systems [2]. Such a pattern can also be observed in biological systems, such as in an aggregation of slime mold *Dictyostelium discoideum* (Fig. 1.1 (b)) [3]. These patterns are not only found in laboratory, but also in nature, for example, the spotted patterns on skin of a leopard and the stripped patterns on skin of a zebra. Zebras use the stripped patterns to confuse predators, e.g., a leopard. Similarly, predators also use their patterns to camouflage prays in hunting. Thus, patterns are important because they enhance the chance of the animal survival [1].

Patterns in nonlinear systems are created by an internal dynamics of the systems, this process of pattern formation is called self-organization. It is noted that self-organization can only occur far from the thermodynamic equilibrium and the structure of such organization is called a dissipative structure [4]. Thus, the system of self-organization is contradicting with the second law of thermodynamics, explaining that systems always reach an equilibrium and the entropy of systems at equilibrium state is maximized. In 1955, Ilya Prigogine, a Belgian physicist, proposed the principle of minimal entropy production to explain why the entropy of self-organizing systems are not maximizing. He explained that the self-organizations occur in an open system, which has a flow of matter and energy between the system and environment that results in non-maximizing of entropy of the system. Therefore, the system does not reach the equilibrium [1, 4]. The complexity and diversity of self-organizing systems can be mathematically explained by using nonlinear equations, such as the reaction-diffusion equations [5].

In 1952, a reaction-diffusion system was proposed by British mathematician named Alan Mathinson Turing in order to understand the processes of morphogenesis, e.g., how a fertilized egg becomes an organism [6]. The reaction-diffusion system is a mathematical model that describes how the concentration of substances changes under influences of two processes: a chemical reaction and diffusion. The reaction-diffusion systems can be applied to model and explain the complexity of pattern formation in

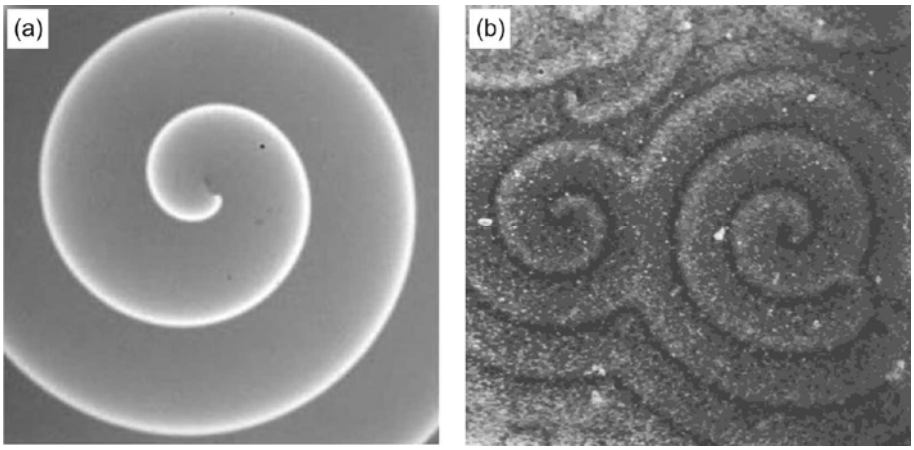

Figure 1.1: Examples of patterns in a chemical and biological system. (a) Spiral wave in the BZ reaction [2], (b) Aggregation of slime mold *Dictyostelium discoideum* [3].

in various scales, for example, calcium wave propagating in a rat retina [7], formation of the bacterial colony [8, 9, 10, 11], and the Turing pattern on animal skins [12, 13].

Among these systems, the pattern formation of the bacterial colony is the simplest biological object for studying and understanding the pattern formation in biological systems [14]. It is also easily controlled because it is purely dominated by physical conditions, such as nutrient concentration and hardness of medium [15].

Recently, there are the studies of pattern formation with various bacterial species, such as *Bacillus subtilis* [8, 16], *Paenibacillus dendritiformis* [9, 17], and *Salmonella typhimirium* [18]. These bacterial species exhibit diverse patterns. Especially, they describe a branching pattern called a fractal pattern, when the colony is grown on a hard medium with poor nutrient [19]. The fractal patter is not only found in a living system, but also in a non-living system. Examples of fractal pattern are shown in Fig. 1.2 [15, 20]. Such a pattern is an example of self-similarity [21]. In colonial formation, the complexity of the colonial growth normally increases as the environmental conditions become less favorable. The bacterial colony responds to adverse conditions by developing a strategy and organizing in a micro-level to cooperate the more efficiency of survival [22]. Various patterns can be observed, depending on both bacterial species and environmental conditions [23, 24, 25].

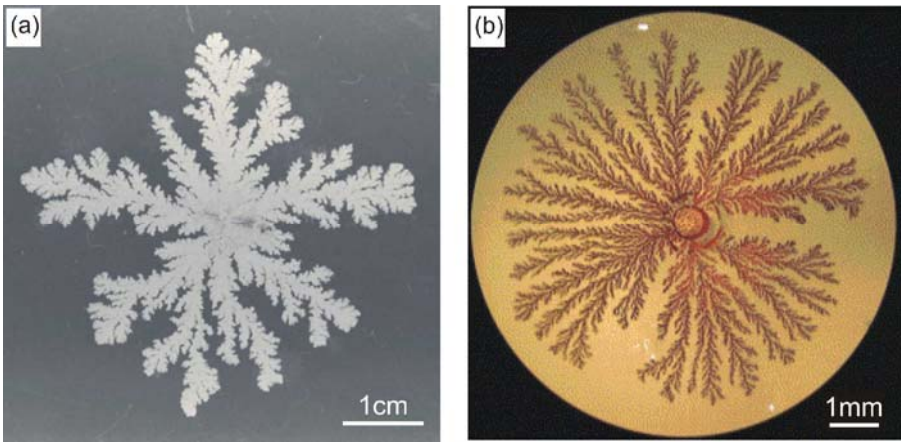

Figure 1.2: Examples of the branching pattern. (a) Fractal pattern of *Bacillus subtilis* colony [15], (b) Branching pattern of copper electrodeposits grown from $CuSO_4$ [20].

This work is focused on pattern formation of bacterial colonies and instabilities in propagating colonial fronts. The bacterial species of *Bacillus subtilis* TISTR 008 is used in experiments. Instabilities in propagating fronts of the bacterial colony is investigated both experimentally and numerically. In this work the reaction-diffusion systems modified from Mimura's model [26] are proposed also in order to explain and confirm the formation of colonial patterns. Furthermore, the formation of the spreading colony in the condition of the soft medium with rich nutrient is investigated.

2. EXPERIMENTAL METHODS

In this section, experimental methods are presented. They consist of a general experimental method (Sec. 2.1), methods for the study of dynamics of the colonial formation (Sec. 2.2), methods of the investigation of instabilities of colonial fronts (Sec. 2.3), and methods for the study of a spreading colony (Sec. 2.4).

2.1 General experimental methods

In this work the used bacterial species was *Bacillus subtilis* TISTR 008. It was obtained from Thailand Institute of Scientific and Technological Research. It is a gram-positive with rod-shaped (0.5 - 1.0 μm diameter, 2 - 5 μm length) as shown in Fig. 2.1. The bacterial species of *Bacillus subtilis* is commonly found in soil and considered to be a motile species [64]. It has been classified as an obligate aerobe. It is able to form a protective endospore via a spore formation process, if the conditions are not appropriated for the growth of bacterial cells, such as starvation and desiccation [65]. An endospore allows this bacterial species to tolerate extreme environmental conditions and is resistant to ultraviolet, gamma radiation, and chemical disinfectants. Normally, *Bacillus subtilis* is not considered as pathogen and toxicity. However, it is a cause of food poisoning, if it is contaminated in food because its spores can survive at high temperature that often used to cook food. Isolated colonies of *Bacillus subtilis* TISTR 008 obtained by streaking on TSA medium are shown in Fig. 2.2. Colonies on TSA medium become opaque and may be wrinkled. The color of colonies is white or cream.

The general experimental method consists of preparation of agar plates (Sec. 2.1.1), preparation of the bacterial starter (Sec. 2.1.2), inoculation method (Sec. 2.1.3), and observation of colonial patterns (Sec. 2.1.4).

2.1.1 Preparation of agar plates

The procedure of preparation of agar plates is summarized in Fig. 2.3(a-c) [9]. A solution for agar plate preparation consists of 5 g of NaCl, 2.397 g of K_2HPO_4, 0.68 g of KH_2PO_4, an amount of Bacto Peptone (Difco Laboratories), and Bacto Agar (Difco Laboratories) in 1 liter of distilled water. The final pH of the solution was 7.0 ± 0.2. The mixture was sterilized by using an autoclave at the temperature of 121°C for 15 minutes, then let it cool down to the temperature of 45°C. Because of evaporation of water in the

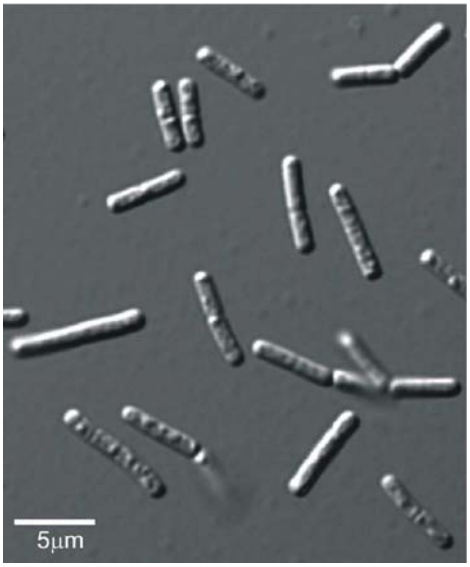

Figure 2.1: Bacterial cells of *Bacillus subtilis* TISTR 008 taken by using a confocal laser scanning microscope.

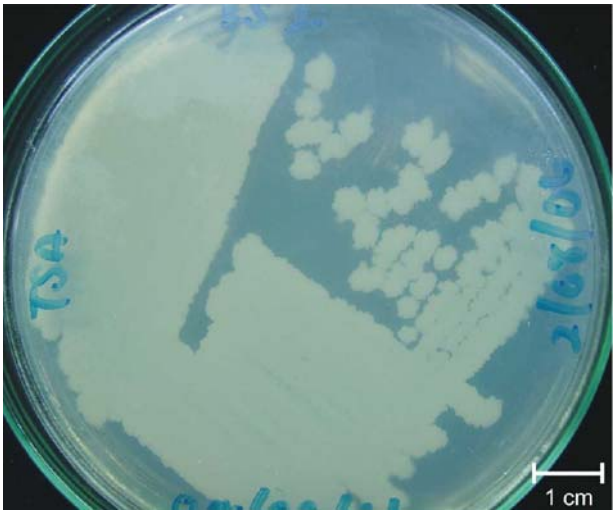

Figure 2.2: Isolated colonies of *Bacillus subtilis* TISTR 008 on TSA medium. The picture was taken by a digital camera after incubation at the temperature of 37°C for 24 hours.

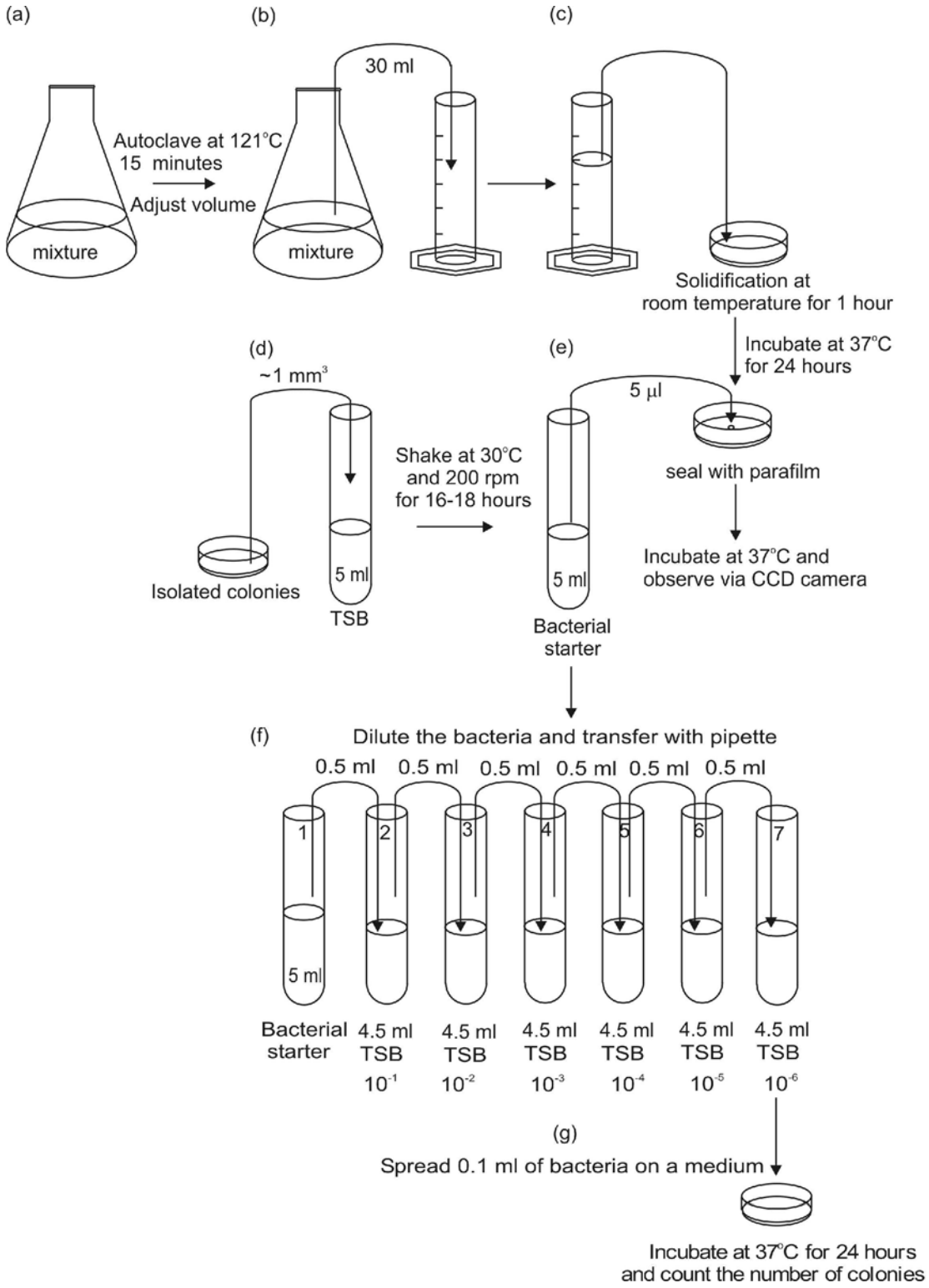

Figure 2.3: The method of preparation of the agar medium, preparation of the bacterial starter, inoculation, and viable count.

mixture (~5 ml), the volume of the mixture was adjusted with sterile distilled water back to 1 liter (Fig. 2.3(a)). Then, 30 ml of the solution was immediately poured into a glass Petri dish (8.8 cm of diameter) (Fig. 2.3(b)). The solution was solidified by keeping at the room temperature of 25°C for 1 hour (Fig. 2.3(c)). The thickness of agar was about 0.5 cm. Then, the agar plates were incubated at the temperature of 37.0±0.3°C and relative humidity of 49.0±2.0% for 24 hours to check the contamination.

2.1.2 Preparation of bacterial starter

The bacterial colonies of *Bacillus subtilis* TISTR 008 were isolated on TSA medium before preparation of bacterial starter. After that, an isolated colony was used to prepare the bacterial starter by picking the isolated colony of about 1 mm, put it into 5 ml of Tryptic Soy Broth (Difco Laboratories) medium contained in a tube (Fig. 2.3(d)). Then, the bacterial tube was shaken at the temperature of 30°C with the rotation speed of 200 rpm for 16 - 18 hours. After that, the suspension of bacterial starter appeared obviously. Normally, the amount of bacterial cells in this liquid culture was approximately (2.0±0.3) x 10^9 cfu/ml.

2.1.3 Inoculation of the bacterial starter on the agar plates

The incubated agar plates were dried at the room temperature for 15 minutes before inoculation. Then, 5 µl of the liquid culture of bacteria was inoculated into the center of an agar plate (Fig. 2.3(e)). The value of the initial cell density calculated by using the method of viable count [66] was about (2.0±0.3) x 10^9 cfu/ml. The inoculated agar plates were closed and sealed with parafilm to keep the humidity inside an agar plate approximately constant. Then, the agar plates were incubated at the temperature of 37±0.3°C and 49.0±2.0% of relative humidity.

2.1.3.1 The method of viable count

The bacterial starter was diluted in Tryptic Soy Broth by using a ten-fold serial dilution method [66] as shown in Fig. 2.3(f). After that, 0.1 ml of each dilution was transferred onto a fresh TSA plate. Then, transferred bacteria were spread on the TSA surface by using a glass spreader. The spread plates were kept in the incubator at the temperature of 37 C for 24 hours. After the incubation, the isolated colonies with a rough boundary appeared as shown in Fig. 2.4. Then, they were counted and averaged from 2-5 sample plates. The optimal number of colonies for a calculation of the initial cell density is in the range of 160-230 colonies.

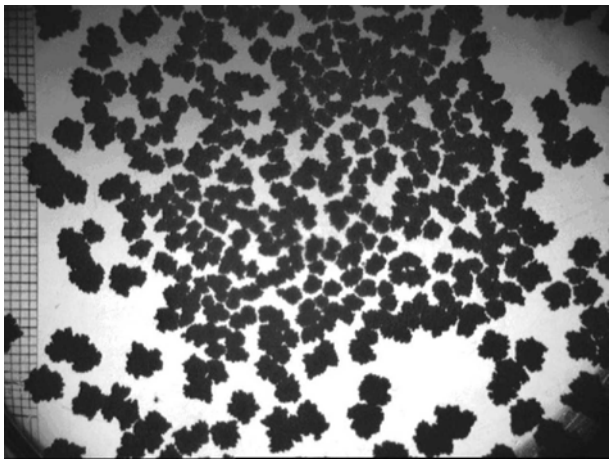

Figure 2.4: Isolated colonies of *Bacillus subtilis* TISTR 008 at dilution of 10^{-6}. The picture was taken by a CCD camera after incubation at the temperature of 37°C for 24 hours.

2.1.3.2 Calculation of the initial cell density

For calculating of the initial cell density, 1 isolated colony implies 1 bacterial cell. A formula for calculation of the initial cell density is

$$X = N(10^{n+1}),$$

where X is the number of bacteria per 1 ml, it is commonly shown in the unit of colony forming unit per milliliter or cfu/ml. N is the number of average colonies obtained from 0.1 ml of diluted bacteria. n is the dilution factor of the bacterial tube diluted to 10^{-n} (see Fig. 2.3(f) also). For example, if the average number of colonies is 187 at the dilution of 10^{-6}, then X will equal to 187 multiplied by 10^{6+1}, i.e., 1.87 x 10^{9} cfu/ml.

2.1.4 Observations of the bacterial colonies

In this part the observation methods of colonial patterns are explained. It can be divided into 2 parts: i) a macroscopic observation and ii) a microscopic observation.

2.1.4.1 Macroscopic observation

Colonial patterns were observed via a charge couple device (CCD) camera (SONY ExwaveHAD) with a magnification of 10 times, and a digital camera (SONY DSC-S85). A schematic diagram of the experimental setup is shown in Fig. 2.5. An array of infrared

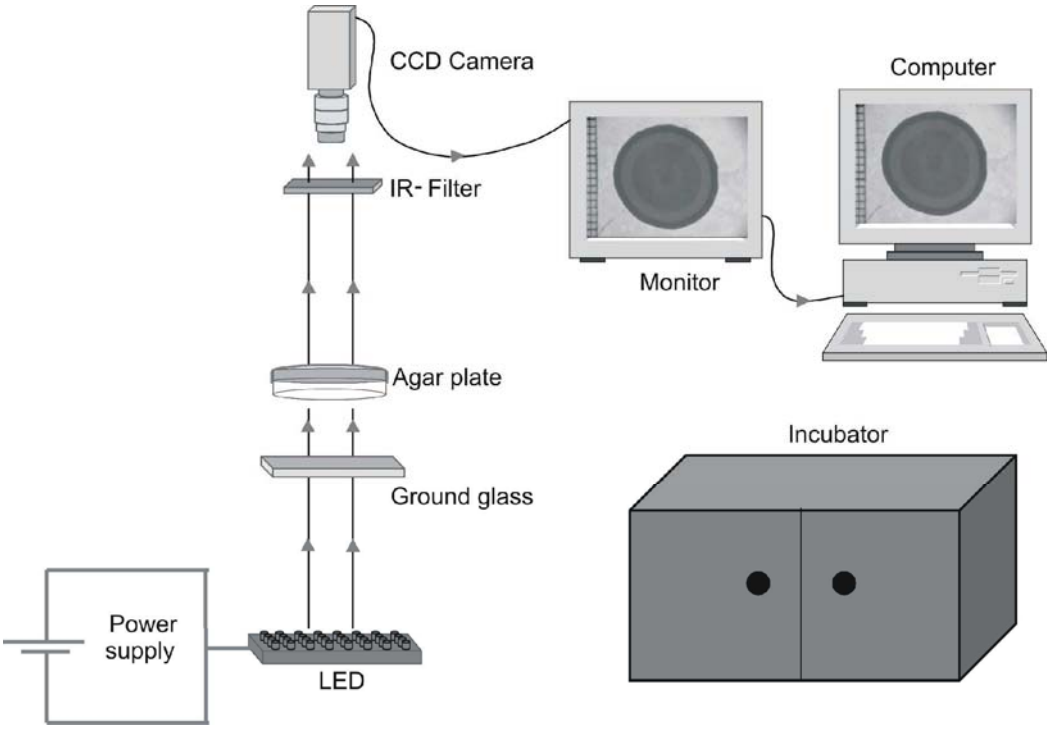

Figure 2.5: Experimental setup for a macroscopic observation of colonial patterns.

light-emitting diodes (IR-LED) is used as an IR light source because it emits a narrow-spectrum of IR light in one direction with a narrow angle. It contrasts to a fluorescent lamp of a light box, which emits light no specific direction. Thus, an image obtained from IR-LED is clearer than that from a light box. The generated infrared light passes through a ground glass used to obtain a homogeneous light, and then the bacterial colony in an agar plate. An infrared filter that allows only the transmission of the IR light was placed before the CCD camera. Then, the images were recorded on a computer hard disk. To observe the colonial pattern, colonial plates were taken out of an incubator to record an image. Then, it was immediately brought back to the incubator to limit the interrupted change of temperature, which affects the bacterial growth.

In order to observe the growth of the colony in real time, a water jacket and a thermostat were applied. The colonial plate was placed in the water jacket where water circulates inside. Water temperature was controlled by the thermostat.

2.1.4.2 Microscopic observation

For microscopic observations, an alignment of cells in a bacterial colony was observed by using a scanning electron microscope (SEM). A confocal laser scanning microscope (CLSM) was used to observe single cells of Bacillus subtilis TISTR 008 [67].

The steps of specimen preparation for using of an SEM are following:

1. Fix the bacterial colony by using the fixative reagent (2.5% glutaraldehyde in 0.1M cacodylate) for 2 hours.
2. Wash the colony 2 to 3 times with 0.1 M cacodylate buffer, then fix again with 0.1% osmium tetroxide (OsO_4) for 2 hours.
3. Dehydrate the sample by using alcohol as the following steps: 30% ethanol for 15 minutes, 50% ethanol for 15 minutes, 70% ethanol for 15 minutes, 80% ethanol for 15 minutes, 85% ethanol for 15 minutes, 90% ethanol for 15 minutes, 95% ethanol for 15 minutes, and absolute ethanol for 30 minutes (2 times).
4. Dry the specimen by using the critical point dry (CPD) technique.
5. Put the sample on a stub with a carbon tape.
6. Coat it with OsO4vapour by using a sputter coater.
7. Observe the alignment of bacterial cells with the SEM.

The preparation of bacterial cells for CLSM technique is following:

1. Prepare the bacteria in 5 ml of TSB medium, density of about $2x10^7$ cfu/ml.
2. Drop the suspension of bacterial cells on the cover slip coated with Poly-L-lysine.
3. Wait for 20 minutes, then observe the bacterial cells with the CLSM.

Note that samples were investigated at Center of NanoImage (CNI), Faculty of Science, Mahidol University.

To avoid the contamination, preparation of agar plates and inoculation of bacterial starter including the viable count were performed in a laminar flow system. The laminar flow

will block microorganisms in order to avoid the contamination. In addition, the aseptic technique was also applied to make sure that the glassware was cleaned. Note that hands should be cleaned by using alcohol before doing an experiment.

2.2 Investigation of dynamics of the colonial patterns

The experiments for the study of dynamics of the colonial growth can be divided into 4 parts: i) variations of peptone and agar concentrations, ii) the variation of initial size, iii) the variation of the initial cell density, and iv) the variation of temperature. To prepare the agar plate and the bacterial starter, the general experimental method described in Sec. 2.1 was applied.

In order to investigate morphological changes due to environmental conditions in the colonial formation of *Bacillus subtilis* TISTR 008, concentrations of peptone and agar were varied. There were 24 conditions of the medium with variations of peptone (0.4 g/l, 10 g/l, 20 g/l, 30 g/l, 40 g/l, and 50 g/l) and agar concentrations (4 g/l, 6 g/l, 10 g/l, and 14 g/l). Other parameters, such as temperature (37°C) and initial cell density (~2×10^9 cfu/ml), were kept constant as shown in Table 2.1. Similarly, the effect of initial size, initial cell density, and temperature on the colonial formation were also investigated. The conditions for investigation of such effects are shown in Table 2.1.

Table 2.1: Conditions for the investigation of dynamics of the colonial front.

Measurement	[Agar] (g/l)	[Peptone] (g/l)	Temperature (°C)	Initial cell density (10^9 cfu/ml)	Inoculated volume (μl)
Variations of agar and peptone concentrations	4, 6, 10, 14	0.4, 10, 20, 30, 40, 50	37	1.760	5
Variation of initial size	6	40	37	1.775	2, 5, 10
Variation of initial cell density	6	40	37	17.750, 1.775 0.178	5
Variation of temperature	6	40	30, 40, 45	1.760	5

2.3 Investigation of instabilities of colonial fronts

In order to study instabilities in propagating fronts, experimental methods can be divided into 2 parts: i) the variation of peptone concentration and ii) the variation of agar concentration. The general experimental method was applied in preparing the agar plate and the bacterial starter. Conditions for the investigation of instabilities are summarized in Table 2.2. There are 5 conditions of the medium for the investigation of instabilities under the variation of agar concentration (6 g/l, 8 g/l, 10 g/l, 12 g/l, and 14 g/l), whereas peptone concentration was fixed at 40 g/l. There are 4 conditions of the medium for the variation of peptone concentration (10 g/l, 20 g/l, 30 g/l, and 40 g/l), with fixed concentration of agar at 6 g/l. Other parameters, such as temperature (37°C), were kept constant.

Table 2.2: Conditions for the investigation of instabilities in propagating fronts.

Measurement	[Agar] (g/l)	[Peptone] (g/l)	Temperature (°C)	Initial cell density (10^9 cfu/ml)
Variations of agar concentrations	6, 8, 10, 12, 14	40	37	1.85
Variations of peptone concentrations	6	10, 20, 30, 40	37	2.23

2.3.1 Measurement of roughness

Fractal concepts can be applied to determine quantitatively the roughness of colonial fronts. The roughness or instability of colonial fronts was determined from fractal dimension. In this work, we used the structured walk or compass dimension method to find fractal dimension of the colonial fronts.

2.3.1.1 Method for generating a contour of the colonial front

The structured walk method requires the contour of the boundary of objects. Thus, the contour of colonial boundary was measured before applying the method. The contour was extract from images of colonies by using the computer program written in the Interactive Data Language (IDL). Colonial patterns obtained from the experiment were 8-bit gray scale images. The threshold value of gray scale corresponding to the boundary of the

Table 2.3: Conditions for the investigation of morphological patterns of the spreading colony.

Measurement	[Agar] (g/l)	[Peptone] (g/l)	Temperature (°C)	Initial cell density (10^9 cfu/ml)	Inoculated volume (μl)
Variations of agar and peptone concentrations	6, 10, 14	10, 20, 30, 40, 50	37	1.940	5

colony was chosen as 0.6 x (maximum gray scale - minimum gray scale) + minimum gray scale.

2.3.1.2 Measurement of fractal dimension

After the contour of the colonial boundary was determined, the structured walk method was applied to find the fractal dimension. In this work, the length of measuring sticks was varied in the range of 1/3 to 1/100 of the colonial diameter.

2.4 Formation of the spreading colony

In order to study the formation of the spreading colony, investigating methods were divided into 4 parts: i) the onset of the spreading colony, ii) the morphological diagram of spreading bacteria, iii) the biochemical test and Deoxyribonucleic acid (DNA) sequencing, and iv) the microscopic observation of the spreading colony.

2.4.1 Onset of the spreading colony

The condition of soft medium (6 g/l of agar concentration) with high concentration (40 g/l) of nutrient was used to investigate the formation of the spreading colony. The incubation temperature was 37°C. The initial cell density was about 2 x 10cfu/ml. The general experimental method described in Sec. 2.1 was applied to prepare the agar plate and bacterial starter.

2.4.2 Morphological diagram of spreading bacteria

The morphological diagram was performed under the variations of agar and peptone concentrations to understand the dynamics of spreading colonies. The conditions

for studying the morphological pattern of colonies are shown in Table 2.3. There were 15 conditions of agar plates with variations of peptone (10 g/l, 20 g/l, 30 g/l, 40 g/l, and 50 g/l) and agar concentration (6 g/l, 10 g/l, and 14 g/l). Other parameters, such as temperature (37°C), were kept constant.

2.4.3 Biochemical test and DNA sequencing

The methods of the biochemical test and the DNA sequencing were applied to confirm that the formation of the spreading colony was not caused from the mutation of bacteria species of *Bacillus subtilis* TISTR 008. The biochemical test and the DNA sequencing with 16S rRNA gene sequence analysis [70] were applied to check the species of bacteria in spreading region (noted as B2) compared with the bacterial cells in stock (B1).

The procedures of 16s rDNA identi cation are following:

1. Prepare the isolated colonies of bacteria B1 and B2 on the TSA plate separately.
2. Choose a single colony (B1 and B2) to be separately cultured in broth medium for 6-12 hours.
3. Centrifuge two bacterial samples with rotation speed of 10,000 rpm for 2-5 minutes.
4. Bring precipitated cells to be dissolved in 100-500 μl TE buffer (Trishydroxymethylaminomethane-Ethylenediamine tetraacetic acid buffer).
5. Shake and then boil the sample for 10-15 minutes.
6. Centrifuge again with the rotation speed of 10,000-12,000 rpm for 5-10 minutes.
7. Collect 50-200 μl the DNA solution to use as a DNA template.
8. Use the polymerase chain reaction (PCR) technique for amplifying DNA.
9. Bring samples into Automated DNA Sequencer 3100-Avant Genetic Analyzer. Samples were investigated at the Central Instrument Facility of faculty of Science in Mahidol University for DNA sequencing and at the Thailand Institute of Scientific and Technological Research for the biochemical test.

2.4.4 Microscopic observation of the spreading colony

To observe the alignment of the spreading colony microscopically, an optical microscope was used. The bacterial colony was prepared on the agar plate of soft agar medium with high nutrient concentration. The incubation temperature was 37°C. The initial cell density was about 2×10^9 cfu/ml. The colonial plate was incubated for 48 hours before observation.

3. NUMERICAL SIMULATION

In order to confirm and describe the pattern formation and instabilities of colonial fronts obtained from the experiment, we decided to use and modify the MSM model [26]. In this work, a 9-point finite difference method [74] was applied to determine the value of diffusion. Since the microscopic observations reveal that cells connected with each other and formed bundles of chains of cells, we assume that the movements of cells is dependent on the density of active cells. To take this assumption, the nonlinear diffusion is applied. We proposed a nonlinear diffusion of active cells as following:

$$d_u = d_1 u^k , \tag{3.1}$$

where an exponent, k refers to the hardness of the medium. u is the population density of active cells. The parameter d_1 is the diffusion of bacterial cells depending on an initial nutrient concentration, v_0. We found that d_1 is a linear function of v_0 as following:

$$d_1 = b_1 v_0 + b_2, \tag{3.2}$$

where b_1 and b_2 are constants. It should be noted that the parameters d_1 and k are respectively corresponding to the nutrient and agar concentrations in our experiments.

Our reaction-diffusion model is shown as following [26]:

$$\begin{aligned}
\frac{\partial u}{\partial t} &= \nabla(d_1 u^k \nabla u) + \varepsilon u v - \frac{a_0 u}{(1 + u/a_1)(1 + v/a_2)}, \\
\frac{\partial v}{\partial t} &= d_v \nabla^2 v - u v, \\
\frac{\partial s}{\partial t} &= \frac{a_0 u}{(1 + u/a_1)(1 + v/a_2)},
\end{aligned} \tag{3.3}$$

where ε, a_0, a_1, a_2, and d_v are constants. u(r,t) and s(r,t) are the population densities of active and inactive bacteria, respectively. v(r,t) is the nutrient concentration. u(r,t) + s(r,t) is the total population density. Here r and t are position vector and time, respectively.

The first equation describes a rate of change of the active cells. In this equation the first term describes the diffusion of cells, whereas the second and last term describes the growth of cells and the rate of a conversion from active to inactive cells, respectively. The second equation describes the consumption rate of nutrient. The last equation describes the rate of the conversion from active to inactive cells.

The condition for studying instabilities in colonial fronts can be divided into 2 parts as summarized in Table 3.1. It is noted that the variation of d_1 with fixing k is corresponding to the variation of nutrient concentration with fixing agar concentration. The other one is corresponding to the variation of agar concentration with fixing nutrient concentration.

Table 3.1: Conditions for the simulation of instabilities of colonial fronts.

Investigation	d_1	k
Variations of d_1	1.2, 1.4, 1.6, 1.8	1.0
Variations of k	1.8	1.0, 1.1, 1.2, 1.3, 1.4

4. RESULTS AND DISCUSSION

In this chapter the results of the experiments for the study of the dynamics of bacterial colony are presented in Sec. 4.1. Morphological diagram of *Bacillus subtilis* TISTR 008 is shown in Sec. 4.1.1. In Sec. 4.1.2, 4.1.3, and 4.1.4, the effect of the initial cell density, the initial size, and the incubation temperature on a colonial pattern are discussed, respectively. The instability of propagating colonial fronts under the variation of agar concentration and that of peptone concentration is explained in Sec. 4.2. The microscopic observations of the smooth and rough colony are discussed in Sec. 4.2.3. The numerical results and the quantity of instabilities are presented in Sec. 4.2.4 and 4.2.5, respectively. In Sec. 4.3, the results of the study of spreading colony obtained in the condition of soft medium with rich nutrient are presented.

4.1 Investigation of the dynamic of bacterial colonies

4.1.1 Morphological diagram of *Bacillus subtilis* TISTR 008

In order to investigate morphological changes due to environmental conditions in colonial formation, two important parameters are varied: the peptone and agar concentrations.

We found that the colony patterns grown on an agar surface change dramatically, if the concentrations of agar and nutrient are varied globally. They are classi ed into ve distinct types, as shown in the morphological diagram (Fig. 4.1): fractal pattern in region A, fine branching pattern in region B, fingering pattern in region C, smooth circular pattern in region D, and spreading pattern in region E.

Region A: The colonies describe a fractal pattern [8, 47]. The nutrient concentration was fixed at 0.4 g/l. Cell division of bacterial cells in this region is low because of the limited nutrient. They only grow and undergo cell division locally. It seems that colonial patterns are not affected by the hardness of the medium.

Region B: When the nutrient concentration is low (10 g/l) and the medium is soft (6 g/l), the colonial fronts are not smooth and form a fine branching pattern. This pattern is also found when the medium is hard (10-14 g/l) and nutrient concentration is high (20-40 g/l). Comparing with the region A, the cell division of bacteria is local similarly, but cell

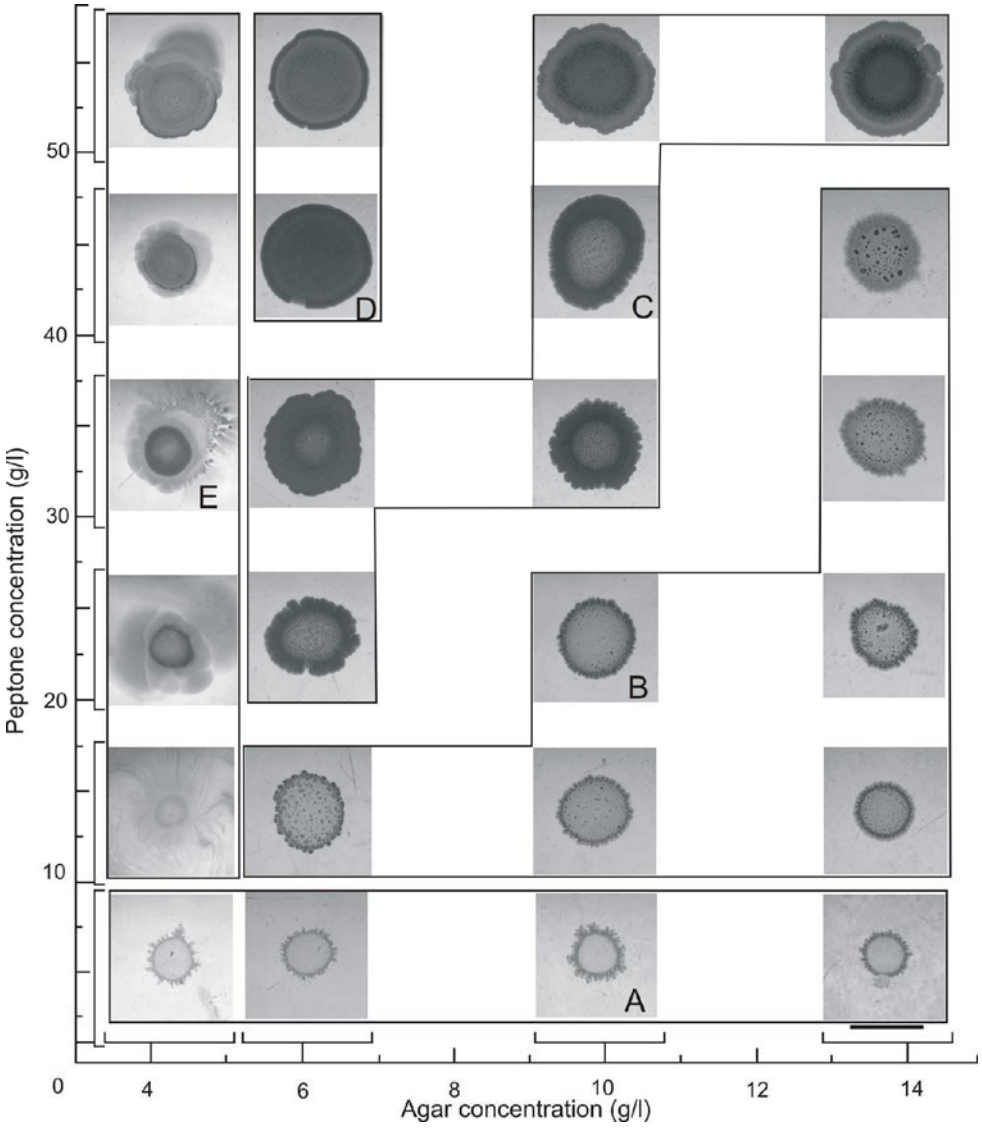

Figure 4.1: Colonial patterns of *Bacillus subtilis* TISTR 008 under the variations of agar concentration and peptone concentration (all pictures were taken at 48 hours). Initial cell density was 1.76 x 10^9 cfu/ml (Inoculated volume was 5 µl). The incubation temperature was 37°C. Scale bar: 1 cm.

density is higher because of higher nutrient concentration. Therefore, the colonies in region B are darker than that in region A.

Region C: The colonies form a fingering front. The colonial patterns become compact. This pattern is found when the nutrient concentration is about 20-30 g/l and the agar concentration is low (6 g/l) or if the agar medium is hard (10 g/l of agar concentration)

with high nutrient concentration (30-50 g/l). The colonies in this region are denser than that in region B obviously because of higher nutrient concentration. The patterns in this region are similar to that in Matsushita's work, i.e., the Eden pattern [8].

Region D: For high nutrient concentration and low agar concentration (soft agar medium with high nutrient levels) the colonial patterns are smooth and form a circular front. The colonies are very dense and the distribution of bacteria inside the colony looks homogeneous. The population density of bacteria cells inside the colony is higher than that in region C. Cell reproduction is high and the colony expands homogeneously, because of soft medium with high nutrient concentration.

Region E: The colonies form a spreading or a swarming pattern. The agar concentration was fixed at 4 g/l (very soft agar medium). The colonial fronts spread quickly because of high moisture of the surface of medium, due to the vapour condensation, which increases the motility of cells.

4.1.2 Variation of initial cell density

The results of effect of the initial cell density on the growth dynamics are shown in Fig. 4.2. The colonial patterns are not explicitly different, but still form a smooth circular

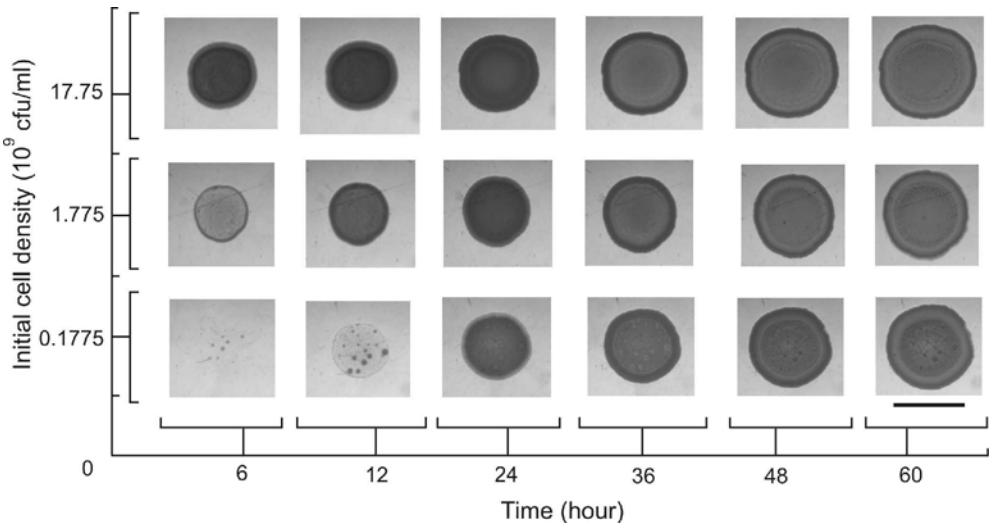

Figure 4.2: Colonial patterns of *Bacillus subtilis* TISTR 008 under the variations of initial cell density. The incubation temperature was 37°C and the concentrations of the agar and peptone were 6 and 40 g/l, respectively. Inoculation volume was 5 µl. Scale bar: 1 cm.

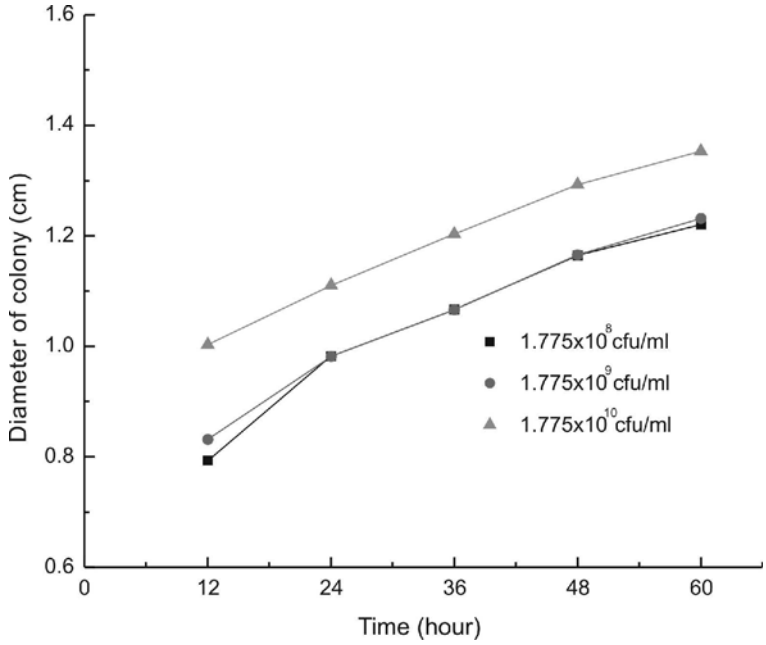

Figure 4.3: Growth rate of colonies under the variation of initial cell density. The agar and peptone concentrations were 6 and 40 g/l, respectively.

pattern although the initial cell density is about 10 times different. The growth rate is determined by the graph of the diameter of colonies as a function of time. In order to determine the value of growth rate, the diameter of colonies in the period of 24-60 hours was used. The value of growth rate is measured quantitatively from the slope of the linear fitted lines as shown in Fig. 4.3. The growth rates are 69.7 $\pm$ 3.0, 76.3 $\pm$ 3.8, and 70.6 $\pm$ 2.9 μm/hour for the initial cell densities of 1.775 x 10^8, 1.775 x 10^9, and 1.775 x 10^{10} cfu/ml, respectively. The growth rates and the final size of the bacterial colony are not linearly different. It can be concluded that the initial cell density does not effect on the growth dynamics of colony.

4.1.3 Variation of initial size

The results of initial size variations, where the cell density was fixed, are shown in Fig. 4.4. The final size of colony is proportional to its initial size, i.e., if the initial size is large, then the final size will be large. The graph of diameter of colonies as a function of time is plotted as shown in Fig. 4.5. The growth rates are 84.3 $\pm$ 0.5, 70.9 $\pm$ 1.5, and 73.5 $\pm$ 1.3 μm/hour for the inoculated volumes of 2, 5, and 10 μl, respectively. In conclusion, the initial size clearly effects on the final size of the colony.

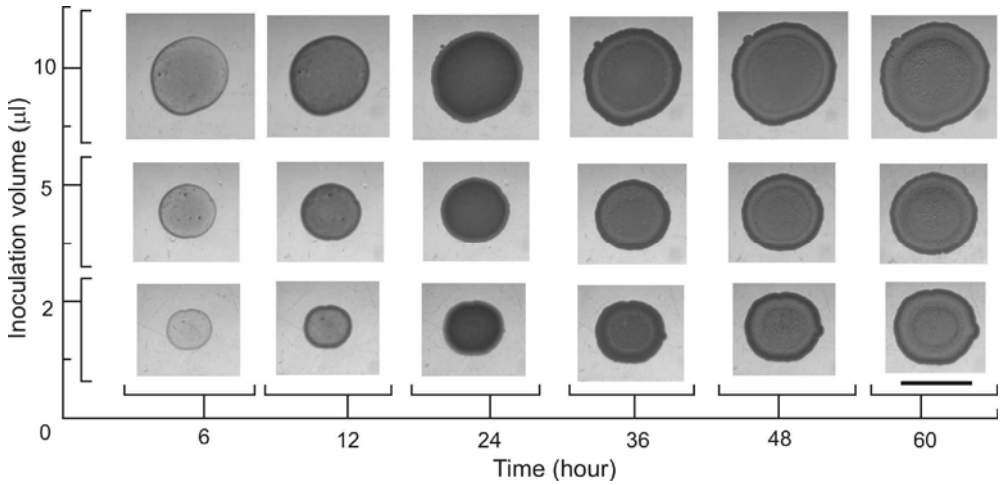

Figure 4.4: Colonial patterns of *Bacillus subtilis* TISTR 008 under the variations of initial size. The inoculated volumes were 2, 5, and 10 µl. Initial cell density was 1.775 x 10^9 cfu/ml. The incubation temperature was 37°C and the concentrations of agar and peptone were 6 and 40 g/l, respectively. Scale bar: 1 cm.

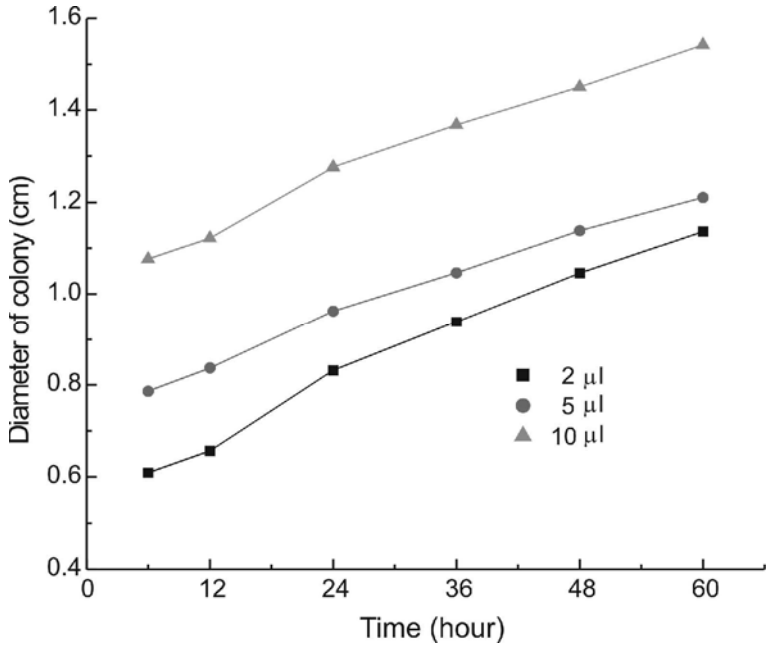

Figure 4.5: Growth rate of colonies under the variation of initial size. The agar and peptone concentrations were 6 and 40 g/l, respectively.

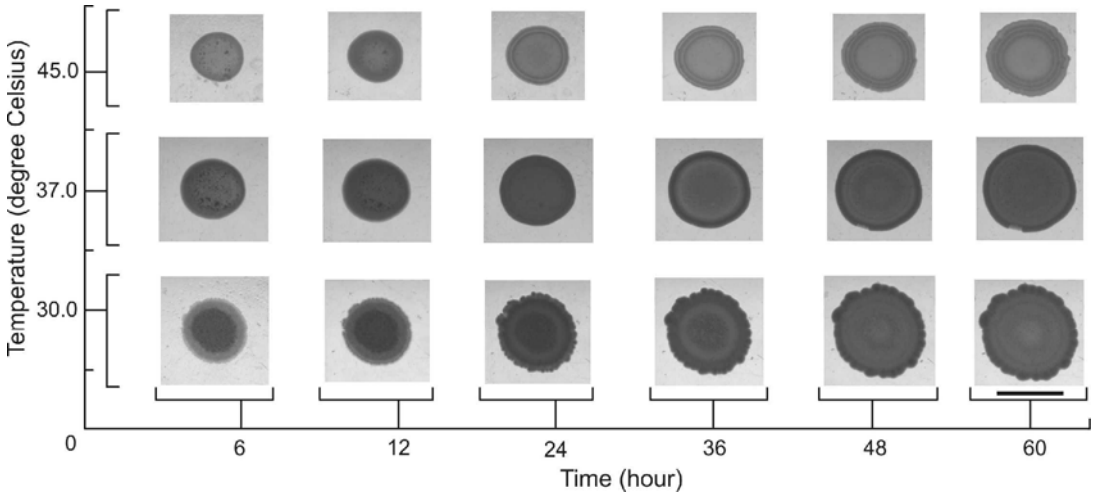

Figure 4.6: Colonial patterns of *Bacillus subtilis* TISTR 008 under the temperature variations. The inoculated volume was 5 µl and the concentrations of agar and peptone were 6 and 40 g/l, respectively. Initial cell density was 1.76 x 10^9 cfu/ml. Scale bar: 1 cm.

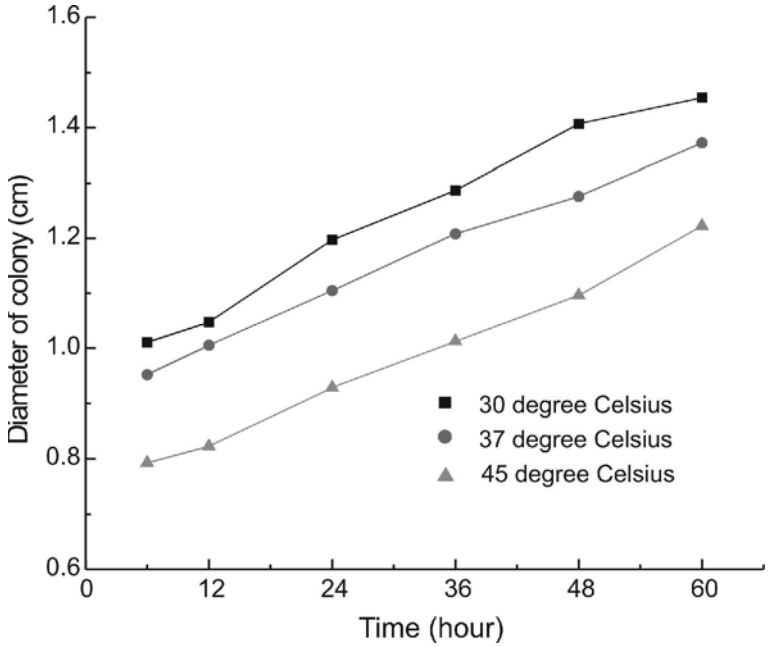

Figure 4.7: Growth rate of colonies under the temperature variation. The agar and peptone concentrations were 6 and 40 g/l, respectively.

4.1.4 Variation of incubation temperature

The results of temperature variations are shown in Fig. 4.6. The colony describes a fingering pattern at incubation temperature of 30°C. For the temperature of 45°C, the colonial pattern is thin and forms many layers inside of the colony. The colonial pattern looks similar to the concentric ring one, which alternately expanded and stopped expanding, observed in Matsushita's work [8]. The growth rate of the colony is quantitatively measured as shown in Fig. 4.7. The growth rates are 86.7 ± 5.6, 77.7 ± 2.3, and 78.7 ± 2.4 μm/hour for the incubation temperatures of 30, 37, and 45 °C, respectively. In conclusion, the temperature effects on the colonial pattern obviously.

4.2 Instabilities of propagating colonial fronts

In this topic the experimental results can be divided into 2 parts. The first one is instabilities under the variations of peptone concentration. The second one is instabilities under the variations of agar concentration.

4.2.1 Variation of the peptone concentration

Instabilities of colonial front under the variation of peptone concentration are shown in Fig. 4.8. If the nutrient concentration is increased with fixing agar concentration,

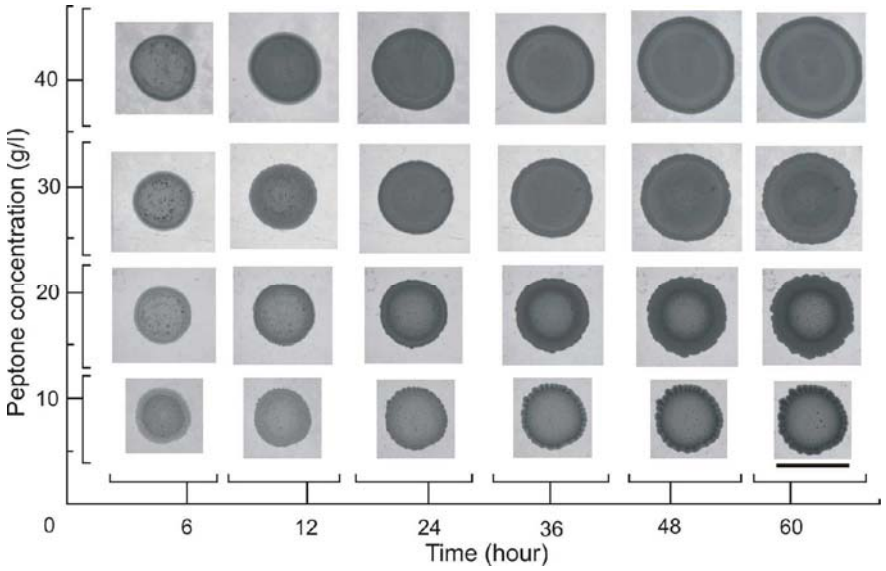

Figure 4.8: Instabilities of the colonial front under the variation of the peptone concentration. The agar concentration was fixed at 6 g/l. Initial cell density was 2.23 x 10^9 cfu/ml Inoculated volume was 5 μl. The incubation temperature was 37°C. Scale bar: 1 cm.

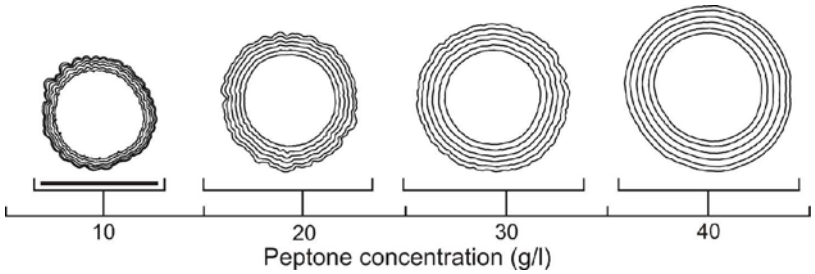

Figure 4.9: Contours of colonial growth under the variation of the peptone concentration with fixed agar concentration (6 g/l). The innermost to outermost contours represent the fronts at 6th, 12th, 24th, 36th, 48th, and 60th hour, respectively. Scale bar: 1 cm.

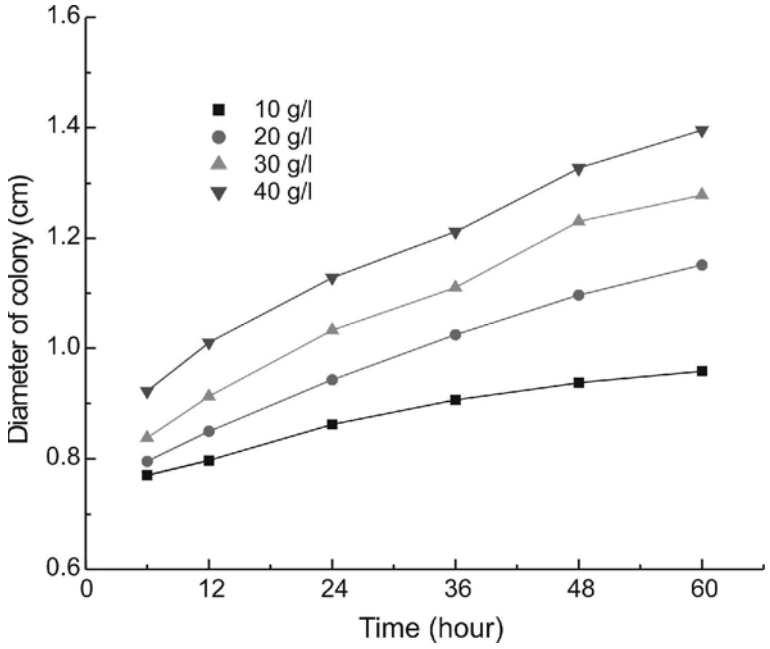

Figure 4.10: Growth rate of colonies under the variation of the peptone concentration and the agar concentration was fixed at 6 g/l.

the colonies show a change from a fingering front with high wave number to a smooth circular front. That is amplitudes of the fingering front decrease with increasing nutrient concentration (Fig. 4.8). The fingering front is slowly obvious with time. The growth rate of colony represented with the space between contours obtained with equal time intervals is quite constant with time as shown in Fig. 4.9. However, the space between contours decreases with decreasing of the peptone concentration. This means that the growth rate

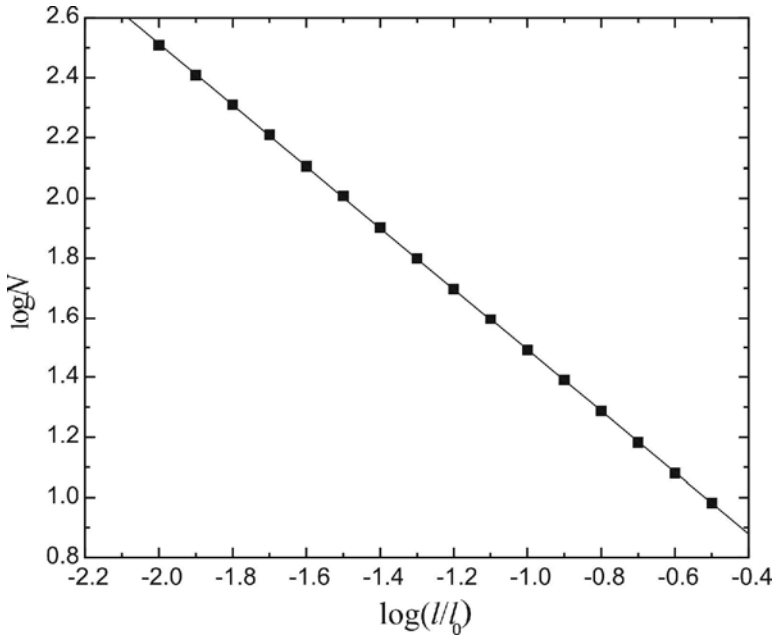

Figure 4.11: An example of log-log plot to find fractal dimension. The agar and peptone concentration are 6 and 10 g/l, respectively. The used colonial boundary is 60th hour.

decreases with decreasing of the peptone concentration. It is corresponding to the result in Fig. 4.10, which is a graph of the diameter of colonies as a function of time. The value of growth rate is measured quantitatively from the slope of the linear fitted lines. The growth rates are 25.0 ± 2.0, 57.3 ± 2.1, 68.7 ± 4.7, and 77.6 ± 2.9 μm/hour for the peptone concentrations of 10, 20, 30, and 40 g/l, respectively. By applying the structured walk method, fractal dimension of colonial front at 60th hour is obtained from the slope of log-log plot as shown in Fig. 4.11. We found that instabilities of the colonial front at 60th hour decrease linearly with increasing of peptone concentration as shown in Fig. 4.12. Fractal dimensions are 1.02187, 1.02018, 1.01232, and 1.00448 for the peptone concentrations of 10, 20, 30, and 40 g/l, respectively. In conclusion, the growth rate of colonies decreases with decreasing of the nutrient concentration. In addition, the final sizes of colony explicitly increase, if the peptone concentration is increased.

4.2.2 Variation of the agar concentration

Instabilities of colonial front under the variation of agar concentration are shown in Fig. 4.13. If the agar concentration is increased (i.e., increasing of the medium hardness), the colonies change from a smooth front to a fingering front. For the agar concentration

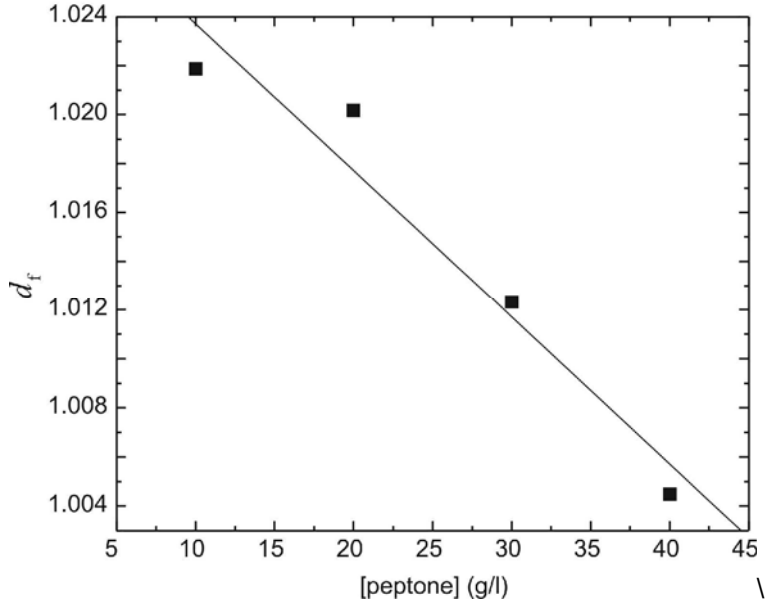

Figure 4.12: Fractal dimension, d_f as a function of peptone concentration.

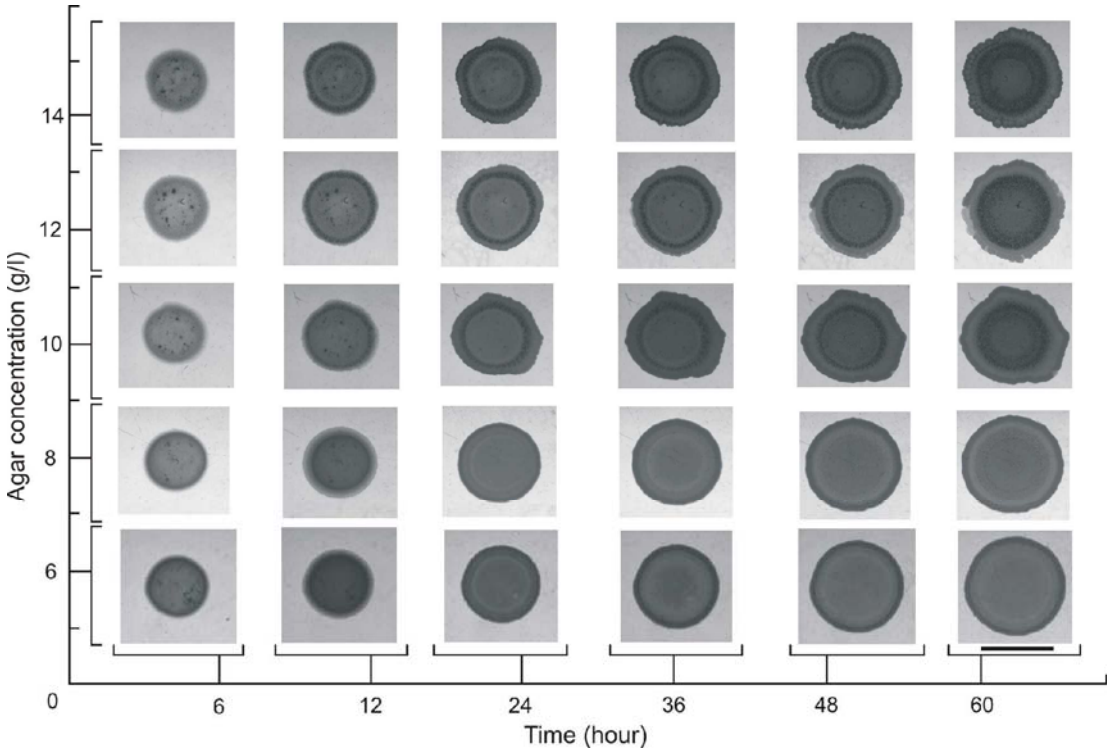

Figure 4.13: Instabilities of the colonial front under the variation of the agar concentration. The peptone concentration was fixed at 40 g/l. Initial cell density was 1.85 x 10^9 cfu/ml (Inoculated volume was 5 μl). The incubation temperature was 37°C. Scale bar: 1 cm.

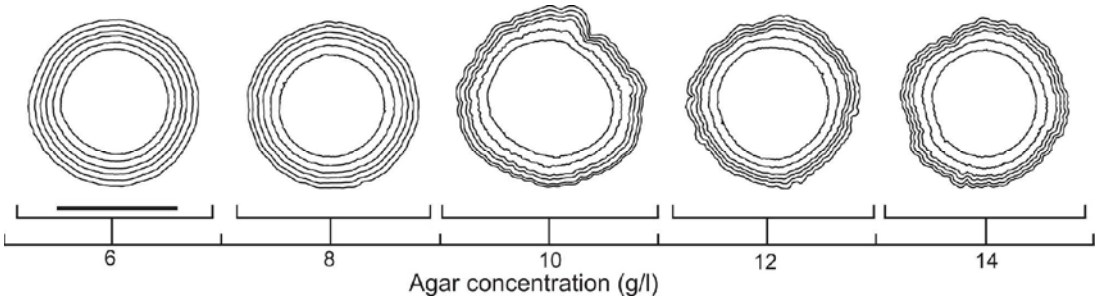

Figure 4.14: Contours of colonial growth under the variation of the agar concentration with fixed peptone concentration (40 g/l). The innermost to outermost contours represent the fronts at 6th, 12th, 24th, 36th, 48th, and 60th hour, respectively. Scale bar: 1 cm.

about 10-14 g/l, the fingering front grows slowly with time, but the growth rate is not constant (Fig. 4.14). It slows down after 24 hour, as the space between contours decreases with time. The growth rate of colonies is quantitatively measured as shown in Fig. 4.15. For the agar concentrations equal to 6, 8, 10, 12, and 14 g/l, the growth rates are 79.0 $\pm$ 2.5, 71.7 $\pm$ 2.7, 52.0 $\pm$ 6.2, 52.9 $\pm$ 5.9, and 50.0 $\pm$ 3.0 μm/hour, respectively. The growth rate of colonies can be divided into 2 groups: high growth rate (6 and 8 g/l of agar concentration)

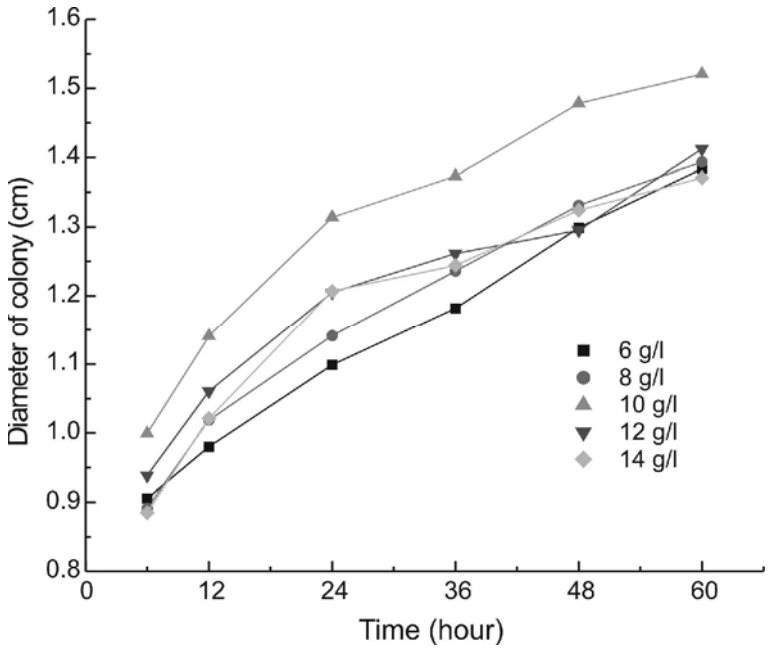

Figure 4.15: Growth rate of colonies under the variation of the agar concentration and the peptone concentration was fixed at 40 g/l.

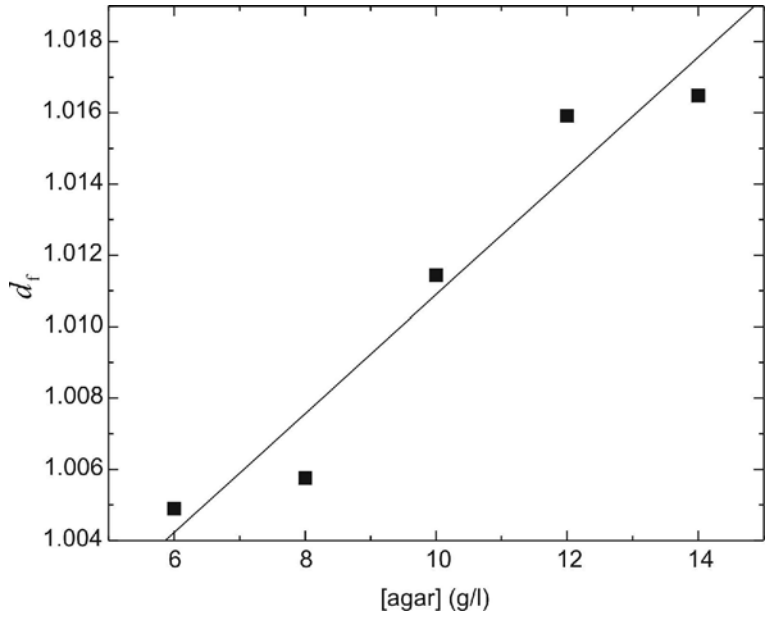

Figure 4.16: Fractal dimension, d_f as a function of agar concentration.

and low growth rate (10, 12, 14 g/l of agar concentration). However, The final sizes of colonies are not so different although the growth rates in 2 groups are different. We found that fractal dimension increases with increasing of agar concentration as shown in Fig. 4.16. That means instabilities of the colonial front increase with increasing of agar concentration. Fractal dimensions are 1.00489, 1.00575, 1.01144, 1.01591, and 1.01649 for the agar concentrations of 6, 8, 10, 12, and 14 g/l, respectively. In conclusion, the growth rate of colonies decreases, if the agar concentration is increased.

4.2.3 Microscopic observations of the alignment of cells of colonial pattern

In this work, the microscopic study is applied to understand an alignment of bacterial cells inside the colony and to investigate the stability and instability of colonial front. The alignments of cells in case of smooth and fingering colonial fronts are investigated by using scanning electron microscope (SEM). It can be divided into 3 parts: i) the smooth circular front, ii) the rough colonial front under the condition of hard medium with rich nutrient, and iii) the rough colony under the condition of soft medium with poor nutrient.

4.2.3.1 Smooth colonial front

The stable colonial front, smooth circular colony, shown in Fig. 4.17 was found on

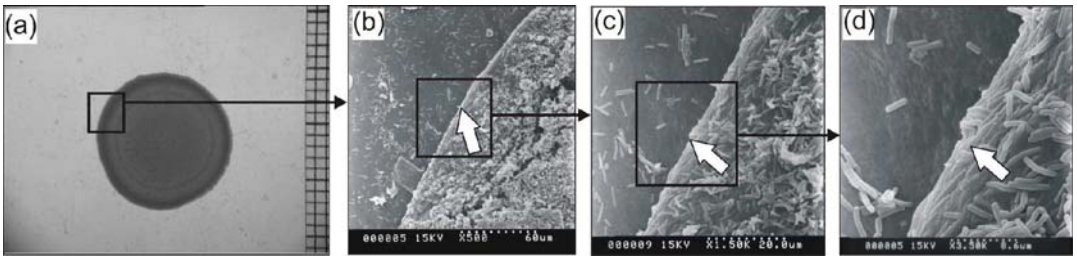

Figure 4.17: Microscopic observations of the stable front of *Bacillus subtilis* TISTR 008 colony. (a) is the picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. The concentrations of agar and peptone are 6 and 40 g/l, respectively. (b), (c), and (d) are the SEM pictures with the magnification of 500, 1500, and 3500, respectively. Initial cell density is 1.78×10^9 cfu/ml.

soft medium (6 g/l of agar) with rich nutrient (40 g/l of peptone). The colony forms a dark ring with a thickness of about 1 mm at the boundary, but the colony inside is brighter. In experiment, the colonial pattern was observed through transmission light of LED light source. It means that if the cells are compact, the image is dark. So, it can be concluded that the cell density at the dark ring is higher than the cell density inside the nutrient concentration. For microscopic observation, the SEM pictures reveal that the boundary of colonial front consists of bacterial cells connected with each other and aligned parallel to the circumference (indicated by white arrows in Figs. 4.17). In contrast, inside the colony the bacterial cells align randomly (lower right corner of Fig. 4.17(c-d)).

4.2.3.2 Rough colonial front under the condition of hard agar medium and rich nutrient

The unstable colonial front, a fingering pattern, at the condition of 10 g/l of agar and 40 g/l of peptone is shown in Fig. 4.18. The cell density of colony at the front is higher than the cell density inside the colony because the front is darker than the inside, similar to the result in Fig. 4.17. Microscopic observations reveal that the colonial front consists of bundles of chains of bacterial cells as shown in Figs. 4.18(h) and (k). These bundles meander crossing each other and form complex alignment. The bacterial cells inside the colony align randomly as shown in Fig. 4.18(i), similar to the experiment in Fig. 4.17(c). At the colonial front, the SEM results show that the bacterial cells are not compact as the stable colonial front because there are spaces between bundles as shown in Fig. 4.18(d) and (g).

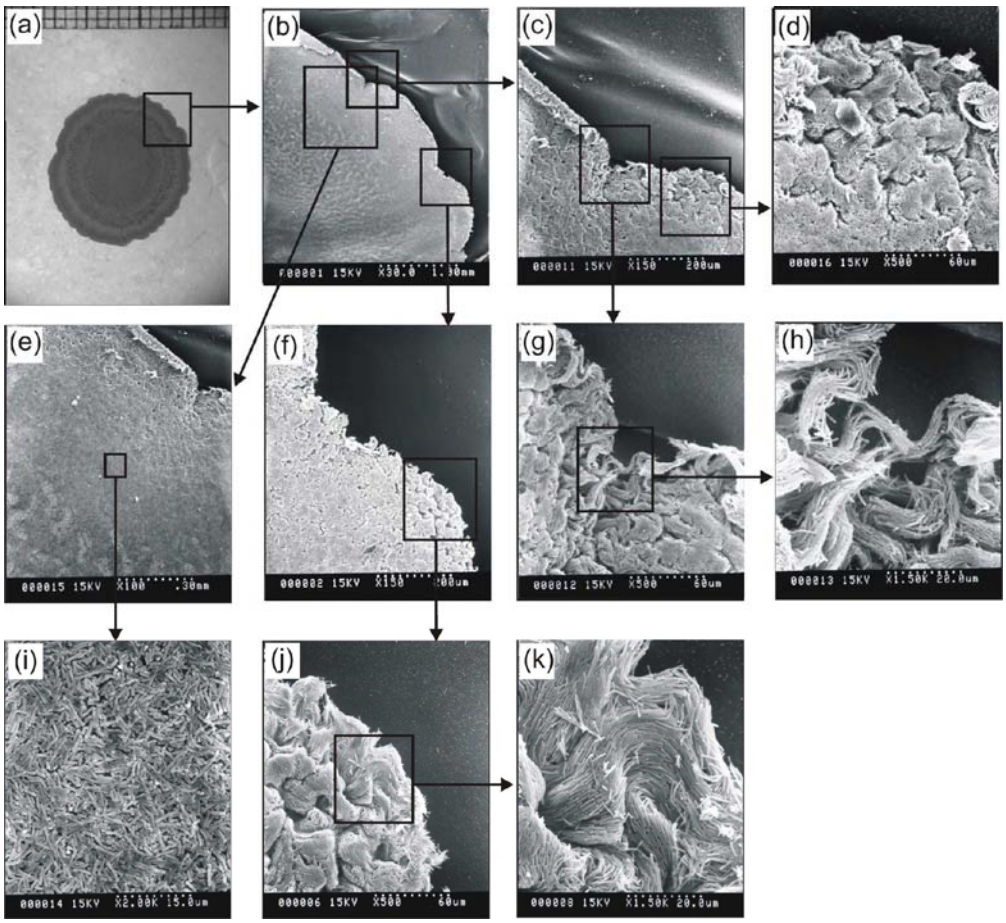

Figure 4.18: Microscopic observations of unstable front *of Bacillus subtilis* TISTR 008 colony under the condition of hard agar medium and rich nutrient. (a) is the picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. The concentrations of agar and peptone are 10 and 40 g/l, respectively. (b), (c), (d), (e), (f), (g), (h), (i), (j), and (k) are the SEM pictures with the different magnification and different part of colony. Initial cell density is 1.86 x 10^9 cfu/ml.

4.2.3.3 Rough colonial front under the condition of soft agar medium and poor nutrient

The unstable colonial front, a fingering pattern, at the condition of 6 g/l of agar and 20 g/l of peptone is shown in Fig. 4.19(a). The front is darker than the inside, similar to the experiment in Figs. 4.17(a) and 4.18(a). The SEM images reveal that bundles of chains of bacterial cells appear similarly to the result in Fig. 4.18.

From the microscopic investigation by using SEM technique, it is plausible to discuss the stability of colonial front in term of cell motility corresponding to expansion rate of front and cell reproduction rate. For the case of the stable colonial front observed under

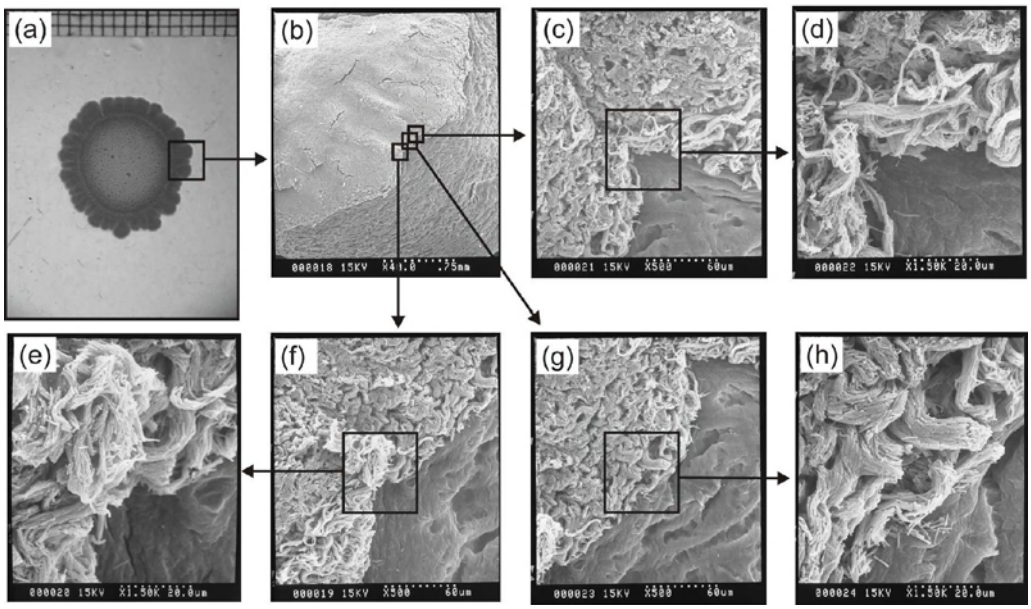

Figure 4.19: Microscopic observations of unstable front of *Bacillus subtilis* colony under the condition of soft agar medium with poor nutrient. (a) is the picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. The concentrations of agar and peptone are 6 and 20 g/l, respectively. (b), (c), (d), (e), (f), (g), and (h) are the SEM pictures with the different magnification and different part of colony. Initial cell density is 1.61 x 10^9 cfu/ml.

the condition of soft medium and rich nutrient, the front expands symmetrically and forms a smooth circular shape. In this case, the expansion rate and cell reproduction rate are compatible. On the other hand, under the condition of low nutrient level or hard medium the SEM images show that the colonial front consists of wavy bundles of chains of bacterial cells. In this case, the expansion rate is lower than that of the first case and does not match the growth rate. Under this reduction in cell motility, cells tend to consume nutrient and multiply themselves locally. Cell reproduction in limited space results in the formation of bundles of chains of cells, which responds to macroscopic instability of colonial front. In general, cell motility is dependent on bacterial density, nutrient concentration, medium hardness, and on the growth rate of cells [15]. Kawasaki et al. remarked also that bacteria can hardly move when either the concentration of nutrient or the density of bacteria are low [61].

4.2.4 Numerical simulation

In this section we demonstrate spatial patterns produced by the total cells u(r,t) + s(r,t). The numerical simulation was performed in two dimensions. It is noted that the varied parameters are d_1 and k, which are corresponding to nutrient and hardness of medium in our experiments. We take d_v = 1.00, ε = 1.00, a_0 = 1.00, a_1 = 0.000416, a_2 = 0.008333, b_1 = 4.0, and b_2 = 2.8. Numerical results can be divided into 2 parts: the variation of d_1 with fixing of k and the variation of k with fixing of d_1.

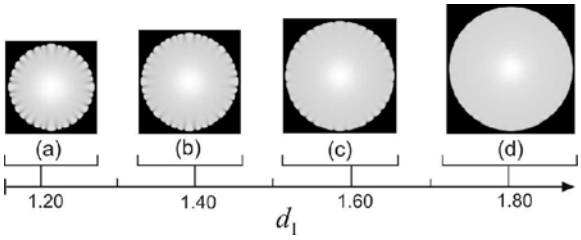

Figure 4.20: Patterns produced by numerical simulations under the variation of d_1. The values of v_0 were 1.00, 1.05, 1.10, and 1.15 for the simulation in (a)-(d), respectively. The value of k was fixed at 1.00. The values of d_v, ε, a_0, a_1, a_2, b_1, and b_2 are 1.00, 1.00, 1.00, 0.000416, 0.008333, 4.0, and 2.8, respectively.

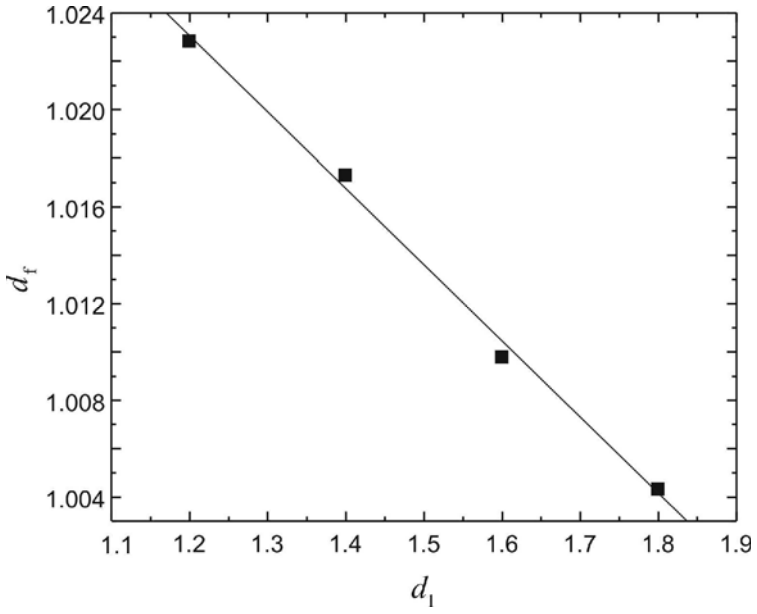

Figure 4.21: Fractal dimension, d_f as a function of d_1.

4.2.4.1 Variation of nutrient, d_1

For small values of both k and d_1 as a function of d_1 (soft medium with poor nutrient), we found the fingering pattern with high roughness of boundary as shown in Fig. 4.20(a). Noted that the white color represents the bacterial density, whereas the black one is the medium. The value of d_1 is increased resulting in the change of fingering pattern to a smooth circular one as shown in Fig. 4.20(d). The final size of colony clearly increases with increasing of d_1. We found that the model can satisfactorily reproduce the experimental

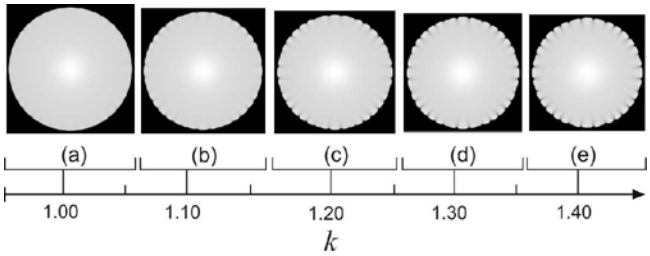

Figure 4.22: Patterns produced by numerical simulations under the variation of k. The value of v_0 and d_1 was 1.15 and 0.18, respectively. The values of d_v, ε, a_0, a_1, a_2, b_1, and b_2 are 1.00, 1.00, 1.00, 0.000416, 0.008333, 4.0, and 2.8, respectively.

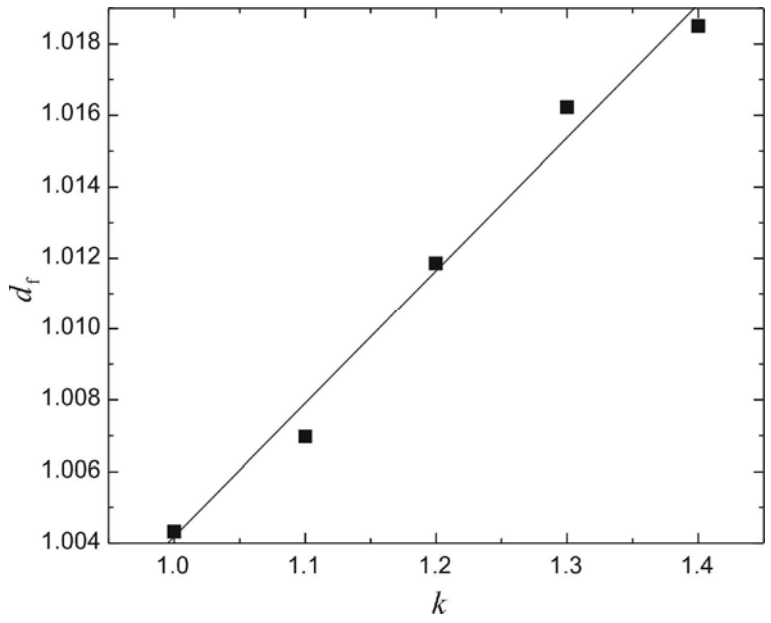

Figure 4.23: Fractal dimension, d_f as a function of k.

results of variation of peptone concentration shown in Fig. 4.8. The fractal dimension

representing instabilities of the colonial front as a function of d1 is shown in Fig. 4.21. We found that the instabilities of colonial front linearly decrease with increasing of d1. Fractal dimensions of colonial patterns are 1.02284, 1.01731, 1.00980, and 1.00433 for the values of d1 of 1.2, 1.4, 1.6, and 1.8, respectively. Instabilities of colonial fronts in variation of dare corresponding to that of peptone concentration in the experiment as shown in Fig. 4.24. It can be concluded that the numerical result of variation of d1 is corresponding to the experimental result of variation of peptone concentration not only the patterns, but also their fractal dimension.

4.2.4.2 Variation of medium hardness, k

In this part the values of d_1 and v_0 are fixed at 1.80 and 1.15 (rich nutrient). For the small value of k (soft medium), the colony represents a smooth circular pattern as shown in Fig. 4.22(a). The colonial pattern changes from the smooth circular front to the fingering pattern, when the value of k is increased. The final sizes of colony are not clearly different with increasing of k. It reproduces the experimental results of variations of agar concentration shown in Fig. 4.13. We found that instabilities of the colonial front are linearly increased with increasing of k as shown in Fig. 4.23. The fractal dimensions of the colonial

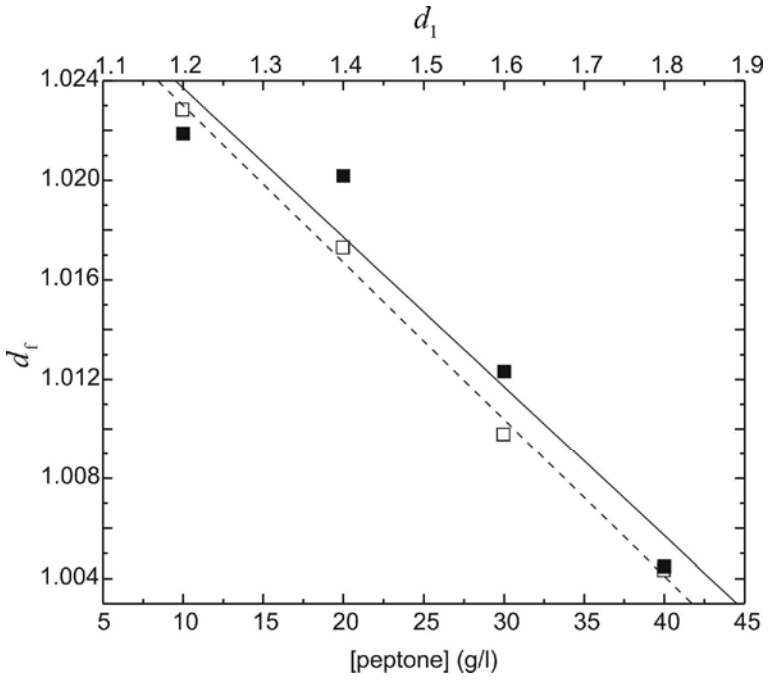

Figure 4.24: Fractal dimension, d_f as a function of peptone concentration and d_1. Black squares represent the experimental results. Open squares represent the numerical results.

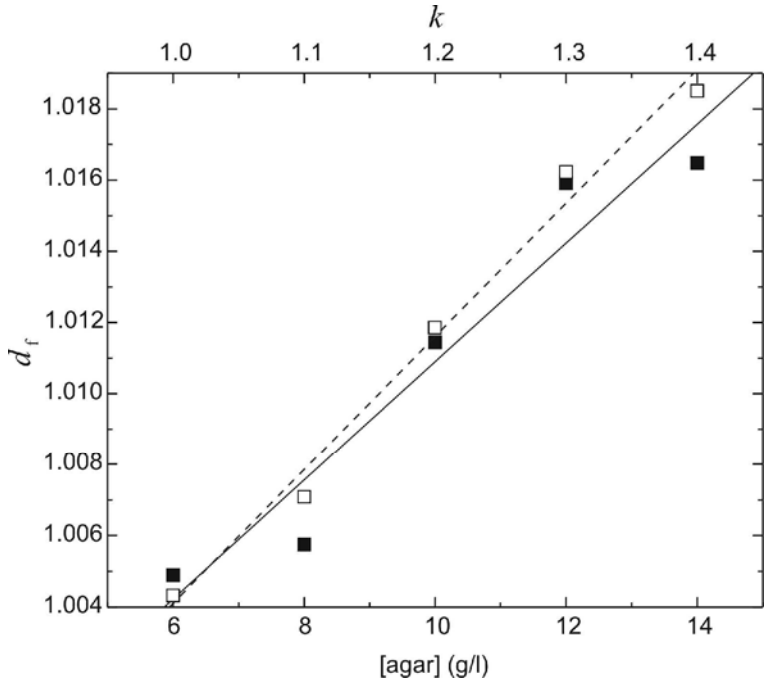

Figure 4.25: Fractal dimension, dfas a function of agar concentration and k. Black squares represent the experimental results. Open squares represent the numerical results.

pattern are 1.00433, 1.00709, 1.01186, 1.01623, and 1.01851 for the values of k of 1.0, 1.1, 1.2, 1.3, and 1.4, respectively. Comparing with the experimental result of variation of agar concentration, we found that instabilities of colonial fronts in variation of k are corresponding to that in experimental one as shown in Fig. 4.25. It can be concluded that the numerical model can reproduce the experimental result of variation of agar concentration.

4.3 Formation of the spreading colony

The results in this section can be divided into 6 parts: i) the onset of the spreading colony, ii) the morphological diagram of spreading bacteria, iii) the biochemical test and DNA sequencing, iv) characteristics of the bacteria of the spreading pattern, v) the alignment of cells of the spreading colony, and vi) swarming of bacterial cells.

4.3.1 Onset of the spreading colony

The dynamics of the spreading colony are shown in Fig. 4.26. Initially, the bacterial species of B. subtilis TISTR 008 is inoculated on the center of agar plate. The agar and peptone concentrations are 6 and 40 g/l, respectively. Then, the colony slowly grows and forms a

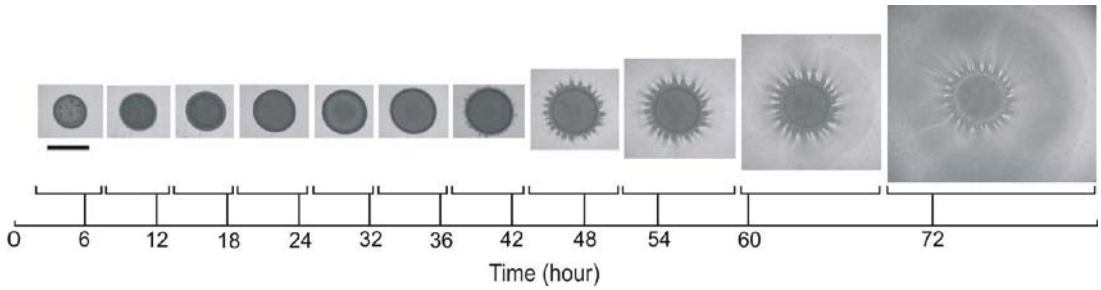

Figure 4.26: Growth of the colony under the condition of 6 g/l of agar concentration and 40 g/l of the peptone concentration. Initial cell density was 2.23 x 10^9 cfu/ml. Inoculation volume was 5 μl. Scale bar: 1 cm.

compact colony with the smooth front for the period of 6-36 hours. Then, the front of the colony bursts and forms a sun-like pattern. The population density of bacterial cells may reach some critical values, when the colony bursts. After that, the bursting parts spread and then cover the agar surface quickly. Finally, the homogeneous thin-film of bacteria appears over the agar surface at 72 hours. This phenomena is called swarming [25, 71], which is one type of the bacterial translocation. It normally occurs, if the surface of medium is moist. In our work, the spreading colony robustly appeared in the condition of soft medium (6 g/l of agar concentration) with rich nutrient (10-50 g/l of peptone concentration). We found that the number of the spreading colony is decreasing with decreasing of peptone concentration.

4.3.2 Morphological diagram of spreading bacteria

After the formation of the spreading colony, bacterial cells in the region of spreading part were streaked in TSA medium to isolate the colonies. The isolated colonies of bacterial cells of the spreading part are shown in Fig. 4.27(a). Comparing with isolated colonies from stock bacteria shown in Fig. 4.27(b), we found a difference between these two types of isolated colonies. The isolated colonies of the spreading part are thinner than that of the stock. It is noted that bacterial cells of the stock and that of spreading part are labelled as B1 and B2, respectively.

In order to understand the growth of B2, the variations of agar and peptone concentrations are applied. The morphological diagram of bacteria B2 is shown in Fig. 4.28. From this diagram, the colonial patterns can be divided into 3 types:

Region A: When the agar medium is soft (6 g/l of agar concentration), colonies are

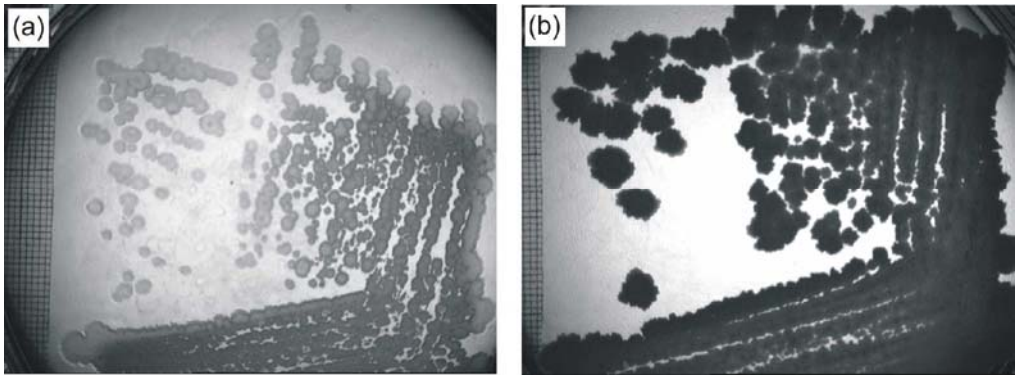

Figure 4.27: Isolated colonies on the surface of Tryptic Soy Agar (TSA) medium. (a) shows an isolated colonies obtained form the spreading part (B2). (b) presents an isolated colonies obtained form the stock (B1). The incubation temperature was 37 C. These pictures were taken at 24 hours after incubation.

busting and spread over the agar surface.

Region B: Colonial patterns describe a compact pattern similar to the one shown in Fig. 4.1 (region C).

Region C: Colonies describes a fine-branching pattern similar to the result shown in Fig. 4.1 (region B).

Comparing with the morphological diagram of B1 shown in Fig. 4.1, the cell density represented by a gray level in the morphological diagram of B2 are not dense although the initial cell density of inoculation are in the same order. There is no spreading colony in the condition of hard medium (10-14 g/l of agar concentration) similar to the morphological diagram of B1 shown in Fig. 4.1. It can be concluded that the spreading of the bacterial colony occurs in the soft medium.

The colonial spreading of B2 was investigated in a function of time in the condition of soft medium with rich nutrient as shown in Fig. 4.29. We found that the colony of B2 forms the smooth circular colony at 6 hours. Then, the colony bursts at 24 hours. Finally, the bursting parts spread quickly over the agar surface. Comparing to the bursting time of B1, the bursting time of B2 (about 24 hours) is quicker than that of B1 (about 48-60 hours).

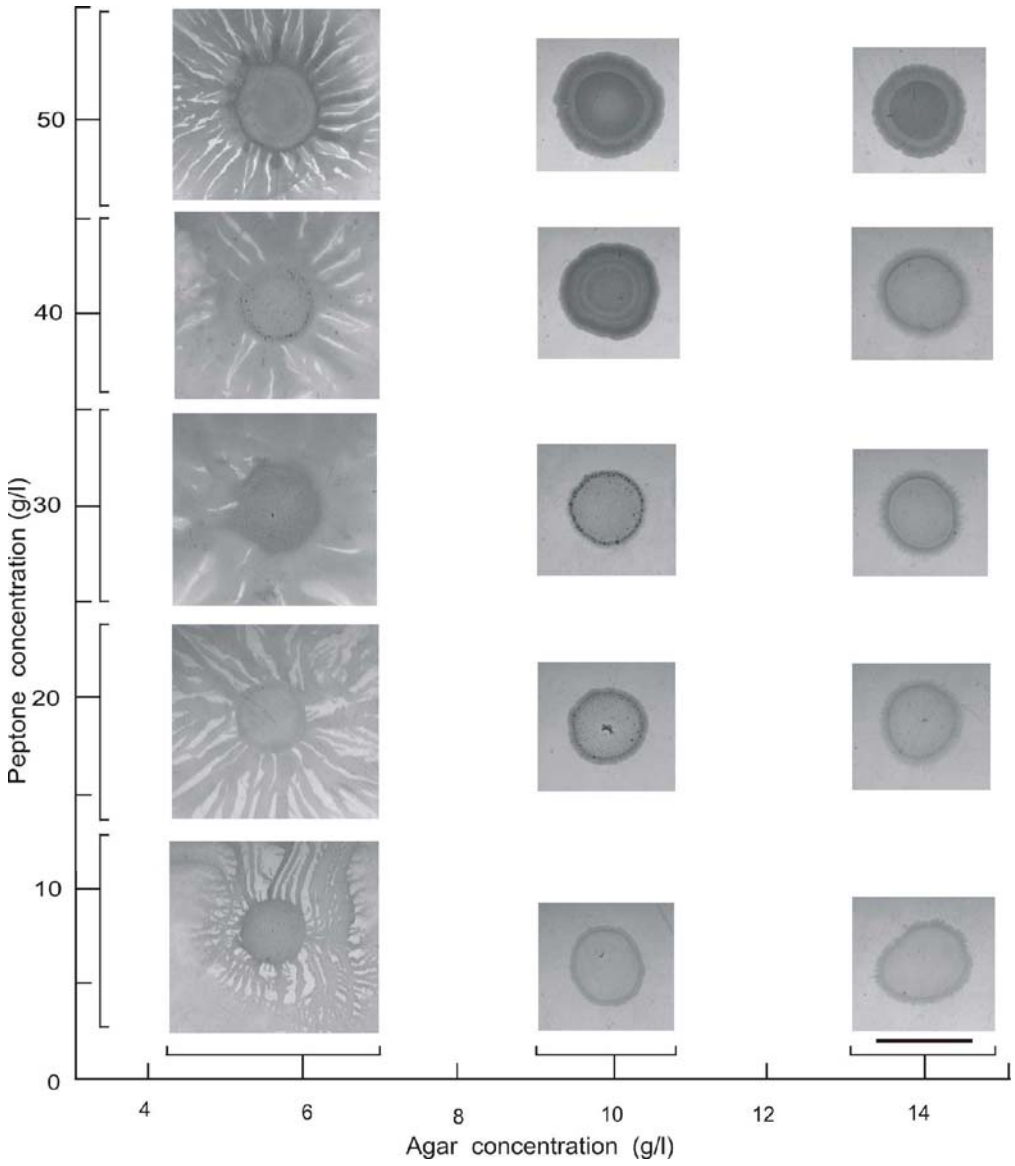

Figure 4.28: Morphological diagram of bacteria B2. The incubation temperature was 37°C. Initial cell density was 1.94 x 10^9 cfu/ml. Inoculation volume was 5 µl. All pictures were taken at 48 hours. Scale bar: 1 cm.

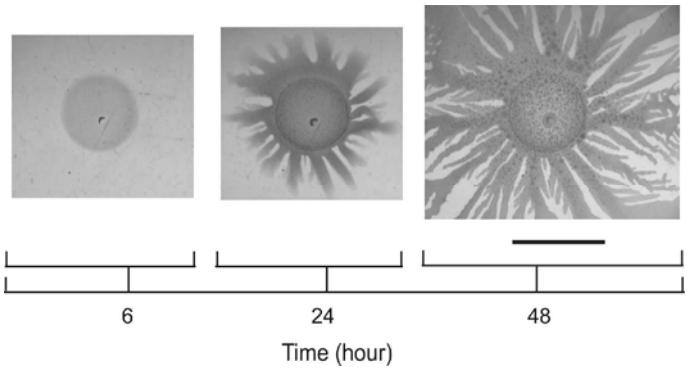

Figure 4.29: Spreading colony on the surface of agar plate with 6 g/l of agar concentration and 40 g/l of peptone concentration.

4.3.3 Biochemical test and DNA sequencing

After reading Ben-Jacob et al.'s publication [68, 69], the suspect of bacterial mutation arise. In their work, the sector formation of expanding bacterial colonies is studied.

Figure 4.30: Result of DNA sequencing of B1 and B2.

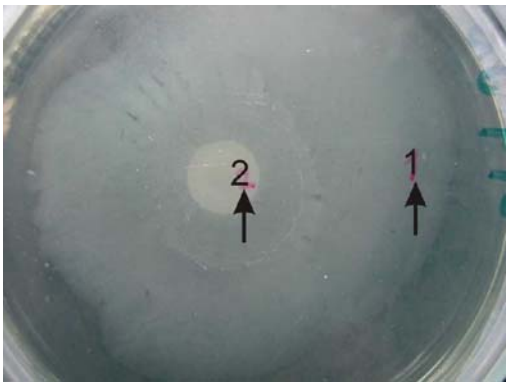

Figure 4.31: Spreading colony on the surface of agar plate with 6 g/l of agar concentration and 40 g/l of peptone concentration. The incubation temperature was 37°C. The number 1 and 2 are corresponding to the bacteria of B2 and B1, respectively. These pictures were taken at 48 hours after incubation.

They suggest that if the mutant bacteria have the same growth dynamics as the normal ones, they will be usually unnoticed after growing of the bacterial colony. Ben-Jacob et al. also suggest that if the bacterial mutants have different growth dynamics, the presence of mutant cells is indicated by the distinguished sector with a different growth pattern. In our results, we found different growth patterns, i.e., changing from the slow growth of the smooth colony to the spreading one. Therefore, in order to prove whether the onset of spreading formation occurred from the mutation of bacterial species or not, the 16s rDNA technique and biochemical test were applied to classify the bacterial cells of B1 and B2. The result of DNA sequencing (Fig. 4.30) reveals that DNA sequences of B1 and B2 are not different. This means that species and strain of B1 and B2 are same. The results of biochemical test (see Appendix III) present that there are some differences, but the species of two types are concluded to be the same species. Thus, it can be concluded that there is no mutation in bacteria, when the spreading colony occurs.

4.3.4 Characteristics of the bacteria of the spreading pattern

In order to investigate the di erent characteristics of B1 and B2, two types of bacteria species were grown on the same medium. The experiments can be divided into 2 parts: i) B1 and B2 obtained from the same medium with di erent part as shown in Fig. 4.31, ii) B1 and B2 obtained from their isolated colonies on TSA medium. In these experiments, the agar and peptone concentrations are 6 and 40 g/l, respectively. In two

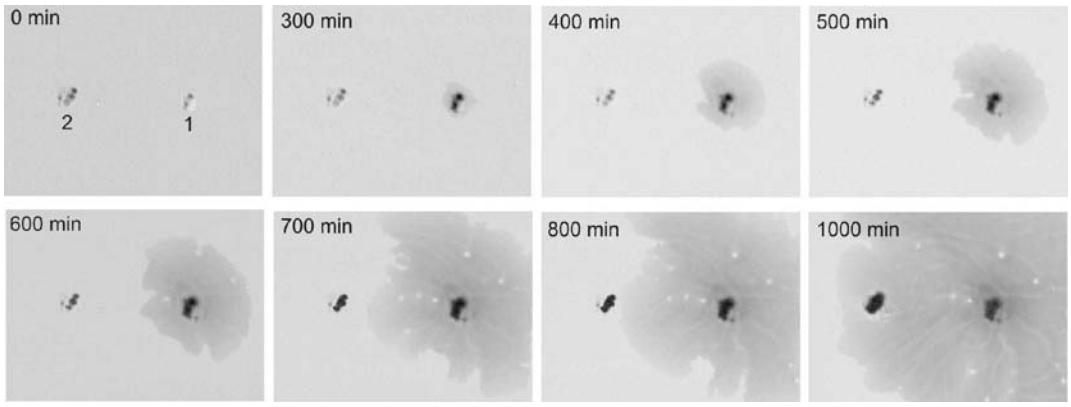

Figure 4.32: Snapshots of colonial growth on the agar plate with 6 g/l of agar concentration and 40 g/l of peptone concentration. B1 and B2 obtained from the same medium. The number 1 and 2 are corresponding to the bacteria of B2 and B1, respectively. The incubation temperature was 37°C. The first picture was taken after incubating for 2 hours.

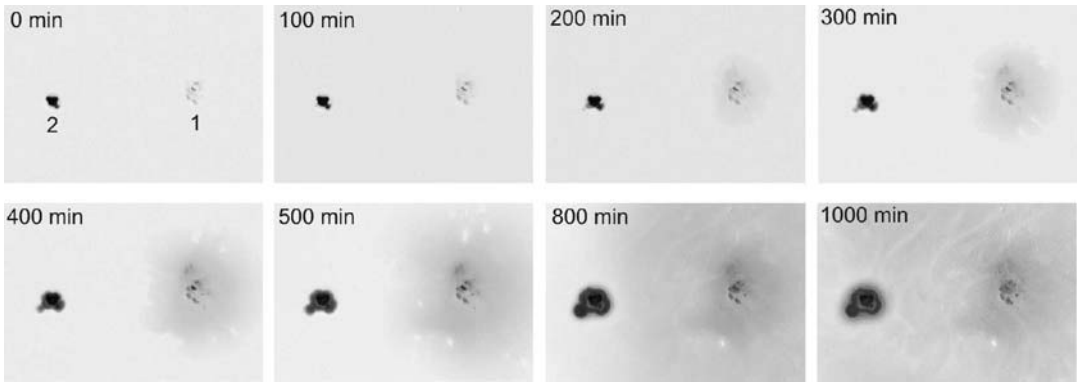

Figure 4.33: The snapshots of colonial growth on the agar plate with 6 g/l of agar concentration and 40 g/l of peptone concentration. B1 and B2 obtained from their isolated colonies. The incubation temperature was 37°C. The number 1 and 2 are corresponding with the bacteria of B2 and B1, respectively. The first picture was taken after incubating for 3 hours.

experiments, B1 and B2 were inoculated by streaking about 2.5 cm far form each other. The experimental results of 2 parts are shown in Fig. 4.32 and 4.33, respectively. The results of two parts represent that there is a lag phase in the period of about 6 hours. Then, the bacterial colony of B2 (labelled as number 1 in Figs. 4.32 and 4.33) grows and spreads

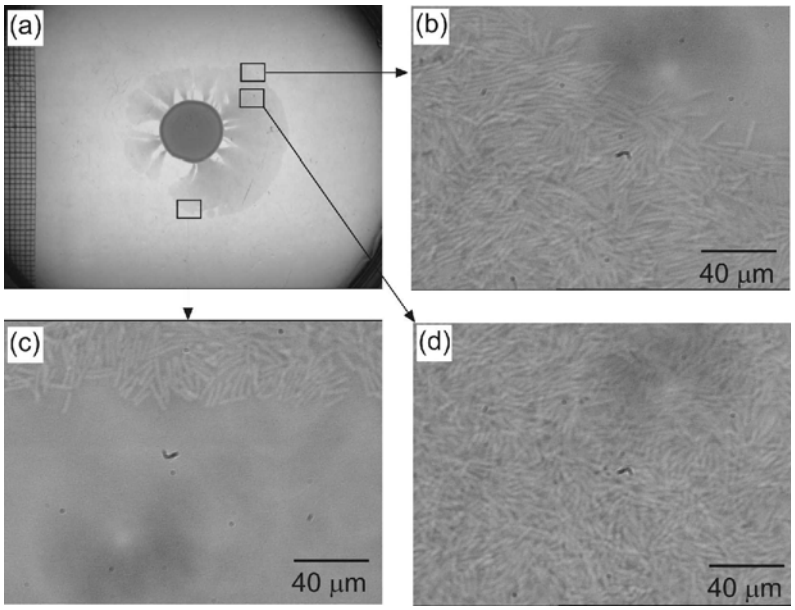

Figure 4.34: Microscopic observations of spreading colony of *Bacillus subtilis* TISTR 008 under the condition of 6 g/l of agar concentration and 40 g/l of peptone concentration. (a) is the picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. (b), (c), and (d) are the pictures obtained from an optical microscope at magnification of 400. Initial cell density is 1.92×10^9 cfu/ml.

quickly, comparing to B1 (labelled as number 2). Finally, bacteria of B2 spread over the agar surface. In these results, we found that B2 are not form a compact colony before spreading, but tend to spread quickly after the lag time (about 6 hours).

4.3.5 Alignment of cells of the spreading colony

The microscopic observation of spreading colony by using an optical microscope is shown in Fig. 4.34. Macroscopically, the cell density represented by a gray level of spreading parts is lower than that of inside of colony clearly as shown in Fig. 4.34(a). Microscopic observations of spreading parts reveal that the alignment of bacterial cells is random (Figs. 4.34(b), (c) and (d)). We found that the bacterial colony in the spreading parts consist of a monolayer of cells. Comparing with the cells inside the colony, the swarming cells are very active and move fast.

4.3.6 Swarming

Swarming is a special form of bacterial surface translocation dependent on the

condition of the surface. This type of translocation was observed in the bacterial species of *B. subtilis*, *P. mirabilis*, *S. marcesens* [71]. It is known that the critical factors of swarming of bacterial cells are the concentration of agar and surface tension. D. B. Kearns et al. reported that *Bacillus subtilis* can exhibit swarming motility on the surface of the medium containing low agar concentration with 0.3 - 1.0% [72]. Corresponding to our work, the colony of *Bacillus subtilis* TISTR 008 is robustly spreading on the condition of soft medium (0.6% of agar concentration). M. Sharma et al. explained that the normal cells (vegetative cells) transition into hyper-agellated swarming ones as the swarming behavior occurred [71].

In 2004, M. B. Connelly et al. [73] studied the swarming Motility in Bacillus subtilis. Bacteria of *B. subtilis* with various strains were used in the study. They found that gene named Epr, a minor extracellular serine protease gene, plays an important role in swarming. They also found that if the strain of *Bacillus subtilis* is different, time for an initiated swarming is different as well.

It can be concluded that the swarming of cells is dependent on the concentration of agar and bacterial species.

5. CONCLUSION AND OUTLOOK

Under variations of agar and peptone concentrations, we found various colonial patterns: a fractal pattern, a fine branching pattern, a fingering pattern, a smooth circular pattern, and a spreading pattern. Microscopic observations reveal that the boundary of a smooth circular colony consists of bacterial cells connected with each other and aligned parallel with the circumference. The colonial front consists of bundles of chains of bacterial cells and these bundles meander crossing each other and form complex alignment in case of a fingering colony. Cell reproduction in limited space resulting in the formation of bundles of chains of cells is a cause of instabilities of colonial fronts.

Instability of propagating front is linearly increased with decreasing of peptone concentration and/or increasing of agar concentration. The proposed model can reproduce the experimental results of instabilities not only patterns, but also the roughness of colonial fronts.

The spreading or swarming colony is robustly obtained in the condition of soft medium with rich nutrient. Time for initiated swarming in *Bacillus subtilis* TISTR 008 is about 48-60 hours.

The outlook of this research is the study of swarming or spreading colony numerically. In order to describe such a behaviour, we suggest that there may have a critical population density of bacterial cells for the onset of spreading colony.

The other outlook is the study of pattern formation formed by cocci bacteria, such as *Staphylococcus aureus*. It is interesting because there is no report about patterns formed by this type of bacteria.

Additionally, reproduction of all patterns in morphological diagram of *Bacillus subtilis* TISTR 008 by using the proposed model can be considered to be a future work.

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ
    - T. Somboon, C. Napasawad, C. Yoosook, and O.-U. Kheowan, “Instabilities in propagating front formed by *Bacillus subtilis* colony.”, Physica D, submitted.
    - T. Somboon, C. Napasawad, C. Yoosook, and O.-U. Kheowan, “Bifurcation of the bacterial translocation in a colony of *Bacllus subtilis* TISTR 008.”, Physica A, in preparation.

2. การนำผลงานวิจัยไปใช้ประโยชน์
    - เชิงพาณิชย์ –
    - เชิงนโยบาย –
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    - Titikan Somboon, Chanita Napasawad, Chalobon Yoosook, and On-Uma Kheowan, Onset of instabilities in propagating bacterial fronts. การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. 13-15 ตุลาคม 2549, ณ โรงแรมรีเจนท์ ชะอำ จ.เพชรบุรี
    - Titikan Somboon, Chanita Napasawad, Chalobon Yoosook, and On-Uma Kheowan, Onset of instabilities in propagating bacterial fronts. การประชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว. 11-13 ตุลาคม 2550 ณ โรงแรมแอมบาสเดอร์ ชลบุรี จ.ชลบุรี
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Abstract: Depending on environmental conditions, colonies of bacterial species Bacillus subtilis TISTR 008 grown on the surface of agar mediums show patterns such as a smooth circular pattern or fingering pattern. The fingering forming or instability of colonial fronts is indicated by the roughnesses of the boundary of the bacterial colony. Hence, the instabilities can be determined quantitatively by means of the fractal dimension of the colony boundary. Experimental results show that the instabilities of propagating colonial fronts are linearly increased with decreasing of peptone concentration and/or increasing of agar concentration. Our microscopic observations reveal that cell reproduction in a limited space resulting in the formation of bundles of chains of bacterial cells causes a nonlinear diffusion and therefore the instability in propagating fronts. The proposed simulation model including the nonlinear diffusion successfully reproduces the experimental results both qualitatively and quantitatively. Furthermore, the instabilities can be discussed in terms of the ratio of diffusion coefficients for nutrient and bacterial cells. We found that the colony front can be stabilized, if this ratio decreases.

Dear Editors,

we are enclosing a manuscript by T. Somboon et al. entitled “Instabilities in propagating front formed by Bacillus subtilis colony, which we would like to submit to Physica D, Special Issue: Emerging Phenomena.

Pattern formation, fascinating phenomena in nonlinear systems, was found in various fields such as, chemistry, physics, and biology. Among these systems, the pattern formation of the bacterial colony is the simplest biological object for studying and understanding the pattern formation in biological systems. It is also easily controlled because its behavior depends directly on physical conditions, such as the nutrient concentration and hardness of the medium.

Although the diversity of colony patterns of Bacillus subtilis has been investigated intensively, however the detailed study about the transition from a simple smooth circular colony to a complicated structural colony is still unrevealed. Therefore, this is the goal of our work. Here we consider an expanding of the colony boundary as a propagating of bacterial front. If a planar front propagates with a uniform velocity, its will remain flat. Otherwise, the front will form a fingering shape, i.e., instability of propagating front. In this paper we focus on the transition from a stable to an unstable propagation of bacterial front. The instabilities of the propagating fronts under variations of medium hardness and nutrient concentration are studied, experimentally and numerically. The reaction-diffusion system with a nonlinear diffusion is proposed in order to explain and confirm the formation of colonial patterns. Finally, we discuss the instabilities in terms of the ratio of diffusion coefficients for nutrient and bacterial cells.

Due to the intensive and systematic investigations of these results we think that Physica D is the appropriate place for publication.

Sincerely yours,

On-Uma Kheowan

Bangkok, March 11, 2009

Instabilities in propagating front formed by Bacillus subtilis colony

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Abstract

Depending on environmental conditions, colonies of bacterial species *Bacillus subtilis* TISTR 008 grown on the surface of agar mediums show patterns such as a smooth circular pattern or fingering pattern. The fingering forming or instability of colonial fronts is indicated by the roughnesses of the boundary of the bacterial colony. Hence, the instabilities can be determined quantitatively by means of the fractal dimension of the colony boundary. Experimental results show that the instabilities of propagating colonial fronts are linearly increased with decreasing of peptone concentration and/or increasing of agar concentration. Our microscopic observations reveal that cell reproduction in a limited space resulting in the formation of bundles of chains of bacterial cells causes a nonlinear diffusion and therefore the instability in propagating fronts. The proposed simulation model including the nonlinear diffusion successfully reproduces the experimental results both qualitatively and quantitatively. Furthermore, the instabilities can be discussed in terms of the ratio of diffusion coefficients for nutrient and bacterial cells. We found that the colony front can be stabilized, if this ratio decreases.

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Instabilities in propagating front formed by *Bacillus subtilis* colony

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Depending on environmental conditions, colonies of bacterial species *Bacillus subtilis* TISTR 008 grown on the surface of agar mediums show patterns such as a smooth circular pattern or fingering pattern. The fingering forming or instability of colonial fronts is indicated by the roughnesses of the boundary of the bacterial colony. Hence, the instabilities can be determined quantitatively by means of the fractal dimension of the colony boundary. Experimental results show that the instabilities of propagating colonial fronts are linearly increased with decreasing of peptone concentration and/or increasing of agar concentration. Our microscopic observations reveal that cell reproduction in a limited space resulting in the formation of bundles of chains of bacterial cells causes a nonlinear diffusion and therefore the instability in propagating fronts. The proposed simulation model including the nonlinear diffusion successfully reproduces the experimental results both qualitatively and quantitatively. Furthermore, the instabilities can be discussed in terms of the ratio of diffusion coefficients for nutrient and bacterial cells. We found that the colony front can be stabilized, if this ratio decreases.

1. Introduction

Recently, much attention has been paid to pattern formation, which is fascinating phenomena in nonlinear systems. Pattern formation was found in various fields such as, chemistry, physics, and biology [1]. Spiral pattern in the Belousov-Zhabotinsky (BZ) reaction is a famous example in chemical systems [2]. Such a pattern can also be observed in biological systems, such as in an aggregation of slime mold *Dictyostelium discoideum* [3]. Patterns in nonlinear systems are created by an internal dynamics of the systems, this process of pattern formation is called self-organization. Such a process can only occur far from thermodynamic equilibrium [4]. Self-organizations occur in an open system, which has a flow of matter and energy between the system and environment that results in non-maximizing of entropy of the system. The complexity and diversity of self-organizing systems can be mathematically explained by using nonlinear equations, such as the reaction-diffusion equations [5]. The reaction-diffusion systems can be ap-

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plied to model and explain the complexity of pattern formation in biological systems in various scales, for example, calcium wave propagating in a rat retina [6], formation of the bacterial colony [7–10], and the Turing pattern on animal skins [11,12]. Among these systems, the pattern formation of the bacterial colony is the simplest biological object for studying and understanding the pattern formation in biological systems [13]. It is also easily controlled because its behaviour depends directly on physical conditions, such as the nutrient concentration and hardness of the medium [9].

In particular, bacteria species *Bacillus subtilis* is known to exhibit various colonial patterns such as, diffusion limited aggregation (DLA)-like, Eden-like, concentric ring-like, and disk-like, depending on the substrate softness and nutrient concentration [7,9,13,14]. Such patterns can be modelled by using reaction-diffusion system [15,16]. In the colonial formation, the complexity of the colonial growth generally increases as the environmental conditions become less favorable. The bacterial colony responds to adverse conditions by developing a strategy and organizing in a micro-level to cooperate the more efficiency of survival [17]. Various patterns can be observed, depending on both bacterial species and environmental conditions [18].

Although the diversity of colony patterns [7,9,13–16] of *Bacillus subtilis* has been investigated intensively, however the detailed study about the transition from a simple smooth circular colony to a complicated structural colony is still unrevealed. Therefore, this is the goal of our paper. Here we consider an expanding of the colony boundary as a propagating of bacterial front. If a planar front propagates with a uniform velocity, its will remain flat. Otherwise, the front will form a fingering shape, i.e., instability of propagating front. In this paper we focus on the transition from a stable to an unstable propagation of bacterial front. The instabilities of the propagating fronts under variations of medium hardness and nutrient concentration are studied, experimentally and numerically. The reaction-diffusion system modified from Mimura's model [16] with the nonlinear diffusion is proposed in order to explain and confirm the formation of colonial patterns. Finally, we discuss the instabilities in terms of the ratio of diffusion coefficients for nutrient and bacterial cells.

2. Experimental method

The used bacterial species was *Bacillus subtilis* TISTR 008. It was obtained from the Thailand Institute of Scientific and Technological Research. This bacterial species is commonly found in soil and able to form a protective endospore via a spore formation process under inappropriate living conditions. The experimental methods were carried out under 2 conditions: i) the variation of peptone concentration and ii) the variation of agar concentration. In the first case, the concentration of peptone were varied for 4 values: 10, 20, 30, 40 g/l, whereas the agar concentration was fixed at 6 g/l. For the later case, there were 5 concentrations of agar: 6, 8, 10, 12, 14 g/l, with fixed peptone concentration at 40 g/l. The temperature was kept constant at 37°C.

A solution for agar plate preparation consists of 5 g of NaCl, 2.397 g of K_2HPO_4, 0.68 g of KH_2PO_4, an amount of Bacto Peptone (Difco Laboratories), and Bacto Agar (Difco Laboratories) in 1 liter of distilled water. The final pH of the solution was 7.0 ± 0.2. The mixture was sterilized by using an autoclave at the temperature of 121°C for 15 minutes,

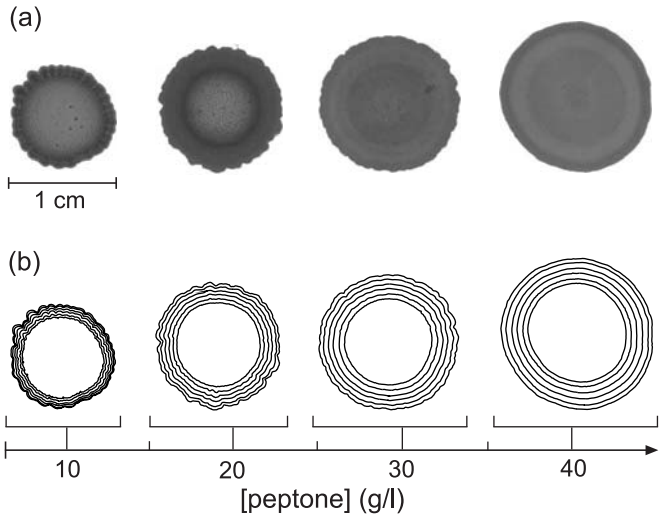

Figure 1. Colonial patterns (a) and the corresponding boundary contours (b) under the variation of peptone concentration. The agar concentration was fixed at 6 g/l. The snapshots (a) were taken at 60th hour, after inoculation. The innermost to outermost contours represent the fronts at 6th, 12th, 24th, 36th, 48th, and 60th hour, respectively. Incubated temperature was 37°C. Initial cell density was 2.23×10^9 cfu/ml.

then let it cool down to the temperature of 45°C. Because of the evaporation of water ($\sim$ 5 ml) in the mixture, the volume of the mixture was adjusted with sterile distilled water back to 1 liter. Then, 30 ml of the solution was immediately poured into a glass Petri dish (8.8 cm of diameter). The solution was solidified by keeping at the room temperature of 25°C for 1 hour. The thickness of agar was approximately 0.5 cm. Then, the agar plates were incubated at the temperature of 37.0±0.3°C and relative humidity of 49.0±2.0% for 24 hours to check the contamination.

Five microliters of liquid culture of bacteria was inoculated into the center of an agar plate. The value of the initial cell density calculated by using the method of viable count [19] was about $(2.0\pm0.3) \times 10^9$ cfu/ml. The inoculated agar plates were closed and sealed with parafilm to keep the humidity inside the agar plate approximately constant. Then, the agar plates were incubated at the temperature of 37±0.3°C and relative humidity of 49.0±2.0%.

Macroscopic colonial patterns were observed via a charge couple device (CCD) camera (SONY ExwaveHAD) with a magnification of 10 times. To observe a colonial pattern, colonial plates were taken out of the incubator to record an image. Then, it was immediately brought back to the incubator to limit the interrupted change of temperature, which affects the bacterial growth. For microscopic observations, an alignment of cells in a bacterial colony was observed by using a scanning electron microscope (SEM).

The roughnesses of colonial fronts were measured by applying the concept of fractal dimension [20–22]. In this work, we used the structured walk method to determine the fractal dimension of the colonial fronts. The length of the measuring sticks was varied in

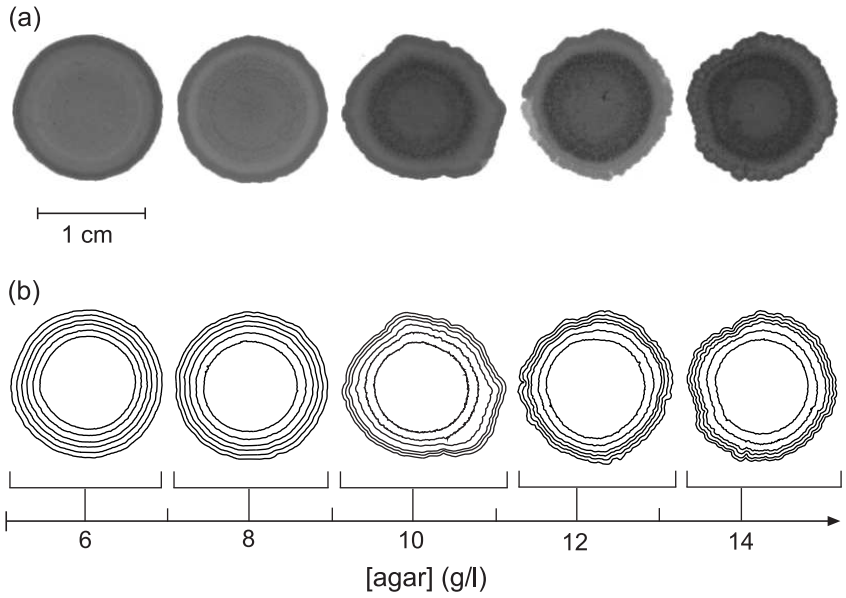

Figure 2. Colonial patterns (a) and the corresponding boundary contours (b) under the variation of agar concentration. The peptone concentration was fixed at 40 g/l. The snapshots (a) were taken at 60th hour, after inoculation. The innermost to outermost contours represent the fronts at 6th, 12th, 24th, 36th, 48th, and 60th hour, respectively. Incubated temperature was 37°C. Initial cell density was 1.85×10^9 cfu/ml.

the range of 1/3 to 1/100 of the colonial diameter.

3. Experimental Results

The experimental results can be divided into 3 parts: i) the instabilities under the variations of peptone concentration, ii) the instabilities under the variations of agar concentration, and iii) the microscopic observation of the bacterial front.

3.1. Variation of the peptone concentration

The instabilities of colonial front under the variation of peptone concentration are shown in Fig. 1. If the nutrient concentration is increased, with fixing of the agar concentration, the colonial front changes from a fingering shape to a smooth circular front as shown in Fig. 1(a). That is the amplitude of the fingering front decreases with increasing of nutrient concentration, and the front eventually becomes smooth when the nutrient concentration is sufficiently high. The growth rate of colony, indicated by the space between contours obtained with an equal time interval, (6 hours) is quite constant with time as shown in Fig. 1(b). However, the space between contours increases with increasing of the peptone concentration. This means that the growth rate is linear proportional to the peptone concentration. The measured growth rates are 25.0 ± 2.0, 57.3 ± 2.1, 68.7 ± 4.7, and

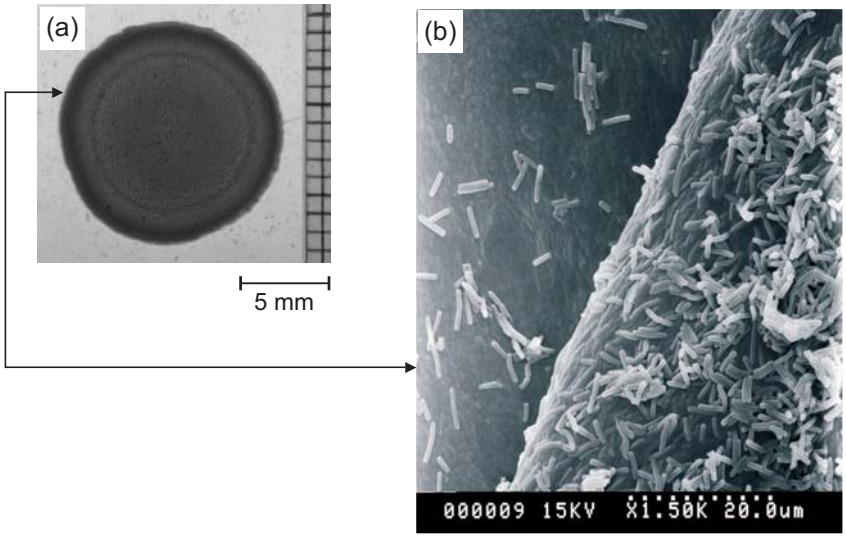

Figure 3. The microscopic observation of a smooth colony. (a) The picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. The concentration of agar and peptone was 6 and 40 g/l, respectively. Initial cell density was 1.78×10^9 cfu/ml. (b) The SEM picture with the magnification of 1500 times.

77.6 ± 2.9 μm/hour for the peptone concentrations of 10, 20, 30, and 40 g/l, respectively. The linear relation between the growth rate and the peptone concentration is growth rate (μm/hour) = 1.692 ((μm/hour)(l/g))*[peptone] (g/l) + 14.850 (μm/hour).

3.2. Variation of the agar concentration

Instabilities of colonial front under the variation of agar concentration are shown in Fig. 2. If the agar concentration is increased (i.e., increasing of the medium hardness), the colonies change from a smooth front to a fingering front as shown in Fig. 2(a). For a rather high agar concentration about 10-14 g/l, the growth rate slows down after 24 hour, as the space between contours decreases dramatically, and the fingering front starts to grow, as shown in Fig. 2(b). However, the final sizes of colonies are not so different, compared to the experiments in Fig. 1, although the growth rates are not constant. This is because the nutrient concentration in this case is excess. Therefore, the population density of bacterial cells is high and the bacteria can move because of population pressure of cells in the colony [16].

3.3. Microscopic observations

In this work, the microscopic study was applied to understand an alignment of bacterial cells in the colonial front. The alignments of cells were investigated by using an SEM technique. The results can be divided into 3 types: i) the smooth circular front, ii) the rough colonial front under the condition of hard medium with rich nutrient, and iii) the

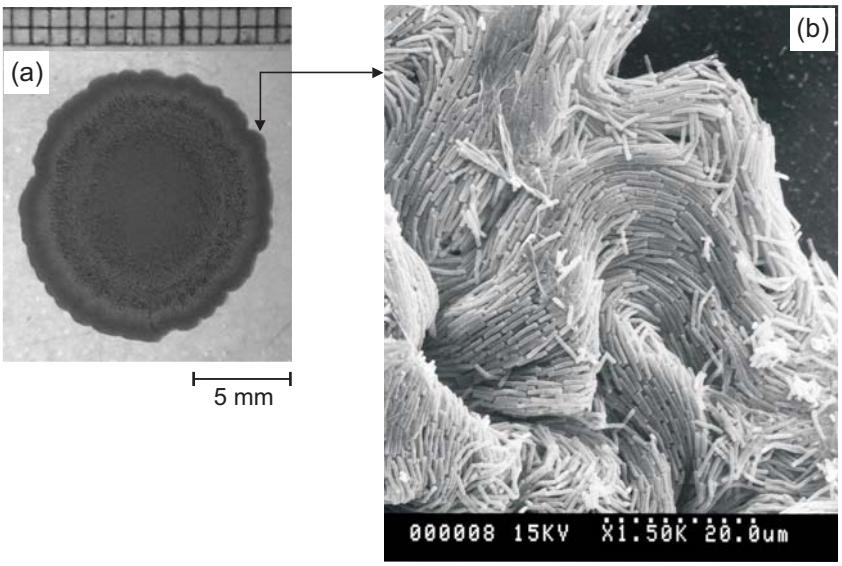

Figure 4. The microscopic observation of a fingering colony. (a) The picture of the colony taken by a CCD camera after incubation at the temperature of 37°C for 48 hours. The concentration of agar and peptone was 10 and 40 g/l, respectively. Initial cell density was 1.86×10^9 cfu/ml. (b) The SEM picture with the magnification of 1500 times.

rough colony under the condition of soft medium with poor nutrient.

The stable colonial front, smooth circular colony, shown in Fig. 3 was found on a soft medium (6 g/l of agar) with rich nutrient (40 g/l of peptone). The colonial front expands symmetrically and the reproduction of bacterial cells is quite high, because of soft agar medium with high nutrient concentration. For microscopic observation, the SEM image (Fig. 3(b)) reveals that the boundary of colonial front consists of bacterial cells connected with each other and aligned parallel to the circumference. In contrast, inside the colony (bottom right corner of Fig. 3(b)) the bacterial cells align randomly. Note that the scattering of single cells on the agar surface around the top left corner of Fig. 3(b) is not part of the dynamics, but it occurred during the process of the SEM sample preparation.

The unstable colonial front, a fingering pattern, at the condition of 10 g/l of agar and 40 g/l of peptone (hard agar medium with rich nutrient) is shown in Fig. 4. The microscopic observation (Fig. 4(b)) reveals that the colonial front consists of bundles of chains of bacterial cells. These bundles fold and meander crossing each other and form complex alignment. The microscopic result of the unstable colonial front at the condition of 6 g/l of agar and 20 g/l of peptone (soft agar medium with poor nutrient) brings out that again the front consists of bundles of chains of bacterial cells (the result is not shown here) similar to the result in Fig. 4.

From the microscopic investigation by using the SEM technique, it is plausible to discuss the stability of colonial front in terms of cell motility corresponding to the expansion rate of front ($\propto 1/[\text{agar}]$) and cell reproduction rate ($\propto [\text{peptone}]$). For the case of the stable

colonial front observed under the condition of soft medium and rich nutrient, the front expands symmetrically and forms a smooth circular shape. In this case, the expansion rate and cell reproduction rate are compatible. On the other hand, under the condition of hard medium the SEM images show that the colonial front consists of wavy bundles of chains of bacterial cells. In this case, the expansion rate is lower than that of the first case and does not match the growth rate. Under this reduction in cell motility, cells tend to consume nutrient and multiply themselves locally. Cell reproduction in the limited space results in the formation of bundles of chains of cells, which responds to macroscopic instability of colonial front. In general, cell motility is dependent on bacterial density, nutrient concentration, medium hardness, and on the growth rate of cells [9].

Kawasaki *et al.* remarked that bacterial cells can hardly move when either the concentration of nutrient or their cell density are low [15]. Under the condition of low nutrient level, bacteria tend to consume nutrient and multiply themselves locally, although the agar medium is soft. Hence they form wavy chains of cells similar to the result shown in Fig. 4(b). However, the density of bundles of chains in this case is lower than that in the condition of the hard medium with high nutrient because of lower nutrient concentration.

4. Numerical simulation

In order to confirm and describe the pattern formation and instabilities of colonial fronts obtained from the experiments, we use the modified reaction-diffusion model proposed by Mimura *et al.* [16]. In this work, a 9-point finite difference method [23] was applied to determine the value of cell density and nutrient concentration. Since the microscopic observations reveal that cells connected with each other and formed bundles of chains of cells. Therefore, it is plausible that at the local maxima of the bundle (high cell density) the front moves forward faster than that at the local minima (low density). We assume that the movements of cells is dependent on the density of active cells, locating mostly near the colony boundary. These active cells move, grow and multiply themselves, whereas inactive cells (inside the colony) do nothing at all but still alive. To apply this assumption, the nonlinear diffusion is used. We proposed a nonlinear diffusion of active cells D_u as following [17]:

$$D_u = d_1 u^k, \tag{1}$$

where an exponent, k refers to the hardness of the medium. u is the population density of active cells. The parameter d_1 is the diffusion of bacterial cells depending on an initial nutrient concentration, v_0. We assume that d_1 is a linear function of v_0 as following:

$$d_1 = b_1 v_0 + b_2, \tag{2}$$

where b_1 and b_2 are constants. This linear approximation agrees with the experimental results in Section 3.1. It should be noted that the parameters d_1 and k are respectively corresponding to the nutrient and agar concentrations in our experiments.

The used reaction-diffusion model [16] is shown as following:

$$\frac{\partial u}{\partial t} = \nabla(d_1 u^k \nabla u) + \varepsilon v u - \frac{a_0 u}{(1 + u/a_1)(1 + v/a_2)},$$

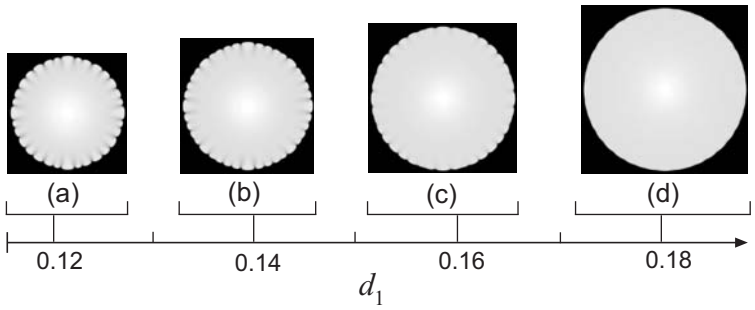

Figure 5. Patterns produced by numerical simulations under the variation of d_1. The values of v_0 were 1.00, 1.05, 1.10, and 1.15 for the simulation in (a)-(d), respectively. The value of k was fixed at 1.00. All patterns were captured at the time step of 800 t.u..

$$\begin{aligned} \frac{\partial v}{\partial t} &= D_v \nabla^2 v - vu, \\ \frac{\partial s}{\partial t} &= \frac{a_0 u}{(1+u/a_1)(1+v/a_2)}, \end{aligned} \tag{3}$$

where ε, a_0, a_1, a_2, and D_v are constants. $u(r,t)$ and $s(r,t)$ are the population densities of active and inactive bacteria, respectively. $v(r,t)$ is the nutrient concentration. $u(r,\, t) + s(r,\, t)$ is the total population density. Here r and t represent position and time, respectively. The used initial condition and boundary condition were obtained from Mimura *et al.* [16]. The numerical simulation was performed in two dimensional space (grid space = 0.5 s.u., array size = 800×800). We use $D_v = 1.00$, $\varepsilon = 1.00$, $a_0 = 1.00$, $a_1 = 0.000416$, $a_2 = 0.008333$, $b_1 = 4.0$, and $b_2 = 2.8$. The varied parameters are d_1 and k.

4.1. Numerical results under the variation of d_1

This simulation condition corresponds to the experiments under the variation of nutrient concentration, with soft agar medium. For a small value of d_1 (poor nutrient), we found a fingering pattern with high roughness as shown in Fig. 5(a). Note that white color represents high bacterial $(u + s)$ density, whereas black color indicates free agar medium. If the value of d_1 is increased, the fingering pattern transforms gradually to a smooth circular one as shown in Fig. 5(d). The final size of colony clearly increases with increasing of d_1. We found that the proposed model satisfactorily reproduces the experimental results in Fig. 1.

By applying the structured walk method, we found that the fractal dimension reflecting the instability of colonial fronts decreases linearly with increasing of d_1, corresponding to the experimental result of variation of peptone concentration as shown in Fig. 6. From this quantitative measurement we can conclude that the colony front can be stabilized, if the nutrient concentration is increased. In addition, the numerical results reproduce the experiments not only the patterns, but also their fractal dimension.

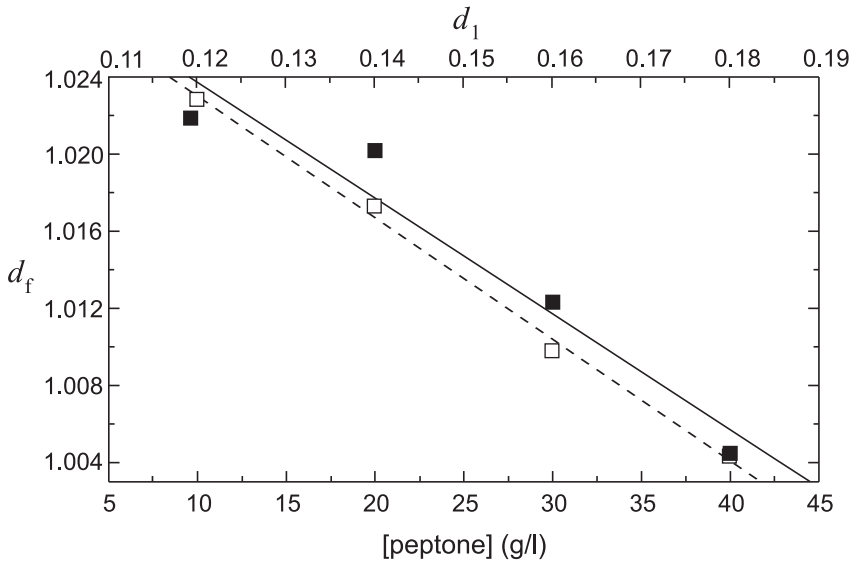

Figure 6. Fractal dimension, d_f of the bacterial fronts as a function of the nutrient concentration (i.e., [peptone] in experiments, indicated by filled squares and d_1 in simulations, indicated by open squares). The solid and dashed lines represent the linear fittings of experimental and numerical data, respectively.

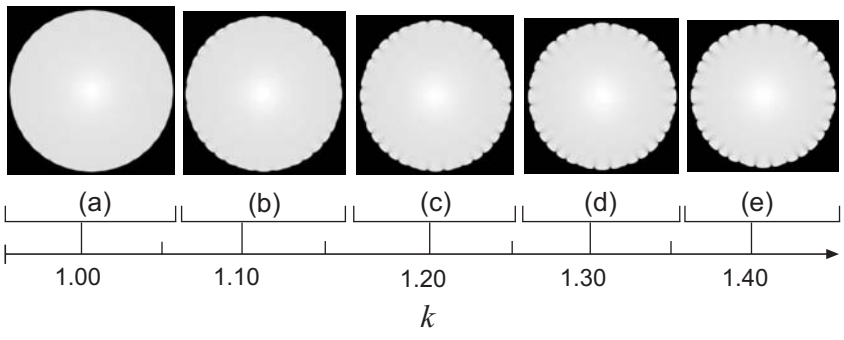

Figure 7. Patterns produced by numerical simulations under the variation of k. The value of v_0 and d_1 was fixed at 1.15 and 0.18, respectively. All patterns were captured at the time step of 800 t.u..

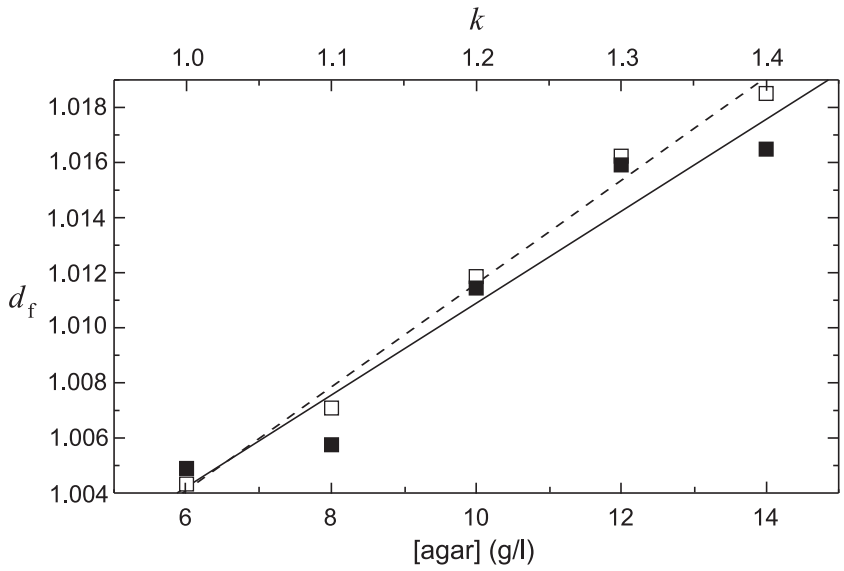

Figure 8. Fractal dimension, d_f of the bacterial fronts as a function of the medium hardness (i.e., [agar] in experiments, indicated by filled squares and k in simulations, indicated by open squares). The solid and dashed lines represent the linear fittings of experimental and numerical data, respectively.

4.2. Numerical results under the variation of k

In this part the values of d_1 and v_0 are fixed at 0.18 and 1.15, corresponding to the experiments under the variation of agar concentration, with rich nutrient. For a small value of k (soft medium), the colony represents a smooth circular pattern as shown in Fig. 7(a). The colonial pattern changes from a smooth circular front to a fingering pattern, when the value of k is increased. The final sizes of colony reduce only slightly with increasing of k. It reproduces the experimental results of variation of agar concentration shown in Fig. 2.

From the measurement of fractal dimension in Fig. 8, we found that the instabilities of colonial fronts linearly increase with increasing of k, corresponding to the results in experimental part. In this case the colony front destabilizes, if the medium hardness is increased. The detail of stabilization and destabilization of the colony front are discussed in Sec. 5.

5. Discussion

This section is devoted for the investigation of the instabilities of the colonial front. The instabilities in propagating reaction-diffusion fronts have been studied numerically by D. Horváth *et al.* [24] in the system of the iodate-arsenous acid reaction. They found that the instability occurs when the ratio of the diffusion coefficient for the reactant (iodate species) to that for the autocatalyst (iodide species) exceeds some critical value. This

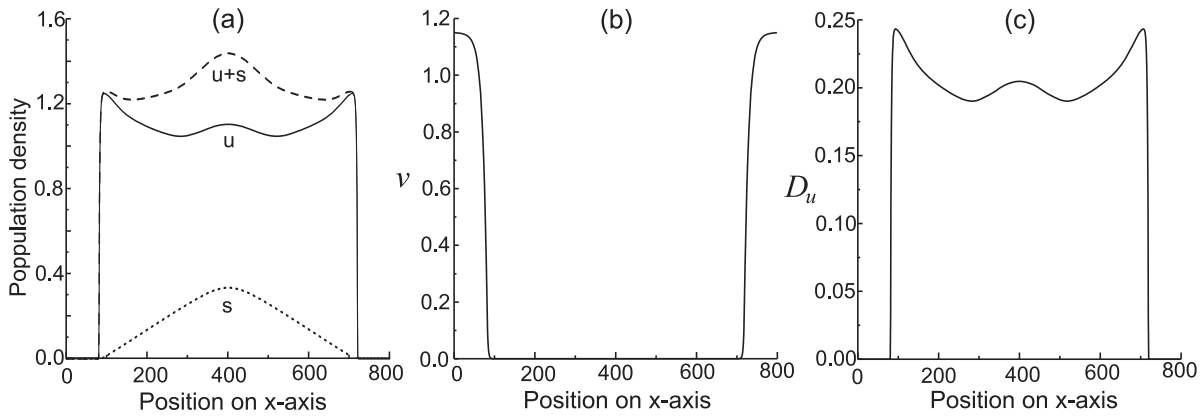

Figure 9. The example profiles of u, s, u+s, v, and D_u as a function of space. The data was obtained from the cross section (at y = 400) of the colony pattern shown in Fig. 7(e).

ratio is shown as following:

$$\delta = \frac{D_A}{D_B}, \tag{4}$$

where δ is the ratio of the diffusivity of reactant to that of autocatalyst. D_A and D_B are the diffusion coefficients of reactant and autocatalyst, respectively. The critical value of δ for the onset of instabilities of the propagating front is 2.9 [24]. The instabilities occur if δ is larger than this value.

Comparing the system of the iodate-arsenous acid reaction with the bacterial colony, here the active cells act as an autocatalytic species, since in the bacterial system a cell consumes the nutrient and then divides itself into 2 cells. The reactant in the chemical system corresponds to the nutrient in bacterial system. Thus, the occurrence of instabilities of colonial front should be also explained by using the ratio of diffusion coefficient in Eq. 4.

For the bacterial colony system, the ratio of diffusion coefficients for nutrient and bacterial cells is defined as

$$\delta = \frac{D_v}{D_u}, \tag{5}$$

where D_v and D_u are the diffusion coefficient of nutrient and active cells, respectively. Since the nutrient molecules are so small and can move around freely through water contained within the agar gel network [7], the value of D_v is taken to be constant (D_v = 1.00). Note that the diffusion coefficient of active cells, D_u in our work is nonlinear, whereas D_B in D. Horváth *et al.* [24] is linear.

In order to calculate the nonlinear diffusion D_u which is spacially nonuniform, we need to substitute $u(r,t)$ into Eq. 1. An example of the cross section profiles of the population densities u, s, u+s, and the nutrient concentration v are shown in Figs. 9(a-b). The maxima of u locate near the colony boundary, whereas the maxima of s locate in the

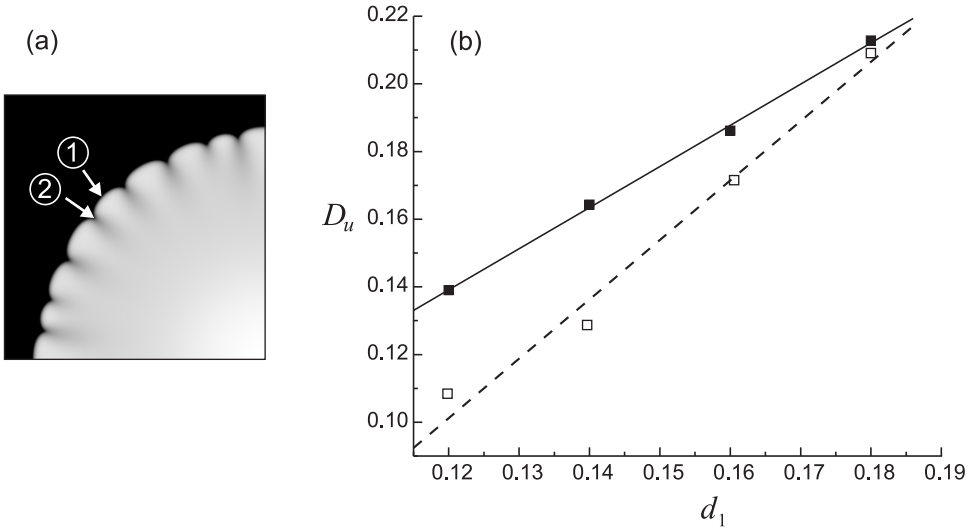

Figure 10. Diffusion coefficient of active cells as a function of d_1. The filled and open squares represent the diffusion coefficients obtained from position 1 (local maxima) and 2 (local minima) of the colony shown in (a), respectively. The solid and dashed lines indicate the linear fitting of the data from position 1 and 2, respectively.

middle. This means that most of the cells near the colony edge are active. Note that in this 2D model, the nutrient layer is very thin and the nutrient under the colony is consumed completely (Fig. 9(b)). The corresponding D_u profile is presented in Fig. 9(c). One can see that the maxima of D_u locate near the boundary of colony, according to the u profile in Fig. 9(a).

However, since the diffusion of the bacterial front is nonuniform as shown in Fig. 10. That is at the local maxima (position 1) the front diffuses fastest, whereas at the local minima (position 2) the front diffuses slowest. Therefore, the actual diffusion coefficient of the front can be determine as the average value of the two positions. For the case of variation of d_1 shown in Fig. 10, we found that the values of D_u increases with increasing of d_1.

After substituting the averaged D_u into Eq. 5, the ratio of diffusion coefficients, δ can be obtained. In our case $D_v = 1.00$ is constant. We found that the value of δ decreases with increasing of d_1 (result is not shown here). This decreasing in δ indicates that the colony front is stabilized, if d_1 is increased. This result agrees with the result of D. Horváth *et al.* [24]. However, the critical values of δ for the onset of the instabilities are slightly different, e.g., for a rather smooth colony in Fig. 5(d) the value of δ is 4.7, whereas D. Horváth *et al* reported that the critical value is 2.9. Actually it is quite difficult to compare the value of δ in our system which is nonlinear to the linear one of D. Horváth *et al.*

Finally we would like to discuss about the range of experimental parameters in comparison with the work of Matsushita *et al.* [7,9,14]. Note that although the concentration

range of agar (5 - 15 g/l) in this work and in Matsushita *et al.* are the same, but our nutrient concentration (10 - 40 g/l) is much higher than that of Matsushita *et al.* (0.2 - 10 g/l). Furthermore, we use different *Bacillus subtilis* strain, TISTR 008, whereas Matsushita *et al.* used *Bacillus subtilis* strain, OG-01. These differences can explain a slightly shift in our results and the results of Matsushita *et al.* However, we focus our study on the transition dynamics from a stable to an unstable propagation of bacterial front, not the diversity of colony patterns.

6. Conclusions

Our experimental results show that the instabilities of propagating front are linearly increased with decreasing of peptone concentration and/or increasing of agar concentration. The microscopic observations reveal that the cell reproduction in limited space resulting in the formation of meandering of bundles of cell chains causes a nonlinear diffusion and therefore the instabilities of propagating front. The proposed model successfully reproduces the experimental results both qualitatively and quantitatively. Furthermore, the instabilities can be discussed in terms of the ratio of diffusion coefficients for nutrient and bacterial cells. We found that the colony front can be stabilized, if this ratio is decreased, in agreement with the previous work.

Acknowledgments

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Bifurcation of the bacterial translocation in a colony of *Bacillus subtilis* TISTR 008.

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Abstract

An abrupt change in the colonial formation of *Bacillus subtilis* TISTR 008 is observed under the condition of soft agar medium with rich nutrient. At first state the bacteria form a thick colonial front which propagates slowly for about 48 hours, then suddenly they form a thin film which spreads quickly over the agar medium. From our experimental results we found that the behaviour in the second state is a swarming, which is a special form of bacterial surface translocation that a group of swarm cells at the rim of the bacterial colony rapidly spreads covering all available agar surface. At the swarming state, the bacteria change from flagellated cells to hyperflagellated ones and the bacterial colony consists of a monolayer of swarming cells which move around actively and randomly.

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I. INTRODUCTION

Depending on both bacterial species and environmental conditions, a bacterial colony can exhibit various colonial patterns [1–6]. The growth conditions are controlled by the initial concentration of the medium components. The agar concentration determines the consistency of the medium, which becomes harder as the amount of agar increases, and the nutrient concentration controls the bacterial reproduction. Depending on these two factors, the colony grows at a higher or lower rate, developing different kinds of patterns [7]. The complexity of the colonial growth normally increases as the environmental conditions become less favorable. The bacterial colony responds to adverse conditions by developing a strategy and organizing in a micro-level to cooperate the more efficiency of survival [1, 6]. Examples of these strategies are the production of extracellular wetting fluid [8] and the secretion of surfactants which change the surface tension resulting in swarming of bacterial cells [10].

Swarming is one type of surface translocation that bacterial cells group and rapidly move across a solid medium [9]. Rapid surface migration was preceded by a cell density dependent lag period, which could be eliminated if actively swarming cells were used as the inoculum. The leading edge of the swarm was characterized by multicellular rafts of highly flagellated cells [10]. As a collective bacterial process, swarming is often associated with biofilm formation and is linked to virulence factor expression in pathogenic bacteria [15]. As the swarming behavior occurred, the normal cells (vegetative cells) transition into hyper-flagellated swarming ones [9]. This type of translocation was observed in many bacterial species such as *B. subtilis*, *P. mirabilis*, *S. marcesens*. It is known that the critical factors of swarming of bacterial cells are the concentration of agar and surface tension [9, 14]. In addition, cell density is critical to swarming as the duration of the lag phase that precedes *P. mirabilis* migration is strongly influenced by inoculum density [14]. In 2004, M. B. Connelly et al. [15] studied the swarming motility in various strain of *Bacillus subtilis*. They found that gene named Epr, a minor extracellular serine protease gene, plays an important role in swarming. They also found that if the strain of *bacillus subtilis* is different, time for an initiated swarming is different as well.

In both Gram-negative and Gram-positive species of swarming bacteria, the transition to the swarming state is accompanied by dramatic changes in cell morphology: swarming cells become filamentous, multinucleoid and hyperflagellated. Flagella were implicated as

the motor for swarming motility by the dramatic increase in the density of flagella on the surface of cells at the swarm edge. Bacillus subtilis produces a lipopeptide antimicrobial surfactant called surfactin [10].

Swarming is a powerful means of rapidly colonising nutrient- rich environments, facilitating colony spread and accelerating biomass production. Several flagellated genera typically swarm, for example, Proteus, Bacillus [11]. Bacterial swimming motility is influential in many pathogenhost interactions, and several pathogens are additionally capable of multicellular swarming migration.

Swarming is neither a starvation response nor an obligatory development stage [14]. It is nonetheless a radical and reversible change in behaviour in response to the environment. The social nature of swarming indicates that extracellular and possibly cellcell signals are central stimuli, as are intracellular physiological parameters and contact with a surface. These signals might be sensed and transmitted by two-component regulatory systems, cytosolic regulators, and even cell-surface flagella [11].

The bacteria *Bacillus subtilis* is known to exhibit the swarming behaviour. They are commonly found in soil and considered to be a motile species [12]. It has been classified as an obligate aerobe. It is able to form a protective endospore via a spore formation process, if the conditions are not appropriated for the growth of bacterial cells, such as starvation and desiccation [13]. An endospore allows this bacterial species to tolerate extreme environmental conditions and is resistant to ultraviolet, gamma radiation, and chemical disinfectants. Normally, *Bacillus subtilis* is not considered as pathogen and toxicity.

In this work the swarming behaviour of the bacteria species *Bacillus subtilis* TISTR 008 under the condition of soft agar medium with rich nutrient is investigated. we focus on the spreading colony which have 2 state: a slow growth of a colony and a rapid one. We attempt to summarize the observed findings of the formation of the swarming colony and also show some experimental ideas for the investigation of the origin of the swarming growth.

II. EXPERIMENTAL METHOD

The used bacterial species was *Bacillus subtilis* TISTR 008 obtained from Thailand Institute of Scientific and Technological Research. A solution for agar plate preparation consists of 5 g of NaCl, 2.397 g of K_2HPO_4, 0.68 g of KH_2PO_4, 40 g of Bacto Peptone (Difco Labo-

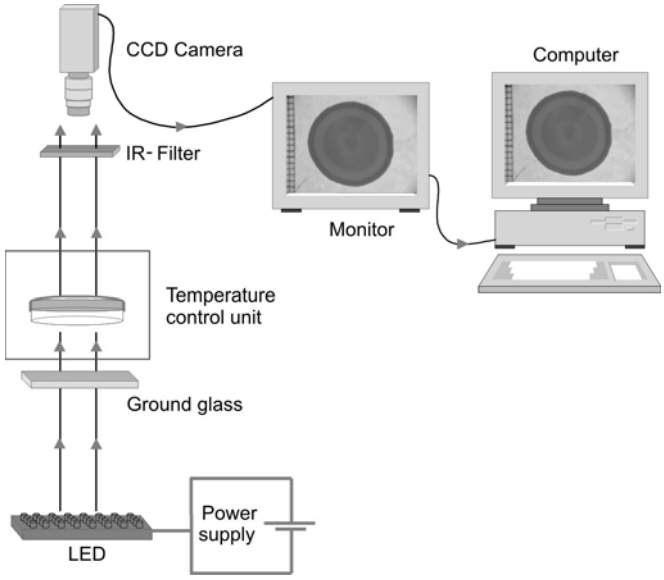

FIG. 1: Experimental setup for a macroscopic observation of colonial patterns.

ratories),and 6 g of Bacto Agar (Difco Laboratories) in 1 liter of distilled water. The final pH of the solution was 7.0 ± 0.2. The mixture was sterilized by using an autoclave at the temperature of 121°C for 15 minutes, then let it cool down to the temperature of 45°C. Because of evaporation of water in the mixture (∼ 5 ml), the volume of the mixture was adjusted with sterile distilled water back to 1 liter. Then, 30 ml of the solution was immediately poured into a glass Petri dish (8.8 cm of diameter). The solution was solidified by keeping at the room temperature of 25°C for 1 hour. The thickness of agar was about 0.5 cm. Then, the agar plates were incubated at the temperature of 37.0±0.3°C and relative humidity of 49.0±2.0% for 24 hours to check the contamination.

5 μl of liquid culture of bacteria was inoculated into the center of an agar plate. The value of the initial cell density calculated by using the method of viable count [16] was about (2.0±0.3) x 10^9 cfu/ml. The inoculated agar plates were closed and sealed with parafilm to keep the humidity inside an agar plate approximately constant. Then, the agar plates were incubated in a water jacket, in which the temperature are controlled by using a thermostat. The temperature inside is 37±0.3°C.

Colonial patterns were observed via a charge couple device (CCD) camera (SONY ExwaveHAD) with a magnification of 10 times. A schematic diagram of the experimental setup is shown in Fig.1. An array of infrared light-emitting diodes (IR-LED) is used as an IR light source because it emits a narrow-spectrum of IR light in one direction with a

narrow angle. It contrasts to a fluorescent lamp of a light box, which emits light in no specific direction. Thus, an image obtained from IR-LED is clearer than that from a light box. The generated infrared light passes through a ground glass used to obtain a homogeneous light, and then the bacterial colony in an agar plate. An infrared filter that allows only the transmission of the IR light was placed before the CCD camera. Then, the images were recorded on a computer hard disk. For microscopic observations, an alignment of cells in a bacterial colony was observed by using an optical microscope.

III. EXPERIMENTAL RESULTS

A. Morphological diagram

In order to investigate the morphological changes due to environmental conditions in colonial formation, two important parameters are varied: the peptone and agar concentrations. We found that the colony patterns grown on an agar surface change dramatically, if the concentrations of agar and nutrient are varied globally. They are classified into 4 distinct types, as shown in the morphological diagram in Fig.2: fingering colony in region A, fine branching colony in region B, fractal pattern in region C, and spreading colony in region D.

Region A: The colonies form a fingering front. The colonial patterns become compact. The colonies in this region are very dense obviously because of high nutrient concentration. The patterns in this region are similar to the Eden pattern in Matsushitas work [12].

Region B: When the nutrient concentration is low (10 g/l) and the medium is soft (6 g/l), the colonial fronts are not smooth and form a fine branching pattern. This pattern is also found when the medium is hard (10-14 g/l) and nutrient concentration is high (20-40 g/l). The bacterial cells in this region undergo cell division locally, similar to that in region A, but cell density is higher because of higher nutrient concentration. Therefore, the colonies in region B are darker than that in region A.

Region C: The colonies describe a fractal pattern. The nutrient concentration was fixed at 0.4 g/l. Cell division of bacterial cells in this region is low because of the limited nutrient. They only grow and undergo cell division locally. It seems that in this region the colonial patterns are not affected by the hardness of the medium.

Region D: The colonies form a spreading or a swarming pattern. The agar concentration

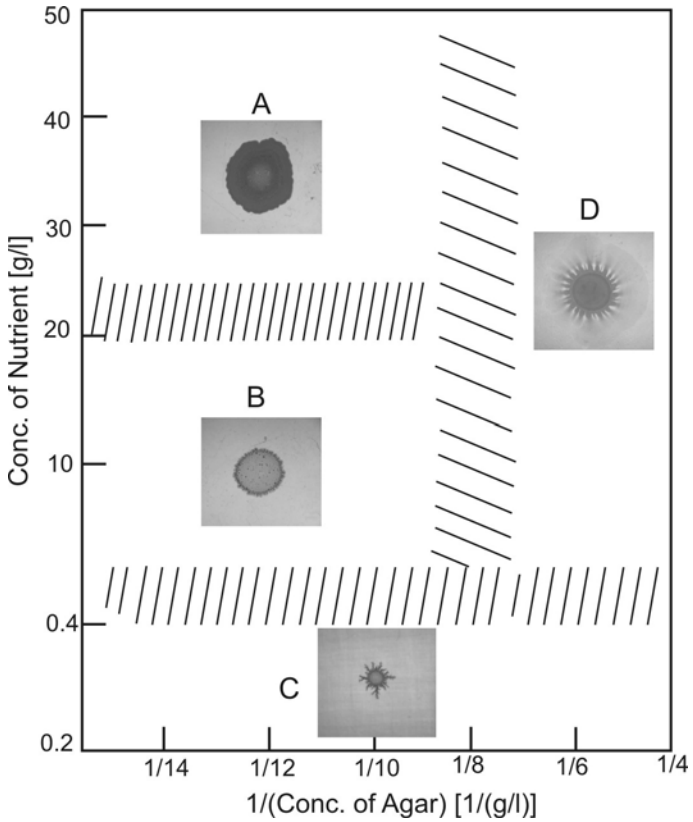

FIG. 2: Colonial patterns of *Bacillus subtilis* TISTR 008 under the variations of agar concentration and peptone concentration (all pictures were taken at 60 hours). Initial cell density was 1.76 x 10^9 cfu/ml. The incubation temperature was 37°C.

was fixed at 4 g/l (very soft agar medium). The colonial fronts spread quickly because of high moisture of the surface of medium, due to the vapour condensation, which increases the motility of cells.

B. Dynamics of a spreading colony

The dynamics of the spreading colony observed in region D are shown in Fig.3. The bacterial colony slowly grows and forms a compact colony with the smooth front for the period of 6-36 hours. Then, the front of the colony bursts and forms a sun-like pattern. The population density of bacterial cells may reach some critical values before bursting of the colonial boundary. After that, the bursting parts spread and then cover the agar surface quickly. Finally, the homogeneous thin-film of bacteria appears all over the agar surface at 72 hours. D. B. Kearns *et al.* reported that *Bacillus subtilis* can exhibit swarming

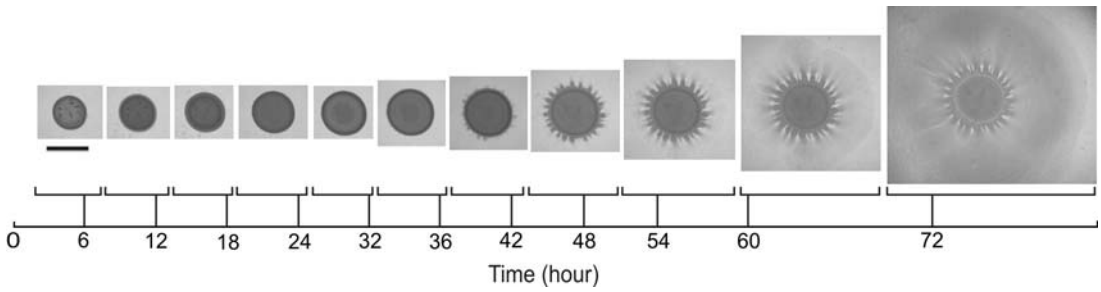

FIG. 3: Growth of a colony in the condition of 6 g/l of agar concentration and 40 g/l of peptone concentration. Incubation temperature was 37°C. Initial cell density was 2.23 x 10^9 cfu/ml. (scale bar: 1cm)

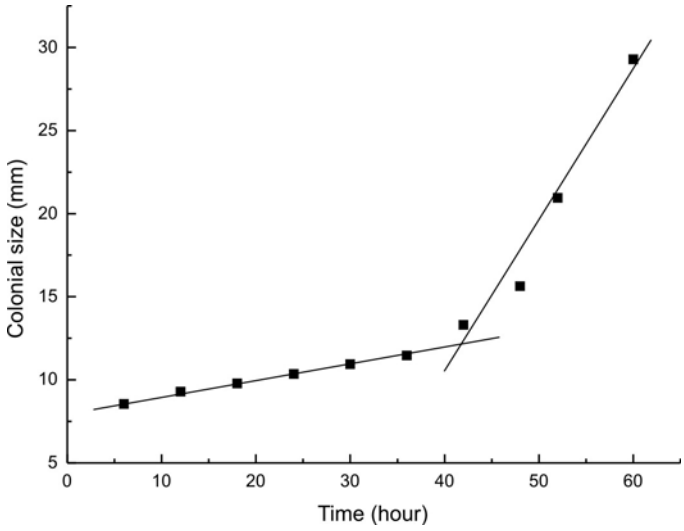

FIG. 4: Colonial size as a function of time.

motility on the surface of the medium containing low agar concentration with 0.3-1.0% [10]. Corresponding to our work, the colony of *Bacillus subtilis* TISTR 008 is robustly spreading on the condition of soft medium (0.6% of agar concentration) with rich nutrient (1-5% of peptone concentration). However, the number of the spreading colony is decreasing with decreasing of peptone concentration and the colony does not show a swarming behaviour when the peptone concentration is very low (0.4 g/l). It is reasonable because the activity of cells decreases with decreasing of nutrient [4].

The colonial size is investigated as a function of time as shown in Fig.3. The result shows that the colonial size slightly increase with time, then the colony grows quickly cover agar surface. There is a switch of propagation velocity of colonial front during the growth of

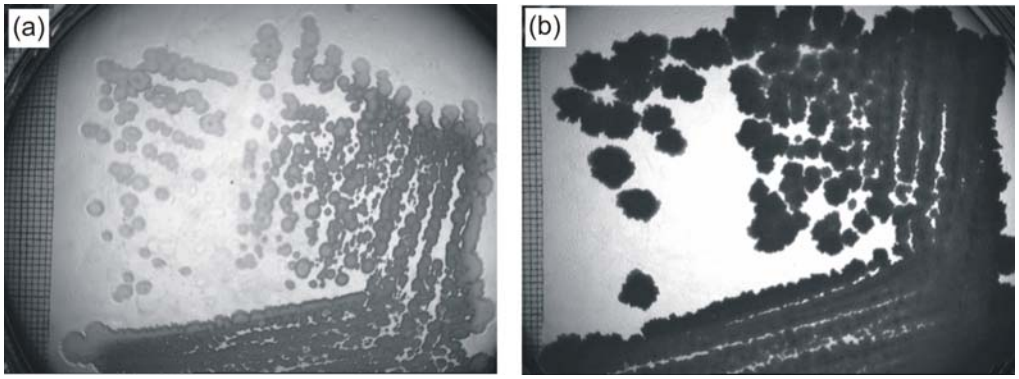

FIG. 5: Isolated colonies on the surface of tryptic soy agar (TSA) medium. (a) shows an isolated colonies obtained form the spreading part (B2). (b) presents an isolated colonies obtained form the stock (B1). The incubation temperature was 37°C. These pictures were taken at 24 hours after incubation.

colony. The velocity of colonial front, which is obtained by linear fitting of a slope in the graph shown in Fig.3, changes form 95.87±2.43 μm/hour to 921.20±130.75 μm/hour.

C. Characteristics of swarming cells

After the formation of the spreading colony is found, we try to understand and investigate how this behaviour occurs. The bacterial cells in the region of spreading part were streaked on TSA medium to isolate the colonies. The isolated colonies of bacterial cells of the spreading part are shown in Fig.5(a). Comparing with the isolated colonies from stock bacteria shown in Fig.5(b), we found a difference between these two types of isolated colonies. The isolated colonies of the spreading part are thinner than that of the stock. It is noted that bacterial cells of the stock and of spreading part are labelled as B1 and B2, respectively.

After reading publications of Ben-Jacob *et al.* [17, 18], the suspect of bacterial mutation arose. In their work, the sector formation of expanding bacterial colonies was studied. They mentioned that if the mutant bacteria have the same growth dynamics as the normal ones, they will be usually unnoticed after growing of the bacterial colony. Ben-Jacob *et al.* also suggest that if the bacterial mutants have different growth dynamics, the presence of mutant cells is indicated by the distinguished sector with a different growth pattern. In our results, we found different growth patterns, i.e., changing from the slow growth of the smooth colony to the spreading one. Therefore, in order to prove whether the onset of spreading formation

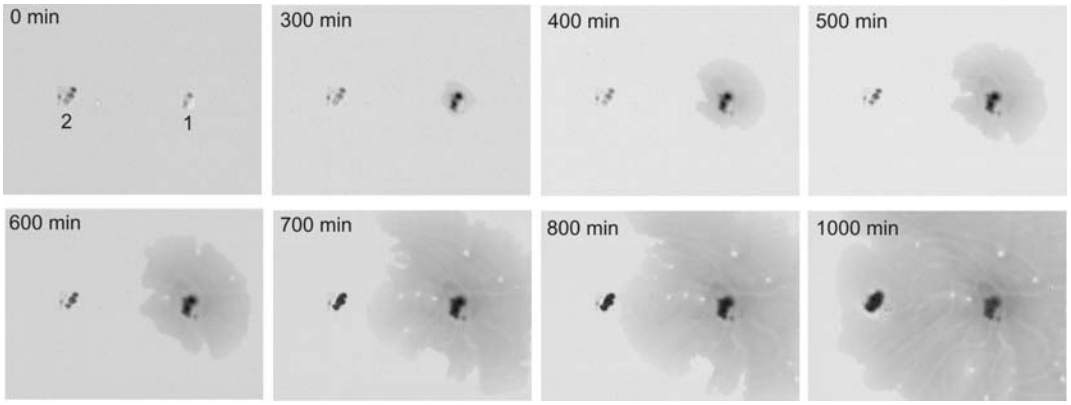

FIG. 6: Snapshots of colonial growth on the agar plate with with 6 g/l of agar concentration and 40 g/l of peptone concentration. B1 and B2 obtained from the same medium. The number 1 and 2 are corresponding to the bacteria of B2 and B1, respectively. The incubation temperature was 37°C. The first picture was taken after incubating for 2 hours.

occurred from the mutation of bacterial species or not, the 16s rDNA technique [19] and biochemical test were applied to classify the bacterial cells of B1 and B2. The result of DNA sequencing reveals that DNA sequences of B1 and B2 are not different. This means that species and strain of B1 and B2 are same. The results of biochemical test present that there are some differences, but the species of two types are concluded to be the same species. Thus, it can be concluded that there is no mutation in bacteria, when the spreading colony occurs.

In order to investigate the different characteristics of B1 and B2, two types of bacteria species were grown on the same medium. The experiments can be divided into 2 parts: i) B1 and B2 obtained from the same medium in the spreading part and the inside one, ii) B1 and B2 obtained from their isolated colonies on TSA medium. In these experiments, the agar and peptone concentrations are fixed at 6 and 40 g/l, respectively. In two experiments, B1 and B2 were inoculated by streaking about 2.5 cm far form each other. The experimental results of 2 parts are shown in Fig.6 and 7, respectively. The results of two parts represent that there is a lag phase in the period of about 6 hours. Then, the bacterial colony of B2 (labelled as number 1 in Figs.6 and 7) grows and spreads quickly, comparing to B1 (labelled as number 2). Finally, bacteria of B2 spread over the agar surface. In these results, we found that B2 are not form a compact colony before spreading, but tend to spread quickly after the lag time (about 6 hours).

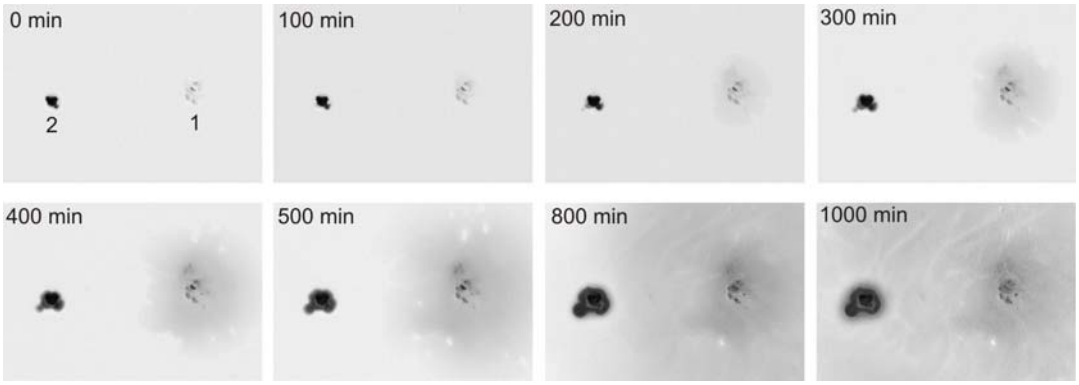

FIG. 7: The snapshots of colonial growth on the agar plate with with 6 g/l of agar concentration and 40 g/l of peptone concentration. B1 and B2 obtained from their isolated colonies. The incubation temperature was 37°C. The number 1 and 2 are corresponding with the bacteria of B2 and B1, respectively. The first picture was taken after incubating for 3 hours.

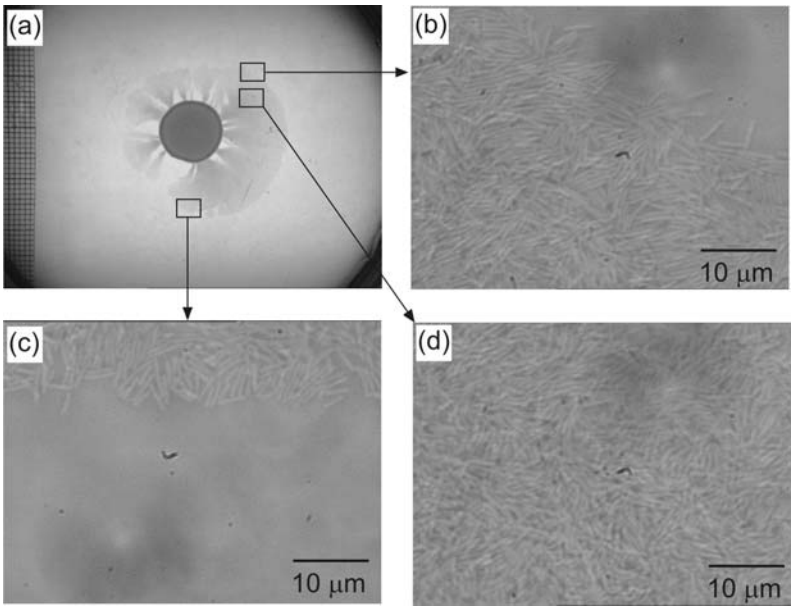

FIG. 8: Microscopic observations of spreading colony of *Bacillus subtilis* TISTR 008 under the condition of 6 g/l of agar concentration and 40 g/l of peptone concentration.

D. Microscopic observation

The microscopic observation of spreading colony by using an optical microscope is shown in Fig. 3. Macroscopically, the cell density represented by a gray level of spreading parts is lower than that of inside of colony clearly as shown in Fig. 3(a). Microscopic observations

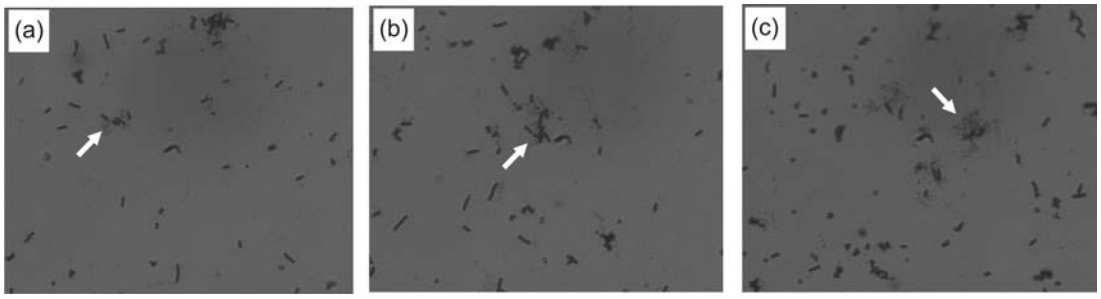

FIG. 9: Bacterial flagella at each state. (a), (b), and (c) represent prespreading state (flagella cells) at 24 hours, colonial bursting state at 36 hours, and spreading one (hyperflagella cells) at 60 hours, respectively.

of spreading parts reveal that the alignment of bacterial cells is random (Figs. 3(b), (c) and (d)). We found that the bacterial colony in the spreading parts consist of a monolayer of cells. Comparing with the cells inside the colony, the swarming cells are very active and move fast.

E. Hyperflagellated cells

The Leifson staining method [20] was used to investigate the swarming colony, since there is a report that the normal cells (vegetative cells) transition into hyper-flagellated swarming ones as the swarming behavior occurred [9, 15]. Thus, the bacterial cells in three states (prespreading, bursting, and spreading) were strained by using Leifson stain [20]. We found that the amount of flagella of bacterial cells increases and the bacterial cells change from flagelled cells to hyperflagelled ones as shown in Fig.9. The bacterial cells in the spreading state become peritrichous resulting in high motility of cells (see also in Fig.9(c)). We can conclude that the cause of swarming and switching in front velocity results from the change in cell phenotype, i.e., flagellated cells change to hyperflagellated ones.

IV. CONCLUSIONS

The spreading or swarming colony is robustly obtained in the condition of soft medium with rich nutrient. Under the condition of 6 g/l of agar concentration and 40 g/l of peptone concentration, an initiated time for swarming in *Bacillus subtilis* TISTR 008 is about 48-60

hours. The normal cells (vegetative cells) transition into hyper-flagellated swarming ones resulting in occurring of the swarming behavior. The outlook of this research can be the study of swarming or spreading colony numerically. In order to describe such a behaviour, we suggest that there may be a critical population density of bacterial cells for the onset of spreading colony and a function that describes the switch of a cell motility.

Acknowledgments

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