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Methods Article

High-throughput evaluation of hemolytic activity through precise measurement of colony and hemolytic zone sizes of engineered *Bacillus subtilis* on blood agar

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Abstract

Biosurfactants have remarkable characteristics, such as environmental friendliness, high safety, and excellent biodegradability. Surfactin is one of the best-known biosurfactants produced by *Bacillus subtilis*. Because the biosynthetic pathways of biosurfactants, such as surfactin, are complex, mutagenesis is a useful alternative to typical metabolic engineering approaches for developing high-yield strains. Therefore, there is a need for high-throughput and accurate screening methods for high-yield strains derived from mutant libraries. The blood agar lysis method, which takes advantage of the hemolytic activity of biosurfactants, is one way of determining their concentration. This method includes inoculating microbial cells onto blood-containing agar plates, and biosurfactant production is assessed based on the size of the hemolytic zone formed around each colony. Challenges with the blood agar lysis method include low experimental reproducibility and a lack of established protocols for high-throughput screening. Therefore, in this study, we investigated the effects of the inoculation procedure and media composition on the formation of hemolytic zones. We also developed a workflow to evaluate the number of colonies using robotics. The results revealed that by arranging colonies at appropriate intervals and measuring the areas of colonies and hemolytic rings using image analysis software, it was possible to accurately compare the hemolytic activity among several colonies. Although the use of the blood agar lysis method for screening is limited to surfactants exhibiting hemolytic activity, it is believed that by considering the insights gained from this study, it can contribute to the accurate screening of strains with high productivity.

Keywords: *Bacillus subtilis*; biosurfactant; blood agar lysis method; mycosbutilin; surfactin

Introduction

Bacillus spp. produce a variety of biosurfactants [1, 2]. Biosurfactants are predicted to be the next-generation surfactants because they are safer and more biodegradable than petroleum-derived surfactants, resulting in a lower ecological impact. They have broad applications in detergents, cosmetics, and pesticides [3]. Therefore, the development of high-production biosurfactant processes as well as the construction of high-production strains are needed. Notable biosurfactants produced by *Bacillus* spp. include surfactin, plipastatin, and iturin, all of which are nonribosomal peptides (NRPs) [4–6]. These biosurfactants are produced through the sequential condensation of fatty acids and amino acids on large modularized proteins, such as NRP synthetase.

Metabolic engineering approaches and mutagenesis have been explored as ways to produce high levels of biosurfactant in *Bacillus* spp. It is challenging to improve NRP synthesis using only metabolic engineering techniques that require enhancing multiple amino acid and fatty acid production pathways, disrupting competing pathways, and increasing the expression of functional NRP synthetase. Therefore, approaches such as obtaining high-production strains from mutants exposed to UV radiation or chemicals have been used [7, 8].

Various methods have been developed to investigate the production of biosurfactants by microorganisms [9, 10]. There are two major methods for evaluating the production of biosurfactants. One involves the detection of biosurfactants contained in the culture supernatant after liquid cultivation of microorganisms, and the other is to directly detect biosurfactants produced by colonies on agar plates.

Methods such as oil spread, drop collapse, and the colorimetric assay can be used to evaluate the quantity of biosurfactants in the culture supernatant. The oil spreading method involves spotting the culture on an oil film formed on water to determine the size of the displaced oil circle, which represents the amount of biosurfactant [11–13]. The drop collapse method determines the amount of biosurfactant by dropping the culture into oil-

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coated wells and observing the collapse and spread of liquid droplets [11, 14]. Both methods require complex steps, such as dropping oil, which makes them unsuitable for evaluating multiple samples. The colorimetric assay has previously provided an accurate quantification of surfactin and other lipopeptides using various dyes [15–17]. For example, bromothymol blue, Victoria pure blue BO, and polydiacetylene have been used as dyes. In either of the above-mentioned dyes, the change in color resulting from the addition of culture supernatant to microtiter plates containing the dye is measured using a plate reader to quantify the biosurfactants by absorbance.

The atomized oil assay, ion-pair plate assay, and blood agar lysis methods are used to detect biosurfactants produced by colonies on plates. The plate assay method is very effective as a primary screening technique because it does not require liquid cultivation; instead, colonies are grown on agar plates, thereby making it easy to operate. In the oil-misting method, oil is sprayed onto colonies on agar plates using an airbrush [18, 19]. The size of the halo that forms around the colony can be used to semi-quantitatively evaluate the amount of biosurfactants. A drawback is the inability to visualize the halo until oil is sprayed. In the ion-pair plate assay method, microorganisms are inoculated onto plates containing methylene blue derivatives [20]. The semi-quantitative evaluation of the amount of biosurfactants is possible through the dark blue halo surrounding the colonies. This technique allows for the detection of anionic biosurfactants. However, the growth of *B. subtilis* was reportedly inhibited by methylene blue derivatives. The blood agar lysis method involves inoculating microbial cells onto blood-containing agar plates and measuring the size of the hemolytic zone around the colonies to determine the amount of biosurfactant [7, 21]. The blood agar lysis method can only be used with biosurfactants that exhibit hemolytic activity. However, this method has an advantage as the hemolytic zone is clearly visible, which aids in area measurement, and temporal changes in the hemolytic zone can also be observed, thereby allowing the measurement of hemolytic activity at the appropriate timing.

Moreover, the blood agar lysis method has been used to screen for *B. subtilis* mutants with increased surfactin production [8, 21]. Challenges with the blood agar lysis method include low experimental reproducibility and a lack of established protocols for high-throughput screening. Therefore, it is currently difficult to evaluate a large number such as tens of thousands of colonies. In the blood agar lysis method, the formation of hemolytic zones is strongly influenced by factors, such as medium composition, cultivation period, and manual techniques, all of which provide obstacles to reproducibility (data accuracy) during multi-sample processing. Therefore, in this study, we thoroughly investigated the factors influencing colony and hemolytic zone formation and incorporated them into the operating conditions of an automated device. We established the feasibility of a screening system capable of simultaneously evaluating a large number of colonies for two structurally distinct biosurfactants: surfactin and mycosubtilin.

Materials and methods

Strain and cultivation conditions

Bacillus subtilis 168 (ATCC 23857) (168-strain) served as the host strain for biosurfactant production. Pre-cultivation was performed in 1 ml of Luria-Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl) with appropriate antibiotics (5 μg/ml chloramphenicol, 5 μg/ml kanamycin, 100 μg/ml spectinomycin, and 0.5 μg/ml erythromycin) in a shaker incubator at 300 rpm and 37°C overnight. For surfactin production, 50 μl of pre-cultivation medium was inoculated in 2.5 ml production medium I [40 g/l soy flour, 5 g/l dipotassium hydrogen phosphate, 0.5 g/l magnesium sulfate heptahydrate, 0.18 g/l calcium chloride dihydrate, 0.025 g/l iron (II) sulfate heptahydrate, 0.022 g/l manganese (II) chloride tetrahydrate, and 30 g/l maltose monohydrate] and cultivated at 37°C and 300 rpm. To produce mycosubtilin, 12.5 μl of pre-cultivation medium was inoculated in 2.5 ml production medium II [40 g/l soy flour, 5 g/l dipotassium hydrogen phosphate, 0.5 g/l magnesium sulfate heptahydrate, 0.18 g/l calcium chloride dihydrate, 0.025 g/l iron (II) sulfate heptahydrate, and 0.022 g/l manganese (II) chloride tetrahydrate] and cultivated at 30°C and 300 rpm.

Bacillus subtilis transformation

Supplementary Table S1 shows all the primers used in this study. The *B. subtilis* transformation method has previously been described [22]. The recombination scheme in this study is shown in Fig. S1. In this study, the introduction of genes was confirmed by PCR (Supplementary Fig. S1b) and sequence analysis.

To create a surfactin-producing strain, the 4-phosphopantetheinyl transferase gene (*lpa-14*) from *B. subtilis* RB14 [23] was inserted into the *sfp* locus of the 168-strain via homologous recombination. This strain was named KB01. The *lpa-14* integration cassette was constructed as follows: the up-homology arm, *lpa-14*, chloramphenicol resistance gene (*cat*), and down-homology arm were amplified from 168-strain genome, synthetic *lpa-14*, pDLT3 plasmid [24], and 168-strain genome by PCR using the sfpU-f1 and sfpU-r, lpa14-sfpU-f-2 and lpa14-cat-r-2, cat-f and cat-r, and sfpD-cat-f and sfpD-r1 primers. The four fragments were combined by overlap extension PCR using primers: sfpU-f2 and sfpD-r2.

Bacillus subtilis ATCC6633 is a naturally occurring mycosubtilin-producing strain [25]. To transfer ATCC6633's mycosubtilin operon (*fenF-mycABC*) to KB01, the kanamycin resistance gene (*kan*) was inserted downstream of the mycosubtilin operon of ATCC6633. The *kan* integration cassette was constructed as follows: the up-homology arm, *kan*, and down-homology arm were amplified from ATCC6633 genome, pAPNCK [26], and ATCC6633 genome by PCR using the mycCU-f1 and mycCU-kan-r, kan-f and kan-T-tag-r, and mycCD-kan-f and mycaCD-r1 primers. The three fragments were combined by overlap extension PCR using primers mycCU-f2 and mycCD-r2. The KB01 strain was then transformed using genomic DNA extracted from the *kan*-integrated ATCC6633. The up-and-down sequences of the plipastatin operon (*ppsABCDE*) of the 168-strain and those of the mycosubtilin operon from ATCC6633 showed high sequence homology [27]. Therefore, the mycosubtilin operon containing the *kan* from ATCC6633 was integrated into the plipastatin operon locus of the 168-strain using homologous recombination. This strain was named KB04. In KB04, DNA sequencing verified that the correct sequence of the mycosubtilin operon was inserted into the target locus of KB01. Finally, the surfactin operon was disrupted in KB04 by homologous recombination with the spectinomycin resistance gene (*spec*). This strain was named KB06. The surfactin operon disruption cassette was constructed as follows: the up-homology arm, *spec*, and down-homology arm were amplified from the 168-strain genome, pJL62 [28], and 168-strain genome, respectively, using PCR with the srfABU-f1 and srfABU-spec-r, spec-f and spec-T-tag-r, and srfABD-spec-f and srfABD-r1 primers. The three fragments were combined by overlap extension PCR with primers srfABU-f2 and

srfABD-r2. To increase mycosubtilin production in KB06, the surfactin operon promoter was inserted before the mycosubtilin operon. This strain was named KB07. The surfactin operon promoter cassette was constructed as follows: the up-homology arm, erythromycin resistance gene, surfactin operon promoter, and down-homology arm were amplified from the ATCC6633 genome, pMUTinHis [29], 168-strain genome, and ATCC6633 genome by PCR using primers mycU-f1 and mycU-erm-r, erm-f and erm-r, PsrfA-erm-f and PsrfA-fenF-r-2, and fenF-f and fenF6633-r1. The four fragments were combined by overlap extension PCR using the mycU-f2 and fenF-r2 primers.

Analysis of surfactin and mycosubtilin

The surfactin and mycosubtilin concentrations in the fermentation medium were analyzed using a high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an NPS ODS-IIIE column (4.6 mm × 33 mm, particle size 1.5 μm; Eprogen, Downers Grove, IL, USA). Analysis was performed using the following conditions: mobile phase, 0.1% (v/v) formic acid in water (A), and 0.1% (v/v) formic acid in acetonitrile (B). The flow rate was 0.7 ml/min with a gradient of 0–5 min (linear gradient from 10% B to 51%); 5–20 min (maintaining at 51% B); 20–20.1 min (linear gradient from 51% B to 10%); and 20.1–35 min while maintaining at 10% B (re-equilibrate); the injection volume was 10 μl and the column temperature was 40°C. Surfactin and mycosubtilin were detected by measuring the absorbance at a wavelength of 205 nm using a UV detector.

Blood agar hemolysis method

Pre-cultivation was conducted in 3 ml of LB medium containing suitable antibiotics in a shaker incubator at 180 rpm and 37°C overnight. Pre-culture cells (100 μl) diluted 1.0×10^5 to 1.0×10^6 times with sterilized water were plated onto NY agar plates [1 g/l nutrient broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), 1 g/l yeast extract (Becton, Dickinson and Company), 40 g/l glucose, 3 g/l NaCl, and 15 g/l agar (Becton, Dickinson and Company)]. The plates were incubated overnight at 37°C for colony development. A blood agar plate was spotted with 2 μl of a single colony suspended in 50 μl of sterilized water for the hemolysis assay. The blood agar contained the following ingredients: 1 g/l nutrient broth (Becton, Dickinson, and Company), 1 g/l yeast extract (Becton, Dickinson, and Company), 40 g/l glucose, 3 g/l NaCl, 15 g/l agar (Becton, Dickinson, and Company), and 40 mL/L defibrinated sheep blood (Japan Bio Serum, Hiroshima, Japan). After spotting, plates were incubated at 35°C, and images were captured every 24 h.

Measurement of colony and hemolytic zone area

The colony and hemolytic zone areas were measured using ImageJ software (https://imagej.net/ij/index.html) [30, 31]. Images were separated into RGB channels using the commands (Image> Color> Split Channels) to calculate colony area. Using the red channel image, a threshold was established (Image> Adjust> Threshold) to select colonies, and a binary image was generated. The colony area was measured using a command (Analyze> Analyze Particles). To clarify the limits of the hemolytic zone for area measurement, the boundaries were highlighted (Process> Find Edges) before being separated into RGB channels. The hemolytic zone area was measured using a red channel following the same procedure as the colony area measurement. If adjacent hemolytic zones came into contact with the binary image, a command (Binary > Watershed) was used to separate them.

High-throughput screening with colony picker

High-throughput colony screening was carried out using the Qpix460 (Molecular Devices, LLC., San Jose, CA, USA) with a 96-pin-picking head (tip diameter: 0.5 mm, pin length: 49.5 mm). The pins were washed with 70% ethanol, water, and 10% bleach before and after picking. The picking colony was automatically selected based on the following criteria: compactness (roundness) ≥ 0.45, axis ratio ≥ 0.45, minimum diameter (mm) ≥ 0.50, maximum diameter (mm) ≤ 5.00, and minimum proximity (mm) ≤0.45. A total of 48 colonies were inoculated on every other pin on each plate to maintain an appropriate distance between the *B. subtilis* colonies.

Results

Construction of biosurfactant-producing *B. subtilis* strains

The widely used *B. subtilis* 168-strain produces neither surfactin nor mycosubtilin. Although the 168-strain contains the surfactin operon, it is unable to produce surfactin due to a mutation in the 4'-phosphopantetheinyl transferase gene (*sfp*), which is essential for the activation of the peptidyl carrier protein domain [32, 33]. As a result, *lpa-14*, a 4'-phosphopantetheinyl transferase gene obtained from the *B. subtilis* RB14 strain [23], was integrated into the genome of the 168-strain. The surfactin-producing strain was named KB01. KB01 yielded 5.5 ± 0.2 g/l of surfactin after 72 h of cultivation (Fig. 1a).

To produce mycosubtilin, the mycosubtilin operon from *B. subtilis* ATCC6633 was inserted into the KB01 strain's genome. This strain KB04 can produce both mycosubtilin and surfactin. Therefore, the surfactin operon in KB04 was disrupted by replacing it with a spectinomycin resistance gene, yielding KB06, which exclusively generated mycosubtilin. KB06 produced 39.8 ± 0.6 mg/l of mycosubtilin after 72 h of cultivation (Fig. 1b). To increase mycosubtilin production in KB06, we changed the mycosubtilin operon promoter to a surfactin operon promoter (PsrfA) derived from the 168-strain, resulting in strain KB07. The KB07 strain significantly increased mycosubtilin production, reaching 1045.7 ± 12.8 mg/l (Fig. 1b).

Evaluation of hemolytic activity of surfactin and mycosubtilin-producing strains

We verified the hemolytic activity of surfactin- and mycosubtilin-producing strains on blood agar plates. In the first experiment, following liquid culture, KB01 and KB07 were directly inoculated onto blood agar plates. Both strains produced hemolytic zones on blood agar plates after 24 h of incubation (Fig. 2a and b). However, because colonies and hemolytic zones overlapped, comparing the sizes of individual colony hemolytic zones proved challenging. Furthermore, colony and hemolytic zone sizes varied because of the density of colonies on the plate.

In the following experiment, we tested the effect on colony and hemolytic zone development by spotting the 168-strain, KB01, and KB07, which were suspended in sterile water, onto blood agar plates. Spotting results showed that the 168-strain did not form a hemolytic zone, whereas hemolysis was seen around the colonies of the KB01 and KB07 strains (Fig. 3a). *Bacillus subtilis* colonies often have complex forms. In this study, all strains had noncircular colony morphologies (Fig. 3a). Therefore, manual measurement of colony and hemolytic zone areas is challenging. In this study, we used image analysis software (ImageJ) to determine the size of the colonies and hemolytic zones. In both KB01 and KB07, the ratio of hemolytic zone area (Ha) to colony area

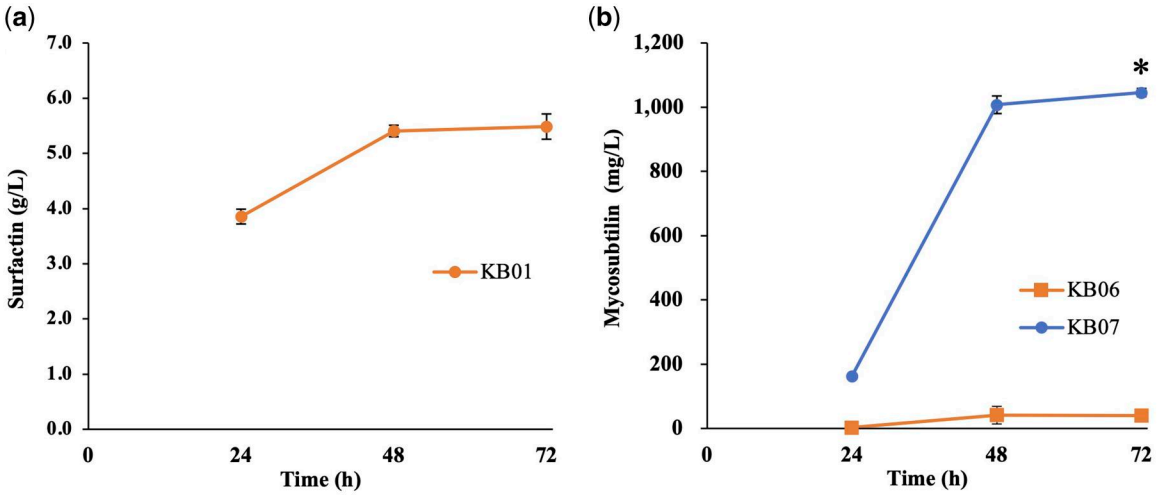

Figure 1. (a) Surfactin and (b) mycosubtilin production using recombinant *B. subtilis* strains. Error bars indicate the standard deviation of three independent experiments. Asterisk represents a statistically significant ($p < 0.01$) difference in mycosbtilin titer after 72 h compared KB06 with KB07 as evaluated by paired comparisons using Student's *t* test

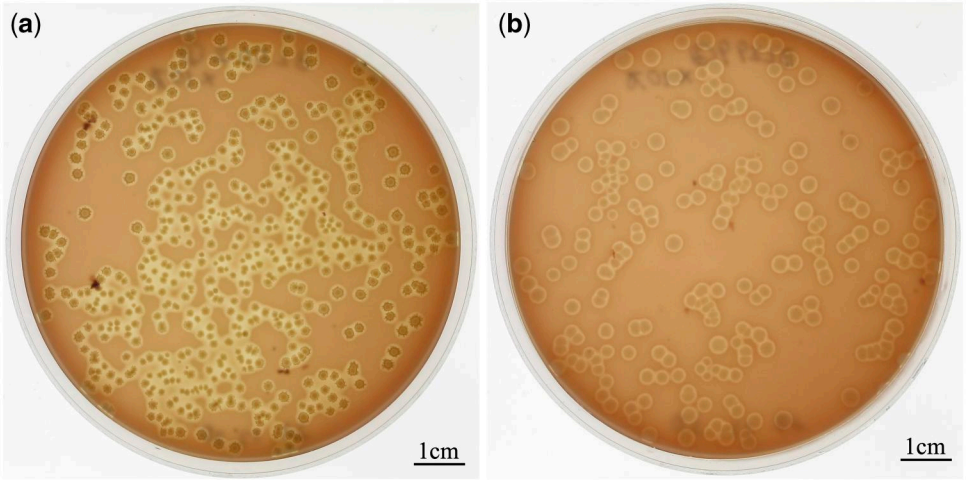

Figure 2. Direct plating of the (a) KB01 and (b) KB07 strains on blood agar plates. Images were taken after incubation at 35 °C for 24 h

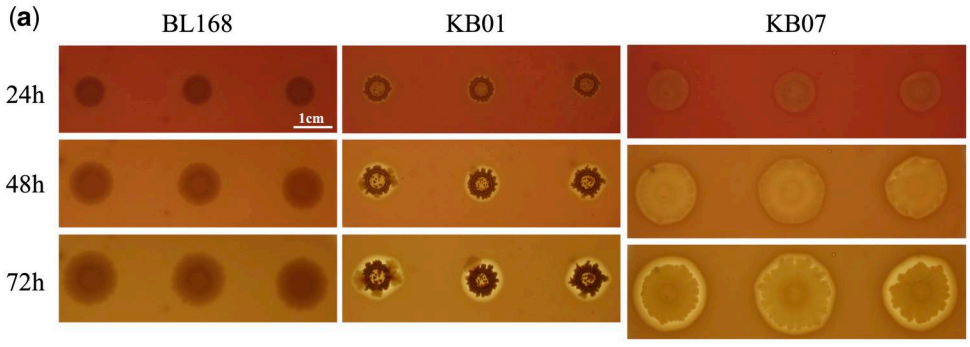

Figure 3. (a) Spotting of strains 168, KB01, and KB07 on blood agar plates. (b) The area ratio was calculated from the colony and hemolytic zone areas measured using ImageJ. For each strain, three independent colonies were selected from donor plates. Colonies were suspended in 50 µl of sterilized water, and 2 µl was spotted on a blood agar plate. The scale bar at the top left applies to all panels

(Ca) increased with incubation time (Fig. 3b). The difference in the Ha/Ca ratio among the three colonies was modest for both strains, allowing for a consistent evaluation of the hemolytic activity of the strains by spotting.

Effect of blood plate composition on colony growth and hemolytic zone formation

Based on prior publications, the blood agar plates in this study used glucose as a carbon source, nutrient broth, and yeast

extract as nitrogen and other nutrient sources [7]. However, there are no reports on how plate composition affects the establishment of hemolytic zones. Therefore, the purpose of this study was to explore plate composition.

Using 40 g/l glucose, 1 g/l nutrient broth, and 1 g/l yeast extract as the standard composition, the effects of changing these quantities on hemolytic zone formation were investigated. In the standard composition, Ha/Ca ratio of KB01 and KB07 was 1.73 and 1.92 after 72-h incubation (Fig. 4a and Supplementary Fig. S2). In the glucose concentration evaluation, it was found that at 4 g/l glucose, the plates became clear, probably due to osmotic effects, making it difficult to evaluate the hemolytic zones (Fig. 4a and b). At 100 g/l glucose, colony growth was suppressed in all strains (Fig. 4b and Supplementary Fig. S2). Compared to the standard composition plate, the 100 g/l glucose plate had similar Ha/Ca ratios for KB01 (1.76) but higher values for KB07 (2.81) after 72 h incubation (Supplementary Fig. S2). When the amount of nutrient broth was evaluated, and compared to the standard composition plate, colonies tended to be smaller at 0.3 g/l nutrient broth plate for all strains, whereas colonies tended to be larger at 10 g/l nutrient broth plate with changes in colony morphology (Fig. 4c and Supplementary Fig. S2). KB07 had 2.0-fold larger colony sizes in 10 g/l nutrient broth at 72 h, but the Ha/Ca ratio (1.49) was not significantly different from the standard composition. In contrast, KB01 demonstrated a 2.8-fold higher Ha/Ca ratio when using 10 g/l nutrient broth when compared to the standard composition. When the amount of yeast extract changed, the colony size and Ha/Ca ratio of both KB01 and KB07 strains showed almost identical outcomes to changes in the amount of nutrient broth. (Fig. 4d and Supplementary Fig. S2).

Development of a high-throughput screening scheme using an automation system

Spotting the strain at equal intervals increased the accuracy of hemolytic zone evaluation (Fig. 3a and b). However, manually suspending and spotting colonies is a time-consuming and labor-intensive procedure. Therefore, we developed a method to accurately determine the hemolytic activity of a large number of colonies by directly inoculating agar plates with colonies selected using an automatic colony picker.

The high-throughput screening method is shown in Fig. 5. Briefly, the strains of interest were cultured in LB medium, and approximately 200–500 colonies were seeded onto NY plates. Subsequently, 48 colonies were inoculated per plate using an automatic colony picker to avoid contact between adjacent colonies (Fig. 6a and b). After 48 h of incubation, the size of the colonies and hemolytic zones was semi-automatically measured using image analysis software (Supplementary Figs S3 and S4).

The average area of 48 KB01 colonies was 0.22 ± 0.04 cm^2, with an average hemolytic zone area of 0.48 ± 0.06 cm^2. The colony

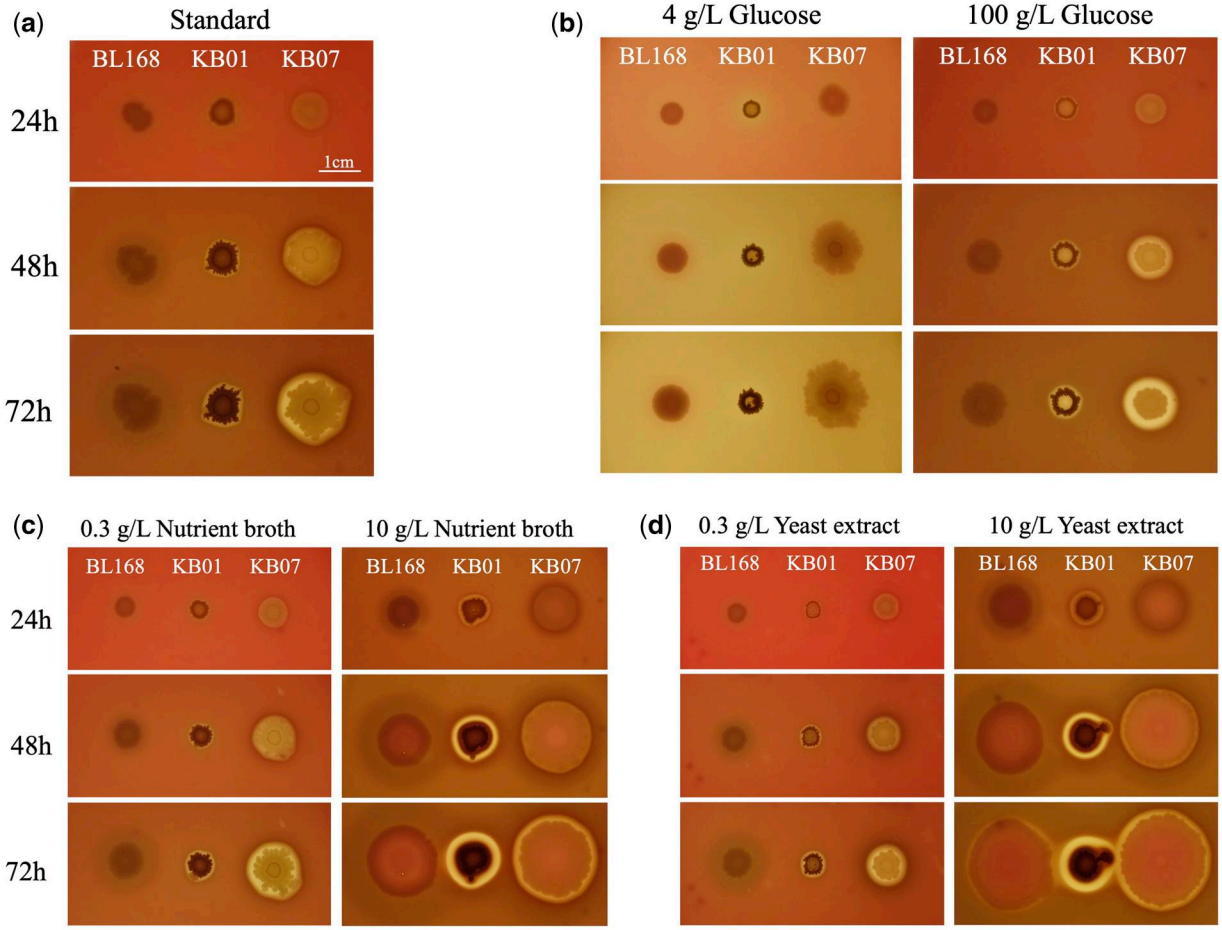

Figure 4. Spotting of strains 168, KB01, and KB07 on blood agar plates with (a) standard (40 g/l glucose, 1 g/l nutrient broth, 1 g/l yeast extract, 3 g/l NaCl, 15 g/l agar, and 40 ml/l defibrinated sheep blood) and modified composition. The amounts of (b) glucose, (c) nutrient broth, and (d) yeast extract were modified with regard to the standard composition. Colonies picked from the donor plate were suspended in 50 μl of sterilized water, and 2 μl was spotted on blood agar plates. The scale bar in panel (a) applies to all panels

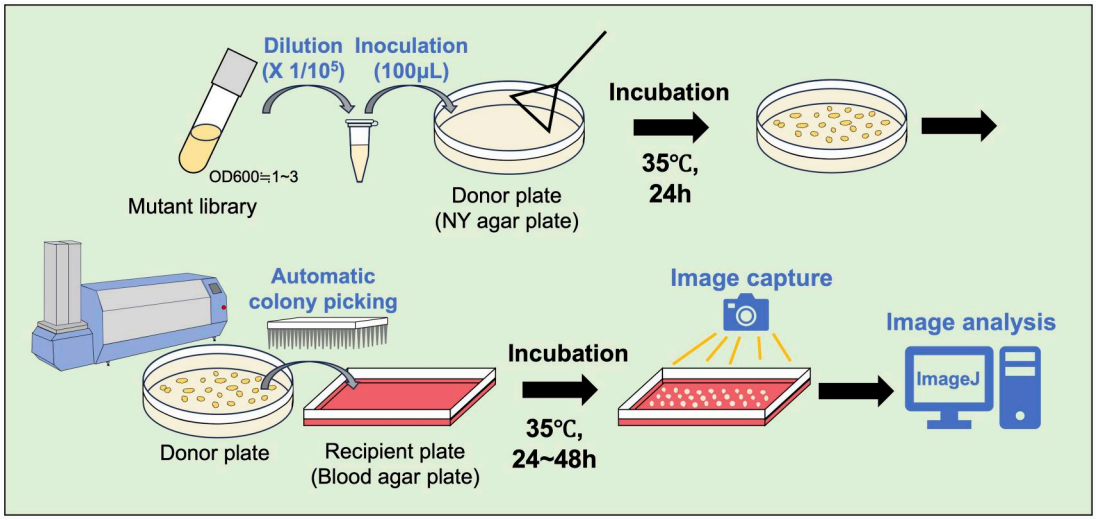

Figure 5. Scheme of high-throughput screening using blood agar lysis method with integrated colony pickers and image analysis. All the elements of this figure were created using PowerPoint for Mac 2019 (Microsoft, Redmond, WA, USA)

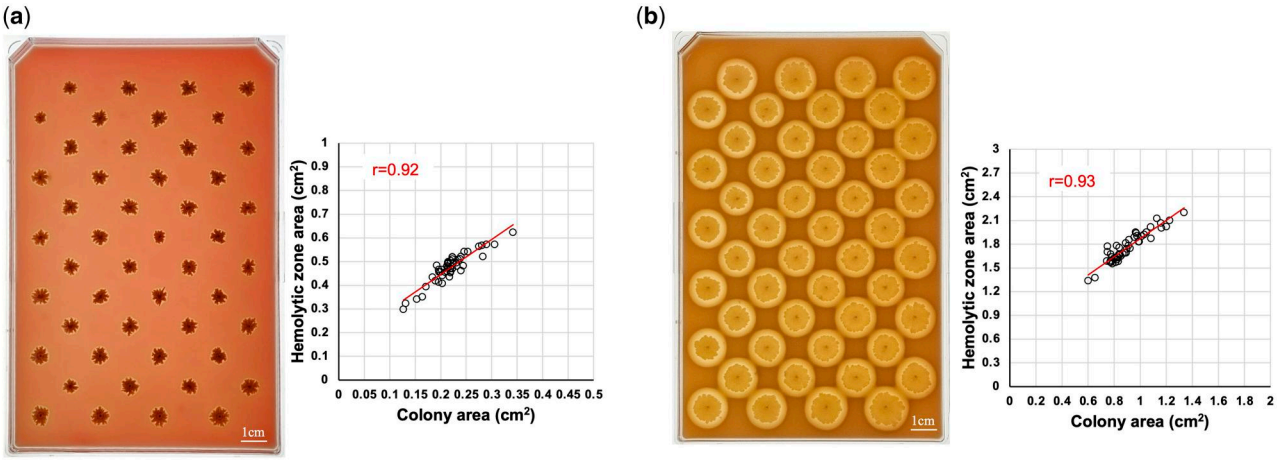

Figure 6. Inoculation of strains (a) KB01 and (b) KB07 on blood agar plates. The graph represents the colony and hemolytic zone sizes for each of the 48 colonies, measured using ImageJ, where r denotes the correlation coefficient. Images were taken after 48 h of incubation

and hemolytic zone areas of the KB01 strain were highly correlated, with a correlation coefficient of 0.92 (Fig. 6a). The mean ratio of hemolytic zone area to colony area was 2.18 ± 0.15, with a coefficient of variation of 0.07. In the KB07 strain, the average area of 48 colonies was 0.91 ± 0.15 cm^2, while the average hemolytic zone area was 1.77 ± 0.19 cm^2. The colony and hemolytic zone areas of the KB07 strain were highly correlated, with a correlation coefficient of 0.93 (Fig. 6b). The KB07 strain had an average ratio of hemolytic zone to colony area of 1.96 ± 0.15, with a coefficient of variation of 0.07. A test of no correlation was conducted between hemolytic zone and colony size in KB01 and KB07. As a result, a significant correlation was found between the hemolytic zone and colony size in both strains, with $p < 0.01$. These findings show that the combination of colony pickers and image analysis software allows for the rapid and accurate evaluation of the hemolytic activity of a large number of colonies.

Discussion

In this study, we investigated the procedure for inoculating bacterial cells onto blood agar plates, as well as the incubation period and plate composition, to develop a high-throughput screening system for biosurfactant-producing strains using blood agar lysis methods. This study indicated that the composition of the plate affects the formation of colonies and hemolytic zones. It was suggested that even with target biosurfactants of low production, optimizing the plate composition could make it easier to observe hemolytic zone. This study also revealed that direct inoculation of *B. subtilis* cells onto blood agar plates made evaluating hemolytic activity difficult because of overlapping colonies and hemolytic zones (Fig. 2). Furthermore, given the diverse morphology of *B. subtilis* colonies, manual measurements of areas of colony and hemolytic zone are difficult. While these aspects had not been mentioned in previous studies, they have been suggested to have a significant impact on the accuracy of screening for highly productive strains in hemolytic activity. Therefore, in this study, two improvements were made to the blood agar lysis method to accurately evaluate hemolytic activity with high throughput. One involved the rearrangement of colonies using a colony picker, and the other was the measurement of colony and hemolytic zone areas through image analysis.

By rearranging the colonies, it became possible to maintain appropriate spacing between colonies, enabling accurate evaluation of hemolytic activity. However, manual colony

rearrangement takes time. Therefore, in this study, automatic colony picker was applied to rearrange colonies for expediting the process (Fig. 5). In previous studies using the blood agar lysis method, Mulligan et al. [7] obtained a *B. subtilis* mutant with a 3.4-fold increase in surfactin productivity through screening 1000 colonies of UV mutants. Bouassida *et al.* [8] obtained *B. subtilis* mutant with over 20% increase in surfactin productivity through screening 300 colonies of mutants. In other screening methods, Zhu *et al.* [17] successfully obtained a *B. subtilis* mutant with a 3-fold increase in production compared to the parent strain, among 27,000 mutants, using a color assay with polydiacetylene vesicles in the culture supernatant. While the time required for obtaining superior strains in previous screening experiments is unclear, the methodology demonstrated in this study could pick 4800 colonies (48 colonies/plate) in 4 h. Owing to inconsistent inoculum cell amount during colony reordering, there was variation in colony sizes. However, by analyzing colony and hemolytic zone areas using image analysis software, a correlation was observed between colony size and hemolytic zone area across 48 colonies (Fig. 6). This suggests that measuring colony and hemolytic zone areas with image analysis software allows for the accurate evaluation of hemolytic activity (Fig. 6a and b). Furthermore, as the time required for image analysis is 2–3 min per image, the analysis of 4800 colonies (100 plates) can be completed within a day.

Conclusions

This study revealed that to accurately evaluate hemolytic activity using the blood agar lysis method, it is necessary to ensure uniform alignment of colonies and precise determination of colony area and hemolytic zone area through image analysis. Furthermore, as the composition of the medium and the incubation time significantly affect the colony area and hemolytic zone area, it is suggested that optimal conditions be explored based on the strain or type of surfactant. Although the use of the blood agar lysis method for screening is limited to surfactants exhibiting hemolytic activity, it is believed that by considering the insights gained from this study, it can contribute to the accurate screening of strains with high productivity.

Author contributions

Takahiro Bamba (Conceptualization [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Writing—original draft [lead]), Rina Aoki (Conceptualization [equal], Investigation [equal], Methodology [equal], Writing—original draft [supporting]), Yoshimi Hori (Investigation [equal], Methodology [equal]), Shu Ishikawa (Investigation [equal], Resources [equal], Writing—review & editing [equal]), Ken-ichi Yoshida (Investigation [equal], Resources [equal], Writing—review & editing [equal]), Naoaki Taoka (Project administration [equal], Supervision [equal], Writing—review & editing [equal]), Shingo Kobayashi (Conceptualization [equal], Project administration [equal], Supervision [equal], Writing—review & editing [equal]), Hisashi Yasueda (Supervision [equal], Writing—review & editing [equal]), Akihiko Kondo (Project administration [equal], Supervision [equal], Writing—review & editing [equal]), and Tomohisa Hasunuma (Project administration [equal], Supervision [equal], Writing—review & editing [equal])

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Conflict of interest statement. The author declares no conflict of interest.

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