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A simple method for rapid screening of biosurfactant-producing strains using bromothymol blue alone

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ABSTRACT

Biosurfactants are deemed as green replacements of synthetic surfactants. Lipopeptides, a class of biosurfactants, have excellent surface active properties along with anti-microbial and anti-tumor activities. Identifying microorganisms producing high titre of lipopeptide is of great importance in reducing production cost as currently lipopeptide production is not commercially competitive. A quantitative colorimetric assay method was developed for detecting lipopeptides and high-throughput screening of lipopeptide-producing strains by mixing bromothymol blue solution (pH 7.2) with lipopeptide-containing samples resulting in an instantaneous and stable color change. This color change is quantified spectrophotometrically at 410 nm and 616 nm showing linearly quantitative response to increased concentrations of 3 major lipopeptides (surfactin, iturin and fengycin) at 0–1 g/l. The assay was used on both cell-free broth and crude extracts of lipopeptide-producing strains and the titre obtained from the assay was supported by HPLC data. This assay can be used for screening lipopeptide-producing strains and quantitatively detecting lipopeptides either in purified form or in cell-free broth.

1. Introduction

Biosurfactants, consisting of lipopeptides, glycolipids and phospholipids, are deemed as green replacements of synthetic surfactants. Lipopeptides, cyclic peptides linked to a fatty acid tail, are mainly produced by *Bacillus* genus (Abdel-Mawgoud et al., 2008; Deleu et al., 2008). Lipopeptides have excellent surface active properties and anti-tumor and anti-viral activities as well as anti-microbial activity against a wide range of microbes (Kosaric, 2001; Yang et al., 2006). Surfactin, a sub-class of lipopeptides, is well known for lowering the surface tension of water from 72 to 27 mN/m and has a critical micelle concentration value as low as 25 μg/ml (Cooper et al., 1981). These properties make them suitable candidates for applications in pharmaceutical and personal care industries (Huang et al., 2006; Mulligan, 2005; Straus and Hancock, 2006). Lipopeptides are still too costly to be produced industrially compared to other surfactant classes mainly due to their low production titre of < 1 g/l (Makkar and Cameotra, 2002). Hence, isolating high titre of lipopeptide-producers is of great importance in lowering their production cost.

The methods popularly used for screening lipopeptide-containing samples include surface tension measurement using Du Noüy ring method, emulsification assay and hemolysis assay (Satpute et al., 2008; Walter et al., 2010). These assays, however, have limitations including scalability of the assays, need of expensive or special equipment, laborious reagent preparation, usage of toxic chemicals and high number of false positive or negative data (Burch et al., 2010; Yang et al., 2015; Youssef et al., 2004; Zhu et al., 2014).

Here we use bromothymol blue (BTB), known to detect lipids and phospholipids in thin layer chromatography (Vioque, 1984), as a rapid and high-throughput screening assay for detection of lipopeptides. Interestingly, the major lipopeptide classes, surfactin, iturin and fengycin, can be differentiated by this assay as they react with BTB showing different colors at concentrations of 1 g/l and below. The presence of lipopeptides can be tested directly from cell-free broth, eliminating the purification step hence saving time.

2. Materials and methods

2.1. Chemicals

Surfactin, iturin, fengycin, BTB, NaCl, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma-Aldrich. Phusion high fidelity DNA polymerase master mix was purchased from Thermo Fisher Scientific.

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2.2. Bromothymol blue assay

BTB solution (0.2 mM) was prepared by dissolving BTB into phosphate-buffered saline (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4 and 1 M NaCl) and the pH of the solution was adjusted to 7.2. The assay was performed in 96 well plates by mixing equal volumes of BTB and surfactant sample unless otherwise specified. The quantitative response was measured using a plate reader (Varioskan Flash, Thermo Fisher Scientific) at wavelengths of 616 nm and 410 nm. Colorimetric response (CR, %) was quantified as previously reported (Satake et al., 1960). Blanks for samples were prepared by mixing BTB with media that the samples were dissolved in. The samples were adjusted to pH 7–8 prior to tests due to sensitivity of the assay to pH changes.

2.3. Screening of biosurfactant-producing microorganisms

The microorganisms isolated from Singapore environment were first treated in 90 °C for 10 min to eliminate non-spore forming microbes. The treated samples were then cultivated in Lennox media at either 30 °C or 50 °C. After 48 h, the broth was collected and centrifuged at 4000 rpm for 15 min. The supernatant (cell-free broth) was transferred to a clean tube for use in various screening assays – Parafilm M test (Kalyani et al., 2014), oil spreading assay (Morikawa et al., 2000), BTB assay and surface tension measurement using Du Noüy ring method. Subsequently, genomic DNA of selected samples was extracted for purposes including species identification and detection for presence of lipopeptide genes. The relevant primers used were stated in Table 1. Briefly, crude biosurfactant samples (1 µl) were ran on TLC plate using a solvent system consisting of chloroform: methanol:water (69:24:2, v/v) along with relevant standards (surfactin, iturin and fengycin at 0.5 g/l). After running, the plate was dried and subsequently sprayed with water for detection of lipopeptides (Peypoux et al., 1991).

2.4. Mutagenesis of strain A

Surfactin-producer *Bacillus subtilis* Strain A was cultivated as described above and was mutated using atmospheric room-temperature plasma machine (ARTP, Tmax Tree Co. Ltd., China) according to manufacturer's instructions. Cell-free broths of selected samples were harvested after 48 h of cultivation. BTB test was performed on cell-free broths. Surfactin standard was dissolved in fermentation media and the equation obtained from the standard curve was used to calculate the titre of the samples from the BTB test. The cell-free broths were extracted as described previously (Kosaric, 2001) for quantification of surfactin titres via HPLC system (Agilent) with a gradient program as described previously (Yang et al., 2015).

Table 1
List of primers.

Gene	Primer	Sequence	Ref.
16s	27f 1492r	5′-AGAGTTTGATCMTGGCTCAG-3′ 5′-GGTTACCTTGTTACGACTT-3′	(Tanner et al., 1999)
Surfactin (sfp)	Sfp-f: Sfp-r:	5′-ATGAAGATTTACGGAATTTA-3′ 5′- TTATAAAAGCTCTTCGTACG-3′	(Porob et al., 2013)
Surfactin (srfC)	Sur3f: Sur3r:	5′- ACAGTATGGAGGCATGGTC-3′ 5′- TTCCGCCACTTTTTCAGTTT-3′	(Kavitha et al., 2012)
Surfactin synthetase (SrfAA)	srfAf: srfAr:	5′- TCGGGACAGGAAGACATCAT-3′ 5′- CCACTCAAACGGATAATCCTGA-3′	(Almoneafy et al., 2014)
Iturin A synthetase C (ItuC)	ituCf: ituCr:	5′- GGCTGCTGCAGATGCTTTAT-3′ 5′- TCGCAGATAATCGCAGTGAG-3′	(Mora et al., 2011)
Iturin A (ItuD)	ItuD1f: ItuD1r:	5′- GATGCGATCTCCTTGGATGT-3′ 5′- ATCGTCATGTGCTGCTTGAG-3′	(Gond et al., 2015)
Bacillomycin (BamC)	Bacc1f: Bacc1r:	5′- GAAGGACACGGAGAGAGTC-3′ 5′- CGCTGATGACTGTTCATGCT-3′	(Abdallah et al., 2017)
Bacillomycin L synthetase B (bmyB)	bmyBf: bmyBr:	5′- GAATCCCGTTGTTCTCCAAA-3′ 5′- GCGGGTATTGAATGCTTGTT-3′	(Frikha-Gargouri et al., 2017)
Fengycin (fenD)	fenDf: fenDr:	5′- GGCCCGTTCTCTAAATCCAT-3′ 5′- GTCATGCTGACGAGAGCAAA-3′	(Khedher et al., 2015)

2.5. Statistics

All data were performed in triplicates and were stated as average ± standard error of the mean (SEM). Results were considered to be statistically significant at $P < 0.05$ using Student's *t*-test.

3. Results and discussion

3.1. Development of bromothymol blue assay

We developed a screening method that was performed by mixing individual lipopeptide standards (surfactin, iturin and fengycin) with BTB. The assay showed a wide drift in colorimetric response (CR, %) from pH 1 to 5 but had relatively stable colorimetric response (CR, %) for pH 10–13.3 and pH 6–8 (Fig. 1a). Most strains propagated healthily for pH 6–8, hence the assay was conducted in this pH range for subsequent experiments. The pH of the BTB assay is versatile and can also be modified to suit the desired optimal pH range of strain growth. Next, the CR was observed to be consistent for all lipopeptide classes for incubation time between 0 s and 2 h (Fig. 1b). After incubation for 24 h, CR for all lipopeptide classes displayed a significant decrease of ~ 20% ($p < 0.005$) as compared to the CR at 0 s (Fig. 1b). The stability of the CR values suggested the ease of performing high throughput studies using the assay as it could be measured anytime between 0 s and 2 h. Subsequent assays were performed without incubation. The spectral scanning of lipopeptide-BTB mixture showed 2 prominent peaks with corresponding increase and decrease at 414 nm and 616 nm respectively increasing lipopeptide concentrations (Fig. 2a, c and e).

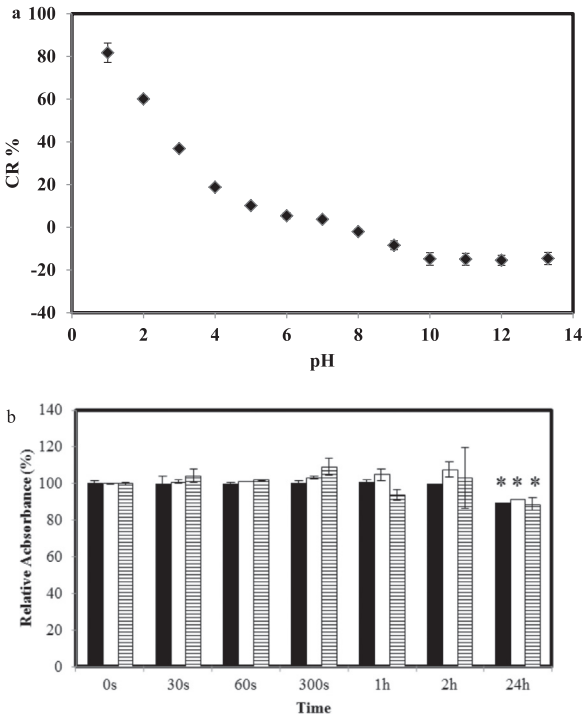

Fig. 1. Influence of pH and time on BTB method. **(a)** Influence of pH on Bromothymol blue assay. Colorimetric response (CR, %) shifted when Lennox medium pH was changed; (b) Stability of absorbance values of lipopeptides standards with time. Surfactin (■); fengycin (□); iturin (–); $p < 0.005$ (*).