



A novel nucleic lateral flow assay for screening of PHA-producing haloarchaea

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ARTICLE INFO

Article history:

Received 23 March 2015

Received in revised form 23 June 2015

Accepted 23 June 2015

Available online 27 June 2015

Keywords:

Biosensor

DNA lateral flow

PHA synthase class III

phaE

PHAs

Halophiles

ABSTRACT

Polyhydroxyalkanoates (PHAs) are important for biodegradable plastic production, and prokaryotes play a very important role in PHA production. PHA synthase is a key enzyme for the polymerization of PHAs. There are four classes of PHA synthase. The *phaC* gene is necessary for the production of all classes of PHA synthase, whereas the *phaE* gene is necessary for the production of class III PHA synthase. This gene is a biomarker for microorganisms that contain class III PHA synthase, such as haloarchaea. Standard techniques for screening of PHA-producing haloarchaea require time for culturing and have poor specificity and sensitivity. Thus, the *phaE* biosensor was developed to overcome these issues. PCR and DNA lateral flow biosensor techniques were combined for construction of the *phaE* biosensor. The *phaE* biosensor has a high specificity for PHA-producing haloarchaea. The lowest amount of genomic DNA of *Haloquadratum walsbyi* DSM 16854 that the *phaE* gene could be detected by the biosensor was approximately 250 fg. The *phaE* biosensor can be applied for screening of PHA-producing haloarchaea from environmental samples. The *phaE* biosensor is easy to handle and dispose. For screening PHA-producing haloarchaea, the *phaE* biosensor requires less time and costs less than the standard methods.

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1. Introduction

Production of polyhydroxyalkanoates (PHAs) is increasing due to their biodegradable characteristics. The properties of PHAs are similar to petroleum-based plastics (Steinbuchel and Fuchtenbusch, 1998). PHAs are environmentally friendly because they can be produced from renewable sources. The consumption of PHAs in European countries was approximately 50,000 to 100,000 tons in 2007 and will increase to 2–4 million tons in 2020 (Crank et al., 2005). Poly(3-hydroxybutyrate) (PHB) is the most common type of PHA that is accumulated by bacteria and archaea (Steinbuchel and Fuchtenbusch, 1998). PHA-producing microorganisms are able to convert carbon sources, such as glucose and fatty acids, to intermediate substances in PHA synthesis pathways. Acetyl-CoA and acyl-CoA are important intermediate substances for PHA biosynthesis (Philip et al., 2007). PHA synthases are key enzymes in PHA biosynthesis and are classified into four groups based on their structures, substrate specificities and subunit components (Rehm, 2003). Classes I and II PHA synthases are encoded by the *phaC* gene. Class III PHA synthase is encoded by the *phaC* and *phaE* genes. Class IV PHA synthase is encoded by the *phaC* and *phaR* genes (Rehm, 2003). The accumulation of PHAs in haloarchaea has been reported in the literature; however, the number of reports on this topic is still limited (Quillaguaman et al., 2010). Haloarchaea require a high concentration of NaCl for growth. Non-

halophilic microorganisms cannot grow in that condition; thus, the cost of sterilization of the equipment, media and bioreactor used for haloarchaea production is low. The salt from the cultured media can be recovered and reused in the PHA production pipeline (Hezayen et al., 2010). Isolation of PHAs from haloarchaea can be performed using hypo-osmotic shock. PHAs leak out of the haloarchaea cells when they are treated with salt-free water. This method reduces the cost of the downstream processes by approximately 40% (Choi and Lee, 1999). *Haloferax mediterranei* is a halophilic archaea that utilizes whey hydrolysate as a carbon source for PHA production. The cost of PHA production is 2.82 euro for 0.29 g⁻¹ L⁻¹ h⁻¹ (Koller et al., 2007). This production cost is approximately 1.4 times lower than that for PHA production by recombinant *Escherichia coli* (c.a. 4.0 Euro) (Koller et al., 2007; Reddy et al., 2003).

In this study, PCR method had been applied for detection of the *phaE* gene of PHA-producing haloarchaea. DNA lateral flow biosensor was a rapid method that had been used for detection of the *phaE* gene amplicon.

2. Material and methods

2.1. Bacterial strains and culture method

Bacillus megaterium DSM 319 and *Ralstonia eutropha* DSM 428 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). *Streptomyces hygroscopicus* BTCC 7028 was obtained from the National Center for Genetic Engineering and

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Biotechnology (BIOTEC). *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *P. fluorescens* ATCC 13525 and *Staphylococcus aureus* ATCC 13565 were obtained from the Department of Microbiology, Faculty of Pharmacy, Mahidol University. Genomic DNA of *Haloquadratum walsbyi* DSM 16854 was obtained from DSMZ. All bacterial strains were inoculated in LB broth at 37 °C with shaking at 100 rpm overnight.

2.2. Primer design for *phaE* gene of PHA-producing haloarchaea

The *phaE* gene sequences of PHA-producing haloarchaea were obtained from the genome database of GenBank. The length of the *phaE* genes of haloarchaea was 507 to 555 bp. The gene sequences were aligned using the iterative refinement method. The evolutionary model selection was based on Akaike information criterion (AIC) and hierarchical likelihood ratio tests (hLRTs) criteria. A phylogenetic tree was constructed using the Bayesian inference method (Yasawong et al., 2011). The conserved region of *phaE* genes was used to design the primers.

2.3. Polymerase chain reaction

Genomic DNA of bacterial cells was extracted using the PowerSoil® DNA isolation kit (Mo Bio, USA). *H. walsbyi* DSM 16854 is a PHA-producing haloarchaea (Burns et al., 2007). *H. walsbyi* DSM 16854 possesses PHA synthase class III and contains the *phaE* gene (Bolhuis et al., 2006). Thus, the genomic DNA of *H. walsbyi* DSM 16854 was used as a positive control for *phaE* amplification. The *phaE* primers were purchased from Bio Basic Canada Inc. The details of the *phaE* primers are shown in Table 1. The size of the PCR product was approximately 224 bp. The PCR reaction was performed in a 50 µL total volume containing 10 ng of DNA, 20 pmol of each primer, 80 µM dNTPs, 1 × reaction buffer (Vivantis, PL1202, Malaysia) and 1.25 U thermostable DNA polymerase (Vivantis, PL1202, Malaysia). The PCR began with an initial step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 10 s, and a final step of 72 °C for 1 min. PCR was performed in a T100™ Thermal Cycler (Bio-Rad, USA). Reproducibility of PCR was examined by testing three replicates of genomic DNA by each assay and repeating the experiment for five times. The amplicon was examined by 1% w/v agarose gel electrophoresis. GeneRuler 100 bp plus DNA ladder (Thermo, USA) was used as the DNA marker for the agarose gel electrophoresis.

2.4. Specificity test of the *phaE* biosensor

The details of the microorganisms used in this study are shown in Table 2. *H. walsbyi* DSM 16854 was used as a positive control, as it is able to produce PHA and contains the *phaE* gene (Burns et al., 2007; Bolhuis et al., 2006). Deionized water was used instead of DNA template for a no template control (NTC) reaction. Negative control group 1 (NC1) included *E. coli* ATCC 25922, *S. aureus* ATCC 13565 and *S. hygroscopicus* BTCC 7028, which are all incapable of producing PHAs and lack the *phaE* gene. Negative control group 2 (NC2) included *B. megaterium* DSM 319, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 9027, *P. fluorescens* ATCC 13525 and *R. eutropha* DSM 428, which are all capable of producing PHAs but lack the *phaE* gene. The *phaE* biosensor procedure contained two steps, amplification and detection. First, the *phaE* gene of PHA-producing haloarchaea was amplified by PCR. Then, the *phaE* amplicon was detected on the HybriDetect DNA lateral

Table 2

Specific details of microorganisms used in this study.

No.	Species	PHA synthase	<i>phaE</i>	Designated
1	<i>B. megaterium</i> DSM 319	Class IV	No	Negative control group 2
2	<i>B. subtilis</i> ATCC 6633	Class IV	No	Negative control group 2
3	<i>E. coli</i> ATCC 25922	–	No	Negative control group 1
4	<i>H. walsbyi</i> DSM 16854	Class III	Yes	Positive control
5	<i>P. aeruginosa</i> ATCC 9027	Class II	No	Negative control group 2
6	<i>P. fluorescens</i> ATCC 13525	Class II	No	Negative control group 2
7	<i>R. eutropha</i> DSM 428	Class I	No	Negative control group 2
8	<i>S. aureus</i> ATCC 13565	–	No	Negative control group 1
9	<i>S. hygroscopicus</i> BTCC 7028	–	No	Negative control group 1

flow biosensor (Milenia Biotec, MGHDI, Germany). The specificity of the *phaE* biosensor was dependent on the amplification step. PCR amplification was positive when the amplicon contained biotin and FITC at its flanking region. The DNA lateral flow biosensor was obtained from Milenia Biotec (Germany) and was set up according to the instructions provided with the kit (Milenia, Germany). Five microliters of PCR products were added to the DNA lateral flow biosensor. The results appeared on the biosensor within one to two minutes. One line on the biosensor (control line) meant that no amplification of the *phaE* gene was detected. The PCR product of *phaE* gene was detected when two lines appeared on the biosensor (control line and test line).

2.5. Sensitivity test of the *phaE* biosensor

The genomic DNA (gDNA) of *H. walsbyi* DSM 16854 was quantified using NanoDrop (Thermo, USA). Two-fold dilutions of the gDNA were performed from 1000 to 125 fg/µL. Each dilution of the gDNA of *H. walsbyi* DSM 16854 was used as a DNA template for the PCR reaction as previously described. The PCR products were examined by 1% w/v agarose gel electrophoresis and the DNA lateral flow biosensor (Milenia Biotec, Germany).

2.6. Testing of the *phaE* biosensor with environmental samples

Soil samples were collected from solar salterns and mangroves. The details of the samples are described in Table 3. Environmental DNA (eDNA) was extracted using the PowerSoil® DNA isolation kit (Mo Bio, USA). Ten nanograms of eDNA was used as the DNA template for PCR. The amplicons were tested on the HybriDetect DNA lateral flow biosensor (Milenia Biotec, Germany).

3. Results

3.1. Primer design for *phaE* gene of PHA-producing haloarchaea

The *phaE* phylogeny of haloarchaea was reconstructed based on the GTR + G model. The *phaE* sequence of *Allochromatium vinosum* was used as an outgroup for the analysis. The posterior probabilities were obtained by performing two separate runs with four Markov chains. Each run was conducted with four million generations and was sampled every 100 generations. The potential scale reduction factor (PSRF) for all parameters was 1.000. The average standard deviation of the split frequencies was 0.001149. A consensus tree was calculated after discarding the first 25% of the iterations as the burn-in. The *phaE* phylogeny of PHA-producing haloarchaea is shown in Fig. 1. The details of the microorganisms used for the phylogenetic analysis are shown in

Table 1

Primers for amplification of *phaE* gene of PHA-producing haloarchaea.

Primer	Sequence (5' → 3')	Length (bp)	5'-labeled	PCR product (bp)
PhaE3A-F	GAGTTCGGYATATCTGGYT	20	Biotin	224
PhaE3A-R	TGCTGACGACGCTCGASYTC	20	FITC	

Table 3
Sampling sites and details of soil samples.

No.	Province	Location	Depth (cm)	Designation
1	Prachuap Khiri Khan	Solar saltern	1	PK1
2	Prachuap Khiri Khan	Solar saltern	10	PK2
3	Chumphon	Solar saltern	1	CP1
4	Chumphon	Solar saltern	10	CP2
5	Chol Buri	Mangrove	1	CB1
6	Chol Buri	Mangrove	10	CB2
7	Chol Buri	Mangrove	20	CB3

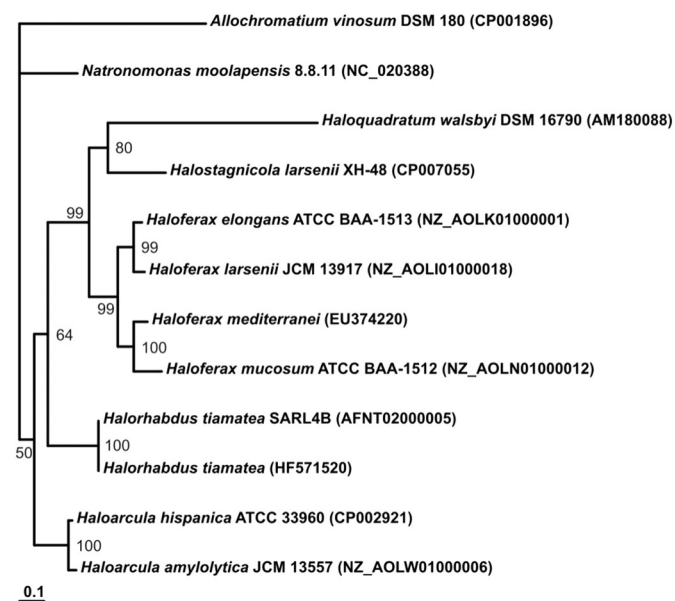


Fig. 1. *phaE* phylogeny was constructed using the Bayesian inference method. The values associated with nodes correspond to the clade credibility support in %.

Table 4. The *phaE* primers were designed manually based on the conserved regions of *phaE* and on *phaE* phylogeny. The conserved region of positions 300–319 was selected as the forward primer, and the conserved region of positions 504–523 was selected as the reverse primer (Fig. 2).

3.2. Polymerase chain reaction

PCR was performed for validation of the *phaE* primers (Table 1). The PCR was optimized to achieve the highest specificity of the primers and the shortest time for the analysis. The PCR successfully amplified 224 bp

of the *phaE* gene of *H. walsbyi* DSM 16854 (Fig. 3), and less than 40 min was required for the PCR analysis. Nonspecific amplification and primer–dimers were not observed.

3.3. Specificity test of the *phaE* biosensor

The PCR products of *phaE* amplification are shown in Fig. 4. No PCR amplification occurred with the non-template control sample or the bacteria of the NC1 or NC2 control groups (Fig. 4). No nonspecific amplification or primer–dimers were observed in any PCR amplifications (Fig. 4). The PCR products were tested on the DNA lateral flow biosensor. The test results are shown in Fig. 5. A positive result was only obtained from the sample that contained the PCR product of the *phaE* gene (*H. walsbyi* DSM 16854). Negative results were obtained for the NTC and the bacteria of the NC1 and NC2 control groups (Fig. 5).

3.4. Sensitivity test of the *phaE* biosensor

The PCR products of *phaE* amplification are shown in Fig. 6. The *phaE* amplicon was detected by the *phaE* biosensor (Fig. 7). The lowest concentration of the *phaE* gene that the biosensor could detect was one copy number.

3.5. Testing of the DNA lateral flow biosensor with environmental samples

The PCR products of *phaE* amplification are shown in Fig. 8. The *phaE* amplicon was detected by the *phaE* biosensor (Fig. 9). The *phaE* gene was found in all eDNA samples, indicating that the soil samples from solar salterns (CP1, CP2, PK1 and PK2) and mangroves (CB1, CB2 and CB3) contained PHA-producing haloarchaea.

4. Discussion

The *phaE* phylogeny consisted of seven genera of haloarchaea that belonged to the family *Halobacteriaceae*. Based on the phylogenetic analysis, *H. walsbyi* could be chosen as a positive control for *phaE* PCR. *H. walsbyi* is a halophilic archaea that requires 14 to 36% (w/v) NaCl for growth. *H. walsbyi* grows at pH 6.0 to 8.5 and 25 to 45 °C. Cells of *H. walsbyi* are square and grow in strictly aerobic conditions. Colonies of *H. walsbyi* on agar plates are iridescent pink (Burns et al., 2007).

PHA-producing microorganisms can be screened by observing the PHA granules that accumulate within the cell. A suitable culture medium and growth condition is needed for the stimulation of PHA formation in the cells. Generally, the PHA granules can be detected under electron microscopy and fluorescence microscopy (Berlanga et al., 2006; Jendrossek et al., 2007). Additionally, the structure of PHAs can be analyzed by GC–MS/MS and NMR (Bonartsev et al., 2013; Elhottova et al., 2000). An indirect technique for screening of PHA-producing halophilic archaea is PCR. The PCR targeted the *phaE* gene of the PHA-

Table 4
Microorganisms for the phylogenetic analysis and the primers designed.

No.	Accession no.	Organism	Habitat	Length (bp)	Reference
1	CP001896	<i>Allochromatium vinosum</i>	Brackish pond	1074	Imhoff et al. (1998)
2	NZ_AOLW01000006	<i>Haloarcula amylyolytica</i>	Salt lake, China	507	Yang et al. (2007)
3	CP002921	<i>Haloarcula hispanica</i>	Solar saltern, Spain	546	Juez et al. (1986)
4	NZ_AOLK01000001	<i>Haloferax elongans</i>	Hamelin Pool, Australia	549	Allen et al. (2008)
5	NZ_AOLI01000018	<i>Haloferax larsenii</i>	Solar saltern, China	549	Xu et al. (2007)
6	EU374220	<i>Haloferax mediterranei</i>	Salt ponds, Spain	549	Torreblanca et al. (1986)
7	NZ_AOLN01000012	<i>Haloferax mucosum</i>	Hamelin Pool, Australia	546	Allen et al. (2008)
8	AM180088	<i>Haloquadratum walsbyi</i>	Solar saltern, Australia	555	Burns et al. (2007)
9	AFNT02000005	<i>Halorhabdus tiamatea</i>	Brine-sediment, Red sea	549	Antunes et al. (2008)
10	HF571520	<i>Halorhabdus tiamatea</i>	Brine-sediment, Red sea	549	Antunes et al. (2008)
11	CP007055	<i>Halostagnicola larsenii</i>	Salt lake, China	555	Castillo et al. (2006)
12	NC_020388	<i>Natronomonas moolapensis</i>	Solar saltern, Australia	546	Burns et al. (2010)

Fig. 2. Multiple sequence alignment of eleven haloarchaeal *phaE* genes. Asterisks represent conserved positions.

1999). However, the Nile red staining has less specificity and sensitivity compared to BODIPY 493/503 dye because the emission spectrum of BODIPY 493/503 is at a lower wavelength than that of Nile red (Kacmar and Srienc, 2005). A fluorescent microscope is an expensive

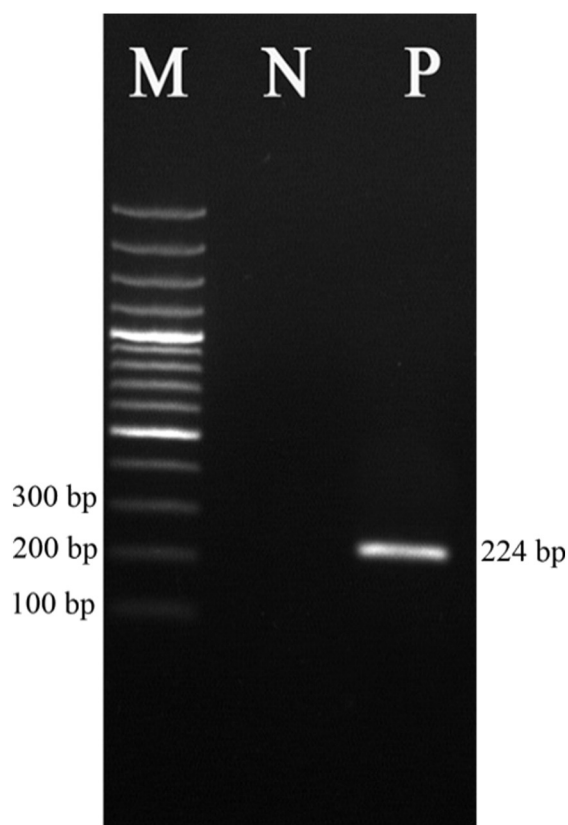


Fig. 3. Testing result of PCR using *phaE* primers with optimized PCR conditions. (M) DNA marker. (N) No template control and (P) The *phaE* amplicon of *H. walsbyi* DSM 16854.

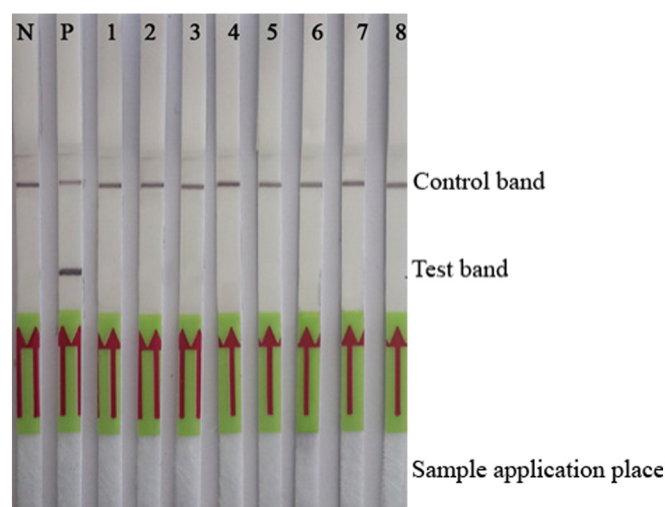


Fig. 5. Detection of the *phaE* amplicon that had been amplified from the specificity testing by DNA lateral flow biosensor. (N) No template control, (P) Positive control (*H. walsbyi* DSM 16854), (1) *E. coli* ATCC 25922, (2) *S. aureus* ATCC 13565, (3) *S. hygroscopicus* BTCC 7028, (4) *B. megaterium* DSM 319, (5) *B. subtilis* ATCC 6633, (6) *P. aeruginosa* ATCC 9027, (7) *P. fluorescens* ATCC 13525 and (8) *R. eutropha* DSM 428.

Solar salterns are widely spread in tropical and subtropical areas worldwide. The solar saltern contains a series of artificial ponds to confine and evaporate seawater. Evaporation of seawater increases the salinity of the ponds. Finally, brine and sodium chloride is precipitated in crystallizer ponds. Sodium chloride is crystallized when the salt concentration is higher than 300 g/L (Oren, 2003). Solar salterns and mangroves are sources of halophilic archaea (Oren, 2003). The PHA-producing haloarchaea, such as *Hyacinthoides hispanica* (Juez et al., 1986), *Haloferax larsenii* (Xu et al., 2007), *H. walsbyi* (Burns et al., 2007) and *Natronomonas moolapensis* (Burns et al., 2010), were isolated from solar salterns. In addition to solar salterns, salt lakes and salt ponds are also rich sources of halophilic archaea. *Haloarcula amylolytica* and *Hs. larsenii* were isolated from salt lakes in China (Yang et al., 2007; Castillo et al., 2006). *Hf. mediterranei* was isolated from salt ponds in Spain (Torreblanca et al., 1986) and is known as the best PHA producer of the family *Halobacteriaceae*. *Hf. mediterranei* can produce up to 65 wt.% PHA with respect to its cell dry mass (Lillo and Rodriguez-Valera, 1990). Halophiles, such as halophilic archaea, are an important source of PHAs. They have a high potential for

device that is necessary for determining the presence of PHA granules after staining with Nile red or BODIPY 493/503. The *phaE* biosensor is an inexpensive technique with a high sensitivity for screening of PHA-producing haloarchaea. Screening of PHA-producing haloarchaea based on the *phaE* biosensor takes less time and is less complex compare to other methods. The *phaE* DNA lateral flow biosensor required less than one minute to obtain the result, while agarose gel electrophoresis required at least thirty minutes. Based on the results of the sensitivity test of the *phaE* biosensor, the lowest amount of the gDNA of *H. walsbyi* DSM 16854 that the *phaE* biosensor can detect is approximately 250 fg.

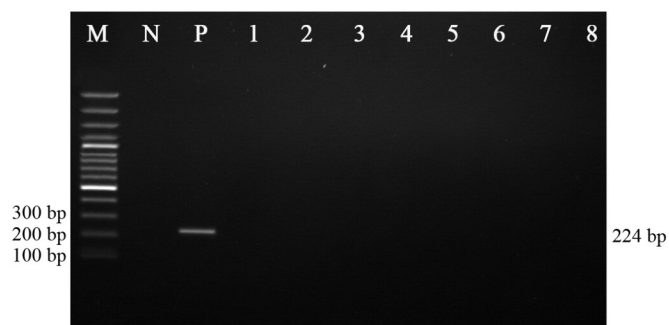


Fig. 4. Specificity testing of the *phaE* PCR. (M) DNA marker, (N) No template control, (P) Positive control (*H. walsbyi* DSM 16854), (1) *E. coli* ATCC 25922, (2) *S. aureus* ATCC 13565, (3) *S. hygroscopicus* BTCC 7028, (4) *B. megaterium* DSM 319, (5) *B. subtilis* ATCC 6633, (6) *P. aeruginosa* ATCC 9027, (7) *P. fluorescens* ATCC 13525 and (8) *R. eutropha* DSM 428.

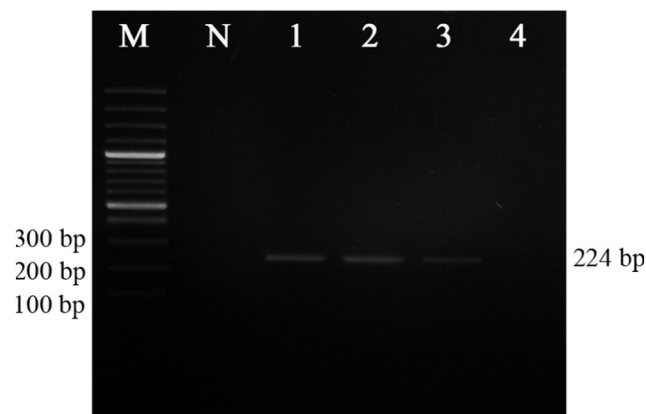


Fig. 6. Sensitivity testing of the *phaE* PCR. (M) DNA marker, (N) No template control, (1) 1000 fg, (2) 500 fg, (3) 250 fg and (4) 125 fg.

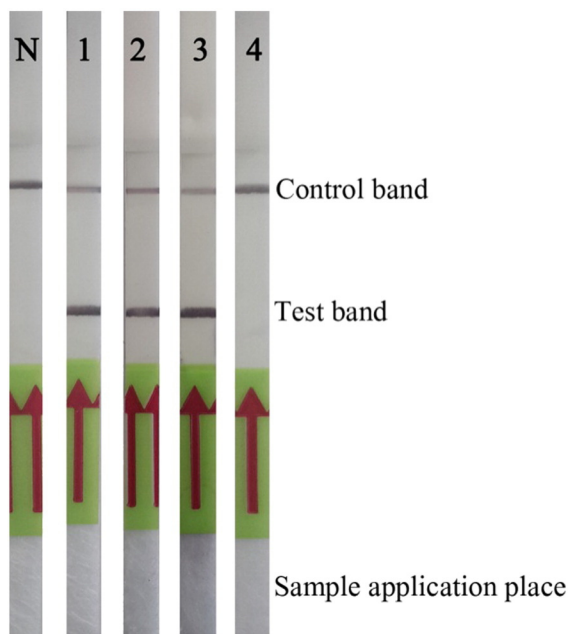


Fig. 7. Detection of the *phaE* amplicon that had been amplified from the sensitivity testing by DNA lateral flow biosensor. (N) No template control, (1) 1000 fg, (2) 500 fg, (3) 250 fg and (4) 125 fg.

supporting industrial scale biopolyester production. However, further studies of the synthesis and mechanisms of control of PHA production in haloarchaea are necessary to improve the rate and yield of PHA production (Quillaguaman et al., 2010). The *phaE* biosensor is a tool for screening PHA-producing haloarchaea. The biosensor can be used for both culturable (Fig. 4 and 5) and non-culturable samples (Fig. 8 and 9). Because of the high specificity and sensitivity of the *phaE* biosensor for PHA-producing haloarchaea, it may be used to accelerate the study of PHA production in halophilic archaea.

The *phaE* biosensor is a novel assay for screening PHA-producing haloarchaea. The *phaE* biosensor combines PCR and DNA lateral flow techniques. The PCR technique improves the sensitivity of the *phaE* biosensor. The *phaE* primers are highly specific to the *phaE* gene, which is necessary for the production of class III PHA synthase. The DNA lateral flow biosensor minimizes the devices needed to examine the PCR product. Detection of the *phaE* amplicon on the DNA lateral flow biosensor requires less time and fewer procedures than agarose gel electrophoresis. The *phaE* biosensor cannot be reused but it can be easily disposed after usage. The *phaE* biosensor can be applied for screening of culturable and non-culturable PHA-producing haloarchaea. The chance

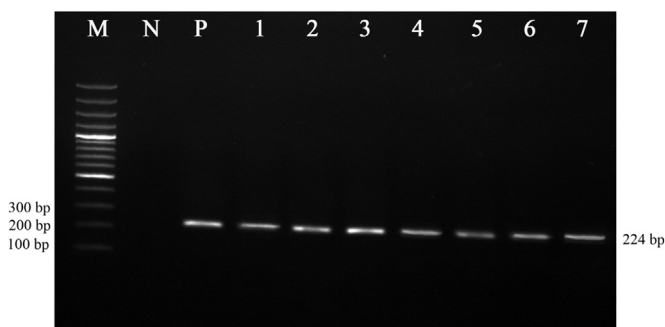


Fig. 8. PCR amplification of *phaE* gene from environmental DNA. (M) DNA marker, (N) No template control, (P) Positive control (*H. walsbyi* DSM 16854), (1) PK1, (2) PK2, (3) CP1, (4) CP2, (5) CB1, (6) CB2 and (7) CB3.

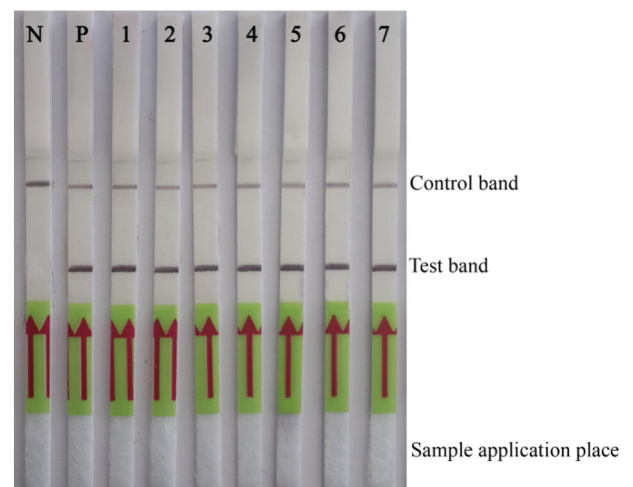


Fig. 9. Detection of the *phaE* amplicon that had been amplified from the environmental DNA by DNA lateral flow biosensor. (N) No template control, (P) Positive control (*H. walsbyi* DSM 16854), (1) PK1, (2) PK2, (3) CP1, (4) CP2, (5) CB1, (6) CB2 and (7) CB3.

of discovering a novel *phaE* gene or PHA-producing haloarchaea may be increased with the use of the *phaE* biosensor. Nevertheless, the *phaE* biosensor can certainly be used as a tool for screening of environmental samples that contain PHA-producing haloarchaea.

Acknowledgments

The authors would like to thank the National Research Council of Thailand (NRCT) for supporting our research work and Mullika Chomnawang for kindly providing the bacterial strains for validation of the *phaE* biosensor.

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