Connecting Three Detection Methods To Screen Polyhydroxyalkanoicates Producing Bacteria In General Nature Region

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Research Article

Keywords: polyhydroxyalkanoates, Nile red, phaC gene, GC Analysis

Posted Date: October 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-937320/v1

Abstract

This study combined the media containing the fluorescent dye Nile red identification method, the genetic detection method of colony polymerase chain reaction (PCR), and gas chromatography (GC) analysis techniques for screening polyhydroxyalkanoates (PHAs) producers isolated from the natural area in Taiwan. Using the three analysis methods can be schemed into different standard operating procedures to complement each other's limitations and shortcomings. We highlight the development of an economic and efficient screening tool that fits each cost process to different environment samples and intensifies the effective discovery of potent PHAs sources. When screening up to 10^5 samples to be analyzed, the scheme of connecting three detection methods was reduced to 97% of the cost than using the combination of two methods of Nile red + GC or PCR + GC methods. Besides, the unit of time consumed was only 6%. With the number of samples over 10^8, the researchers need to consider the overall cost, and only the combination of Nile red and PCR methods without GC will lower the cost to 88% and 57% of the time rather than using all three methods, with the true positive rate of 30.7%.

Introduction

Plastics are by design durable, inexpensive, lightweight, and resistant to degradation and have thus, over time, been found even in the deepest marine trenches with no human habitation. According to the UN Environment Programme report, about 8 million tons of plastic waste end up in the oceans each year and are damaging the ecosystem and wildlife. The intensity of environmental plastic pollution has generated much concern about the usage and promotion of polymers from renewable resources over recent years.

Polyhydroxyalkanoates (PHAs) are biologically produced macromolecules (polyesters with molecular weights from 5×10^4 to 2×10^6 Da) (Anderson and Dawes 1990) with a wide range of properties. PHAs are synthesized by many microbial strains under nutrient depletion or unbalanced growth conditions such as the presence of excess carbon source and limitation of at least one essential nutrient (Steinbüchel 1991). These polymers are stored in the cytoplasm in the form of granules as a carbon and energy reserve for the cell (Steinbüchel 1991; Madison and Huisman 1999; Park *et al.*2012). They are generally biodegradable and fairly biocompatible, especially in the ocean (Suzuki et al. 2021), making them attractive as biomaterials; besides, bacteria producing PHAs can be found in a variety of environments. Several research articles and reviews have been published on PHAs (Cuiet al. 2011; Chien et al. 2007; Ng and Sudesh 2016; Kolle 2017; Xu et al. 2014). Despite the continuous efforts, PHAs-based bioplastic remains commercially unattractive because of challenges such as a high production cost and difficult processing. Therefore, finding and improving efficient microbial PHAs producers is a continuous process.

The procedure for screening PHAs producers mainly involves detection of the vivo PHAs granule by microscope staining method in the past (Lee 1996). The detection of genes coding for the enzymes for PHAs synthesis (Sheu et al. 2000) and staining with lipophilic dyes are the most popular methods (Kitamura and Doi 1994; Spiekermann et al. 1999). Nevertheless, analysis of environmental samples is labour-intensive and time-consuming. Genetic screening involves the colony PCR based amplification of a fragment of the *phaC* gene using degenerate primers.

In this study, we attempted to link three detection methods to design a fast bio-prospection process. Bacteria can be co-culture with the fluorescent stain Nile Red on Petri dishes to identify PHAs positive colonies with UV light excitation and still be alive. The process does not need bacterial pad printing and a traditional straining process. Photograph of the stained colonies containing Nile Red–PHAs fluorescent complex is a simple screening target as it allows confirmation of the characteristic bright glow of the UV excitation, and is thus suitable as the first step of this process involving three detection methods. Degenerate primers for *phaC* gene based on multiple sequence alignment results and were designed for the second step of colony PCR to detect *phaC*, the gene for PHAs synthase. In the final step, gas chromatography (GC) Analysis can accurately confirm the types and yield of PHAs at the target bacterium. We collected samples from nine different natural regions in Taiwan. This is the first study to accurately analyze and verify the most accurate and economic standard operating procedure for the detection and evaluation of the potential of those microbes to produce PHAs. We hypothesize that natural habitats harbor a diverse cultivable bacterial community capable of producing PHAs that may be a new source of industrial PHA production.

Experimental

Sources of samples and microbial cultivation

A total of 35 samples, including nine freshwater and 27 compost solids with seawater samples were collected from various natural intertidal regions and spa habitats in Taiwan. These samples were subjected to primary screening for the isolation of PHA producing bacteria. Strain suspensions were diluted with sterile water and seawater from 101 to 104 final concertation and respectively plated on NA+4 % glucose, marine+3% glucose, and R2A agar+2% glucose and incubated at 26∘C for one week in the thermostatic chamber to form single colonies or in liquid cultures. Before culturing, glucose solution and medium were sterilized separately and then combined. The positive control PHAs producing strains used were *Bacillus megaterium* BCRC 10608, *Cupriavidus necator* BCRC 13036, 148738, 17389 and *Haloferax mediterranei*. The negative control PHAs product strains were *Saccharomyces cerevisiae* BCRC 20270, *Escherichia coli* DH5α, and *Pseudomonas putida. E. coli* DH5α be purchased from Protech Technology Enterprise Co., Ltd., Taiwan (Ma et al. 2012). *P. putida, G. phthalatica and H. mediterranei* were isolated from the sludge of sewage treatment plant, paper mill plant and Cigu Salt Fields for previous studies in Taiwan. And the rest of the reference strains purchased from BCRC. All these microbes information on their culture condition were obtained from BCRC.

Co-culturing on media containing fluorescent lipophilic dye and direct identification

Stock sterilized solutions of Nile Red dyes were prepared in DMSO and added to the autoclaved agar medium to give a final concentration of 0.5 mg/mL dye. Strains samples (50 µL) were mixed by spread-plate method onto the plates. After one week of incubation at 26∘C, the plates were exposed under UV light and the fluorescent colonies were appraised as positive for PHA presence (Spiekermann et al. 1999).

Genotypic detection by colony PCR

Synthesis of oligonucleotide primers.

Three degenerate primers were designed and synthesized according to the multiple sequence alignment results. The sequences were 5'- atcaacaagggt ttctacaatccct ccgacct -3' (designated phaCF1), 5'- gtcgttcaat ccggtgaggc tggcgcaaccc -3' (designated as phaCF2,) and 5'- aggtagttgt ttgaccccaa aaaagggtag ttttcca -3' (designated as phaCR4).

The sample colony were scraped by pipette tips from the Petri dishes and then used as a DNA template for PCR. Optimized colony PCR reaction mixture contained 1X PCR SuperMix (Bio-Helix, MB200-P100), 10 µM of each primer, 1.2 U DNA polymerase in a 25 µL PCR reaction mixture. Colonies approximately 1 mm in diameter were picked up with a sterilized toothpick and directly transferred to the PCR tube as DNA templates. The thermal cycle program, run on a gradient PCR system (TakaRa PCR thermal cycler) involved 1 cycle at 95°C for 10 min and 30 cycles at 95°C for 30 s, 50°C for 20 min, 72°C for 2 min, and then incubation at 72°C for 5 min, and a final incubation at 4°C. Detection of PCR-amplified DNA fragments was observed by agarose gel (1%; Merck) electrophoresis. Ten microliters of each amplification mixture and the molecular mass marker (GenKB LC DNA Ladder 0.2-14.0 kb, GeneMark) were resolved by agarose gel electrophoresis, stained using a safe view dye, and finally visualized by UV illumination (QUANTUM-ST4-1100, Biotech).

PCR Amplification of the 16s/23s Spacer Sequences for the identification of bacterial species

PCR amplification materials were the same as mentioned in the above section. Considering that the diversity of bacterial species makes PCR difficult to amplify, 3 sets of Primers (27F/1525R: Lane et al 1991, 8F2/806R: Relman et al 1992, D1modF/16S1RR-B: Weisburg et al 1991 and Anneke et al 1995) are used for 16S rDNA PCR amplification. Cycling conditions were denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The purified PCR products that are successfully amplified and longer in length were modified for automated sequencing.

Analysis of PHAs Production by Gas Chromatography and NMR Spectroscopy

Two milliliters of cell culture were centrifuged at 13,000 rpm for 10 min, the pellet was resuspended with distilled water, transferred to a pressure tube, and dried by lyophilization to a constant weight. Samples were then treated with 2 mL of acidified methanol (containing 15% concentrated H_2SO_4) and 2 mL of chloroform at 100°C for 140 min. The samples were kept at room temperature, then 1 mL of distilled water was added and vortexed for 1 min (Guell and Winterburn 2019). After separation, the bottom chloroform

layer was injected into GC-FID equipped with a DB-WAX column (30 m, 0.25 mm, 0.25 µm, Agilent). Samples were analyzed under a temperature regime starting at room temperature, increasing to 80°C at hold time of 2 min and run time of 2 min, to reach a final temperature of 240°C at a rate of 10°C min^{-1} for hold time of 1 min and run time of 19 min. Standard (2.5-50 mg/mL) used were of commercial 3HB and 2HB grade (Sigma-Aldrich, Germany) to construct a standard curve.

Liquid NMR experiments on extracted PHAs samples were performed on an Oxford 300 MHz NMR Mercury Plus spectrometer (Varian, USA) with a 5-mm probe. Deuterated chloroform (99.8%, Sigma Aldrich) was used as the solvent. 1H NMR spectra were recorded using recycle delay of 4 s and 32 scans (Colombo et al. 2017).

Results And Discussion

Nile Red and colony PCR screen PHAs producers

According to the report of Spiekermann et al.(1999), the lipophilic dye of Nile red can be dissolved in DMSO and added to the culture medium for live bacterial staining of PHAs. If neither dye nor DMSO affects the growth rate or cell density, then this method can successfully discriminate between PHAs negative and PHAs positive strains in live cells. Furthermore, the method can directly provide results after the colonies are formed and no additional staining process is needed; thus the next stage of identification process can be performed, making the method is simple and efficient. Compared with the traditional microscopy for observing single bacteria, this method can deal with a large number of bacteria simultaneously, and it does not require colony transfer in advance because of the death due to the dyeing process. We tested the production of PHAs by adding 2%-4% glucose to three different media, the NA, R2A, and Marine. The spectrogram was taken under UV light at 312 nm, and colonies with strong fluorescence were identified as positive.

The experiment results are shown in Figure 1. Some of the colonies produced a compound that exhibited stronger fluorescence, and these corresponding results were observed in all three media. Subsequent GC analysis confirmed that some bacteria indeed produce PHAs. Table 1 shows that among 35 samples randomly collected from nine natural areas in Taiwan, were plated on a total of 386 Petri dishes with different dilution multiples, and 33 positive colonies were finally selected and further identified. After GC analysis, it was confirmed that Nile red could discriminate PHAs produced from emission fluorescence with an average accuracy rate of 39.4%. The combination of the three media can cover most of the bacteria that grow in a natural environment, such as freshwater, seawater, and soil, it shows that the co-culture dye staining method using Nile red can be applied to add the general media for screening of various strains.

Table 1
The Sensitivity of Screening Method Using Nile Red Dye Co-Culture Media

Culture Medium type	Nile Red positive fluorescence	GC recheck analysis	True positive rate
R2A	17	8	47.1
Marine	8	3	37.5
NA	8	2	25.0
Total	33	13	39.4

The biosynthesis pathway of PHAs requires a crucial enzyme PHA synthase that is encoded by the gene *pha*C for polymerization. Studies have reported (Sheu et al. 2000) the use of degenerate primers to amplify the gene fragment from PHA-positive bacterial strains such as *Ralstonia eutropha*. We used *pha*C gene sequences from known PHA-positive bacteria to align the degenerate primers and evaluate the unique sequence combinations and recognized the strains which have the ability to produce PHA. Designing a mixture of primers is a useful strategy for amplifying several possible bases of a gene sequence of a similar gene between related organisms. Two sets of new primers were used to PCR amplify the *pha*C gene and were confirmed by electrophoresis. Figure 2 shows the use of different primers to detect samples having *PhaC* sequence strains and the control group. The result shows that using of *pha*CF2 and *pha*CR4 primers could efficiently amplify *pha*C gene from positive control strains including *P. putida*, *C. necator*, and *H. mediterranei*. Thus, these sets of primers for PCR could confirm at the genetic level whether the strain had the ability to produce PHAs. After Nile Red screening, for the second step of identification, the colonies were picked out, and then using the PCR detection method, we could identify whether these environmental strains contain PHAs synthesis gene. The result in Figure 2 shows that some samples have the *phaC* gene sequence. Compared with the marker, the DNA size was about 200 bp and the same as the positive control, but

the bands were weak bright on the electrophoresis gel. Among the randomly selected 25 positive strains of the Nile red method, 10 strains were positive after PCR inspection, and finally, GC retest confirmed that four strains indeed produced PHAs, thus the true positive rate of PCR was 40%. The reason for a false positive result of the Nile red staining method (Spiekermann et al.1999) is that Nile red cannot distinguish between bacteria that accumulate PHA granules and those that accumulate lipid compounds, or some Gram-positive bacteria with thick lipid cell membranes. The PCR reaction also involves various factors such as amplification efficiency and annealing of primers. In addition, although PCR analysis confirms the presence of the *pha*C gene, it is possible that the gene is present but cannot be induced by glucose or due to other phenotype problems.

GC Analysis and NMR Identification of PHAs Types

Checking whether the bacteria produce PHAs is an important step to verify if the screened bacteria have PHAs production capacity. In recent years, various methods have been used for this purpose, but the problem of false positivity still exists. The production of PHAs by the bacteria is very important to subsequent fermentation research and for commercial applications, so their production must be confirmed. Common methods for accurately identifying PHAs produced by bacteria include GC, NMR, and FTIR (Dai et al.2008; Alsafadi and Mashaqbeh 2017 Liu and Chen 2007). The object must be ground into powder when using FTIR equipment for analysis, however, PHA is a low melting point polymer, the heat energy of the grinding process may melt the compound makes the powdering steps more complex and difficult, thus often GC or NMR should be used. Choosing GC or NMR equipment for screening PHAs bacterial identification the cost must be considered. Since the cost of an NMR equipment is as high as 520,000 US dollars per unit, while that of a GC is 100,000 US dollars, for this experiment, we used GC to determine general PHAs for the primary bacterial analysis, and unknown PHAs compounds were rechecked by NMR. Fig. 3 shows the use of standard products to construct a GC quantitative curve and prove linear regression.

The products extracted from the single colony of wild bacteria were analyze using acidified methanol, and the concentration of PHAs produced under the medium added 2%-4% glucose was between 0.043 to 0.245 mg/mL (Fig. 4). When an unknown peak appeared in GC results (Fig. 4.b), we use NMR to identify the compound structure. The result is shown in Fig. 5. This corroborates with the report by Chang et al. (2018), which states that poly(3-hydroxybutyrate) (3HB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (3HB-co-3HV) widely exist in the natural environment in the PHAs-producing bacteria, and subsequent strains were identified by 16s RNA analyses.

Identification of wild PHAs-production species using 16S RNA sequence analysis

The comparison of 16S rRNA genomic sequence for species identification needs to consider five parameters. All E values of this experiment were 0, so, these were omitted in Table 2. The Table results shows that *Bacillus* is the most detected in the natural environment by screening process to establish and the percentage was followed 75% (No.1-6 and 8). 16S RNA gene sequence analysis reveals that the screened *B. cereus*, *B. proteotyticus*, and *B. thuringiensis* species have high genetic similarity, as well as both the concentration and the type of PHAs products by glucose induction. PHAs product concentration of *Vibrio* from marine samples was lower than that by *Bacillus*, further conforming to past researches (Mohapatra et al. 2017; Odeniyi and Adeola 2017). The PHAs yield induced by glucose is presented in Table 2; under no special fermentation control conditions, the types of PHAs produced include 3HB and 3HV. In addition, in the culture and domestication process, wild colonies are susceptible to death and can be used with difficultly for industrial applications. Thus, after a high concentration of carbon source-stimulation for the long-term growth, the bacteria move to the stationary phase, and may even enter the death phase. Therefore, it is important to restore the normal C/N ratio in the medium when the organism is transferred to the liquid culture process or after subculturing.

Table 2
The use of 16S rRNA to Identify the Species of Positive PHAs Producers

Screening medium	No.	Strain identification	Accession	Max score	Total score	Query Cover	Ident	PHAs conc. (mg/ml)	PHAs types
R2A	1	Bacillus cereus	NR115526.1	2675	2675	97	99.73	0.246	3HB-co-3HV
		Bacillus proteotyticus	NR157735.1	2675	2675	97	99.73		
	2	Bacillus proteotyticus	NR157728.1	2663	2663	97	99.52	0.187	3HB-co-3HV
		Bacillus thuringiensis	NR043403.1	2660	2660	97	99.66		
	3	Bacillus cereus	NR115714.1	2686	2686	97	99.86	0.175	3HB-co-3HV
	4	Bacillus cereus	NR074540.1	2686	2686	97	99.80	0.138	3HB-co-3HV
		Bacillus proteotyticus	NR157735.1	2680	2680	97	99.73		
	5	Bacillus thuringiensis	NR114581.1	1382	1382	97	99.60	0.076	3HB
		Bacillus toyonensis	NR121761.1	1382	1382	97	99.60		
	6	Bacillus albus	NR157729.1	1389	1389	97	99.74	0.044	3HB
		Bacillus proteotyticus	NR157734.1	1389	1389	97	99.74		
		Bacillus cereus	NR074540.1	1389	1389	97	99.74		
	7	Enterobacter tabaci	NR146667.2	1341	1341	98	98.66	0.043	3HB
NA	8	Bacillus cereus	MG027673.1	2669	2669	100	100	0.105	3HB
Marine	9	Vibrio alginolyticus	NR121709.1	1349	1349	96	98.42	0.061	3HB
Max Score: The highest alignment score from that database sequence									
Total Score: The total alignment scores from all alignment segments									
Query Cover: The percent of query covered by alignment to the database sequence									
Ident: The highest percent identity (Max ident of all query-subject alignments)									

Economic Analysis of the Three Methods and Four Schemes

The Economic analysis of operation time and cost to three detection methods is shown in Table 3. Utilizing a solid plate for the separation of a single bacterial, a colony is a necessary step for any detection method, and capacity per batch can use up to 50 Petri plates. Because Nile red can be added into medium during the pre-step of making agar plates directly, the cultivation time only needs to be extended to dying PHAs-producing bacteria to accumulate products and involves an additional photography process. Therefore, Nile red identification method has the largest capacity per batch and the lowest cost of the equipment requirements and consumables. At the level of molecular biology, the second highest cost involves the development of the PCR method for the presence of *phaC* gene, and the per unit expense of GC analysis is the highest. Although the average analysis time of one sample in GC column is only 30 min, the overall analysis time still needs extra steps including subculturing the single colony into liquid media for expansion and freeze drying and the acidified methanol reaction. So not only is the Equipment cost the highest, but the analysis time is also the longest. However, it can analyze accurately the PHAs products types or production of bacteria, therefore, it is necessary to use this step to

confirm the final product. Thus, the GC detection method is the final identification standard under the minimum detection limit for building the standard curve. The sensitivity of the analysis result was 100%, and the accuracy of Nile red and PCR methods was 39.4% and 40.0%. Thus, the accuracy of the experimental results of the two methods is similar.

Table 3
Economic Analysis of Operation Three Detection Method process

Step	Operation item	Spending time (hours)	Major Equipment	Equipment price (USD)	Equipment depreciation (USD /hr)	Material cost (USD /sample)	capacity per batch	true positive rate %
Culture Analytes	Collected sample pretreatment spread plate method growth bacterial single colonies	72	Laminar airflow bench Autoclave and Incubator	10,500	0.1199	0.997	50 plates per batch; 300 colonies per plate	-
Nile red	Prepare chemical Extend colonies growth time UV illumination visualizing imagine	25	UV light Gel Image System	10,000	0.1631	0.387	300 colonies per plate;1 plate per picture	39.4
PCR	PCR UV illumination visualizing imagine	6	PCR machine, Gel Image System and Horizontal Electrophoresis System	20,000	0.3262	1.912	96 samples per PCR;16 samples per Electrophoresis gel	40.0
GC	liquid Culture bacteria amplification Lyophilisation Methylation GC analysis	76	Refrigerated centrifuge and GC machine	128,000	2.9224	2.734	10 samples per pretreatment;1 sample per GC test	100

There were variations in the strain pattern and quality of bacteria collected from the natural environment. The number of bacteria analyzed in 1 g of samples fluctuated between 10^1 and 10^8. Only using one detection method of the three methods cannot appropriately screen the targets. We could build an economical model considering the requirements and sensitivity, and the above three inspection methods can be organized into four schemes of detection permutation about A, B, C, and D, as shown in Table 4. We ignored special situations in different regions and presumed that the collected environmental samples contain 1% PHAs producing bacteria. Under the small size of sample screening, per unit amount bacteria were 10^3; linking three methods of the scheme D could get an accurate result and the time and expense average cost of only 62.5% while 34.5% when the two methods were connected, as shown in schemes B and C. When the colonies up to 10^5 bacteria per unit, scheme D can reduce time and expense costs down to 10.7% and 3%, respectively, than when using the average of schemes B and C. Fig. 6 shows an exponential trend in the increase in the overall cost as the cost of analysis increases after the bacterial count increases more than 10^3. Moreover, as the number of colonies under analysis exceeds 10^5 bacteria, the time consumed will decrease to 0.014 as per scheme D, while that in schemes B and C was 0.2 and 0.26, respectively. The scheme D only spent 6% rather than the average of schemes B and C. For detecting a large number of bacteria to build a big analysis database over 10^8, the researchers need to consider the overall cost. Executing the low-cost of scheme

A is economically viable. It is only 12% of the total cost and 57% of time cost in contrast with schemes A and D at 10^8 of the sample size. The screen result can eliminate a large number of negative PHAs strains, however, the PHAs producers in all positive identified presumed strains gave only 30.7% true positivity rate, lower than by using Nile red or PCR method individually. This is caused by the superimposition of the two error rates of both Nile red and PCR methods.

Table 4
The Organized of Four Detection Permutation Schemes

scheme	Operating count				Time consuming (hr)	Equipment cost (USD)	Consumables cost (USD)	Total costs (USD)	sensitivity
	Culture Analytes	Nile red	PCR	GC					
10^2 colonies									
A	1	1	1	-	103	14.7	3.3	18.0	30.7
B	1	1	-	1	173	223.1	4.1	227.2	100
C	1	-	2	1	160	223.0	5.6	230.5	
D	1	1	1	1	179	225.1	6.0	231.1	
10^3 colonies									
A	1	1	1	-	103	14.7	3.3	18.0	30.7
B	1	1	-	3	325	643.9	9.6	653.5	100
C	1	-	11	3	366	661.4	30.2	691.6	
D	1	1	1	2	179	225.1	6.0	231.1	
10^4 colonies									
A	1	1	3	-	115	18.6	7.1	25.7	30.7
B	1	1	-	26	2073	5483.4	72.5	5555.9	100
C	1	-	105	25	2602	5474.5	270.1	5744.6	
D	1	1	3	1	191	229.0	9.9	238.8	
10^5 colonies									
A	7	7	27	-	841	141.8	61.3	203.1	30.7
B	7	7	-	254	19983	53533.8	704.1	54237.9	100
C	7	-	1042	250	25756	54703.0	2682.8	57385.8	
D	7	7	27	7	1373	1614.7	80.5	1695.2	

Scheme A = Nile red + PCR;Scheme B = Nile red (NR) + GC□Scheme C = PCR + GC□Scheme D = NR + PCR + GC. Total costs do not include salary and wages, indirect costs, and other non-operating expenses. The calculation of equipment cost is based on the project, using the equipment at the time of depreciation.

Conclusions

This study analyzed the true positive rate of the Nile Red method to identify PHAs producers to be 39.4%. This method can handle a large scale of bacteria samples and has acceptable sensitivity, and lower equipment requirements and consumables when screening samples from natural regions. The true positive rate of The PCR detection method was 40%. The third step of GC Analysis could accurately confirm the production of PHAs types and the yield. The development of three different detections was systematized an experimental SOP for screening bacteria was prepared in accordance with economy and accuracy. When screening a natural area, the researcher can evaluate which scheme to use according to the requirements for equipment and consumables to meet the assessed economy and accuracy. This study cost model shows that when screening samples are up to 10^5, the three detection methods

combined accrue 3% of the total cost and 10.7% of the time than by combining two methods; besides the unit of time consumed is only 6%. While screening over 10^8 microbes might pose a challenge to the implementation of three detection methods in a small research project, the scheme that combines two detection methods without GC is accrued only 12% of the total cost and 57% of time cost in contrast with using all the three methods. This process also exhibited the elasticity of amplification and adjustment. Nile Red staining method was used to start with, and we can used more different media to fit PHAs bacteria in their original growth environment. In addition to analyzing the *phaC* gene, PCR detection can also analyze other related biosynthesis or metabolic genes simultaneously. The systematic SOP of the original screening medium and culture conditions in this screening method can supply basic reference data for subsequent testing of fermentation products. Those strong points will be beneficial to continue to search for better PHA-producing strains in the follow-up, which will accelerate the development of optimal conditions for the PHAs industrial production.

References

1. Alsafadi D, Mashaqbeh OA (2017) A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) from olive mill wastewater by Haloferax mediterranei. N Biotechnol 34:47–53. https://doi.org/10.1016/j.nbt.2016.05.003
2. Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:50–72
3. Bergmans MCA, Groothedde JW, Schellekens FPJ,et al (1995) Etiology of Cat Scratch Disease: Comparison of Polymerase Chain Reaction Detection of Bartonella (Formerly Rochalimaea) and Afipia felis DNA with Serology and Skin Tests. J Infect Dis 171:916–923. https://doi.org/10.1093/infdis/171.4.916
4. Chang CK, Wang HMD, Lan JCW (2018) Investigation and Characterization of Plasma-Treated Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrateco-3-hydroxyvalerate) Biopolymers for an In Vitro Cellular Study of Mouse Adipose-Derived Stem Cells. Polymers 10:355–368. https://doi.org/10.3390/polym10040355
5. Colombo B, Favini F, Scaglia B et al (2017) Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. Biotechnol Biofuels 10:201–216. https://doi.org/10.1186/s13068-017-0888-8
6. Cui HL, Yang X, Gao X, Xu XW (2011) Halogranum gelatinilyticum sp. nov. and Halogranum amylolyticum sp. nov., isolated from a marine solar saltern, and emended description of the genus Halogranum. Int J Syst Evol Microbiol 61:911–915. https://doi.org/10.1099/ijs.0.024976-0
7. Chien CC, Chen CC, Choi MH et al (2007) Production of poly-beta-hydroxybutyrate (PHB) by Vibrio spp. isolated from marine environment. J Biotechnol 132:259–263. https://doi.org/10.1016/j.jbiotec.2007.03.002
8. Dai Y, Lambert L, Yuan Z et al (2008) Characterisation of polyhydroxyalkanoate copolymers with controllable four-monomer composition. J Biotechnol 134:137–145. https://doi.org/10.1016/j.jbiotec.2008.01.013
9. Guell AF, Winterburn J (2019) Increased production of polyhydroxyalkanoates with controllable composition and consistent material properties by fed-batch fermentation. Biochem Eng J 141:35–42. https://doi.org/10.1016/j.bej.2018.10.004
10. Lane DJ, Stackebrandt E, Goodfellow M et al (1991) 16S/23S rRNA Sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic Acid Techniques in Bacterial Systematic. John Wiley and Sons, New York, pp 115–175
11. Lee, SY (1996) Review bacterial polyhydroxyalkanoates. Biotechnol Bioeng 49:1–14. https://doi.org/10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P
12. Liu WK, Chen GQ (2007) Production and characterization of medium-chain-length polyhydroxyalkanoate with high 3-hydroxytetradecanoate monomer content by fadB and fadA knockout mutant of Pseudomonas putida KT2442. Appl Microbiol Biotechnol 76:1153–1159. https://doi.org/10.1007/s00253-007-1092-8
13. Kitamura S, Doi Y (1994) Staining method of poly (3-hydroxyalkanoic acids) producing bacteria by Nile blue. Biotechnol Tech 8:345–350
14. Koller M (2017) Production of polyhydroxyalkanoate (PHA) biopolyesters by extremophiles. MOJ Polym Sci 1:1–19. https://doi.org/10.3390/bioengineering4040088
15. Madison LL, Huisman GW (1999) Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

16. Ma TY, Lin TH, Hsu TC et al (2012) An improved method of xylose utilization by recombinant Saccharomyces cerevisiae. J Ind Microbiol Biotechnol 39:1477–1486. https://DOI 10.1007/s10295-012-1153-6
17. Mohapatra S, Maity S, Dash HR et al (2017) Bacillus and biopolymer: Prospects and challenges. Biochem Biophys Rep 12:206–213. https://doi.org/10.1016/j.bbrep.2017.10.001
18. Nikodinovic-Runic JU, Guzik M (2013) Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoate) production using bacteria. Adv Appl Microbiol 84:139–200. https://doi.org/10.1016/B978-0-12-407673-0.00004-7
19. Ng LM, Sudesh K (2016) Identification of a new polyhydroxyalkanoate (PHA) producer Aquitalea sp. USM4 (JCM 19919) and characterization of its PHA synthase. J Biosci Bioeng 122:550–557. https://doi.org/10.1016/j.jbiosc.2016.03.024
20. Odeniyi OA, Adeola OJ (2017) Production and characterization of polyhydroxyalkanoic acid from Bacillus thuringiensis using different carbon substrates. Int J Biol Macromol 104:407–413. https://doi.org/10.1016/j.ijbiomac.2017.06.041
21. Park SJ, Kim TW, Kim MK et al (2012) Advanced bacterial polyhydroxyalkanoates: towards a versatile and sustainable platform for unnatural tailor-made polyesters. Biotechnol Adv 30:1196–1206. https://doi.org/10.1016/j.biotechadv.2011.11.007
22. Relman DA, Schmidt TM, MacDermott RP et al (1992) Identification of the Uncultured Bacillus of Whipple's Disease. N Engl J Med 327:293–301. https://doi.org/10.1056/NEJM199207303270501
23. Sheu DS, Wang YT, Lee CY (2000) Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microbiology 146:2019-2025. https://doi.org/10.1099/00221287-146-8-2019
24. Spiekermann P, Rehm BH, Kalscheuer R et al (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 171:73–80. https://doi.org/10.1007/s002030050681
25. Suzuki M, Tachibana Y, Kasuya KI (2021) Biodegradability of poly(3-hydroxyalkanoate) and poly(ε-caprolactone) via biological carbon cycles in marine environments. Polym J 53:47–66. https://doi.org/10.1038/s41428-020-00396-5
26. Weisburg WG, Barns SM, Pelletier DA et al (1991) 16S Ribosomal DNA Amplification for Phylogenetic Study. J Bacteriol 173:697–703. https://doi.org/10.1128/jb.173.2.697-703.1991
27. Xu F, Huang S, Liu Y et al (2014) Comparative study on the production of poly(3-hydroxybutyrate) by thermophilic Chelatococcus daeguensis TAD1: a good candidate for large-scale production. Appl Microbiol Biotechnol 98:3965–3974. https://doi.org/10.1007/s00253-014-5524-y

Figures

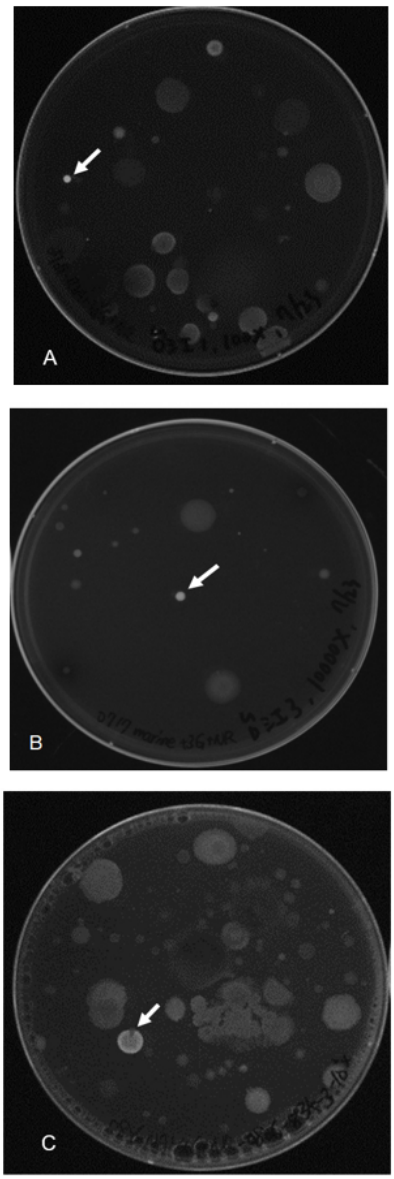

Figure 1

Fluorescent Nile red (0.5 μg Nile red/mL) staining bacterial colonies of wild samples grown on (A) R2A agar medium containing 2% glucose, (B) marine agar medium containing 3% glucose, (C) NA medium containing 4% glucose. PHA positive colonies exhibited a stronger fluorescence.

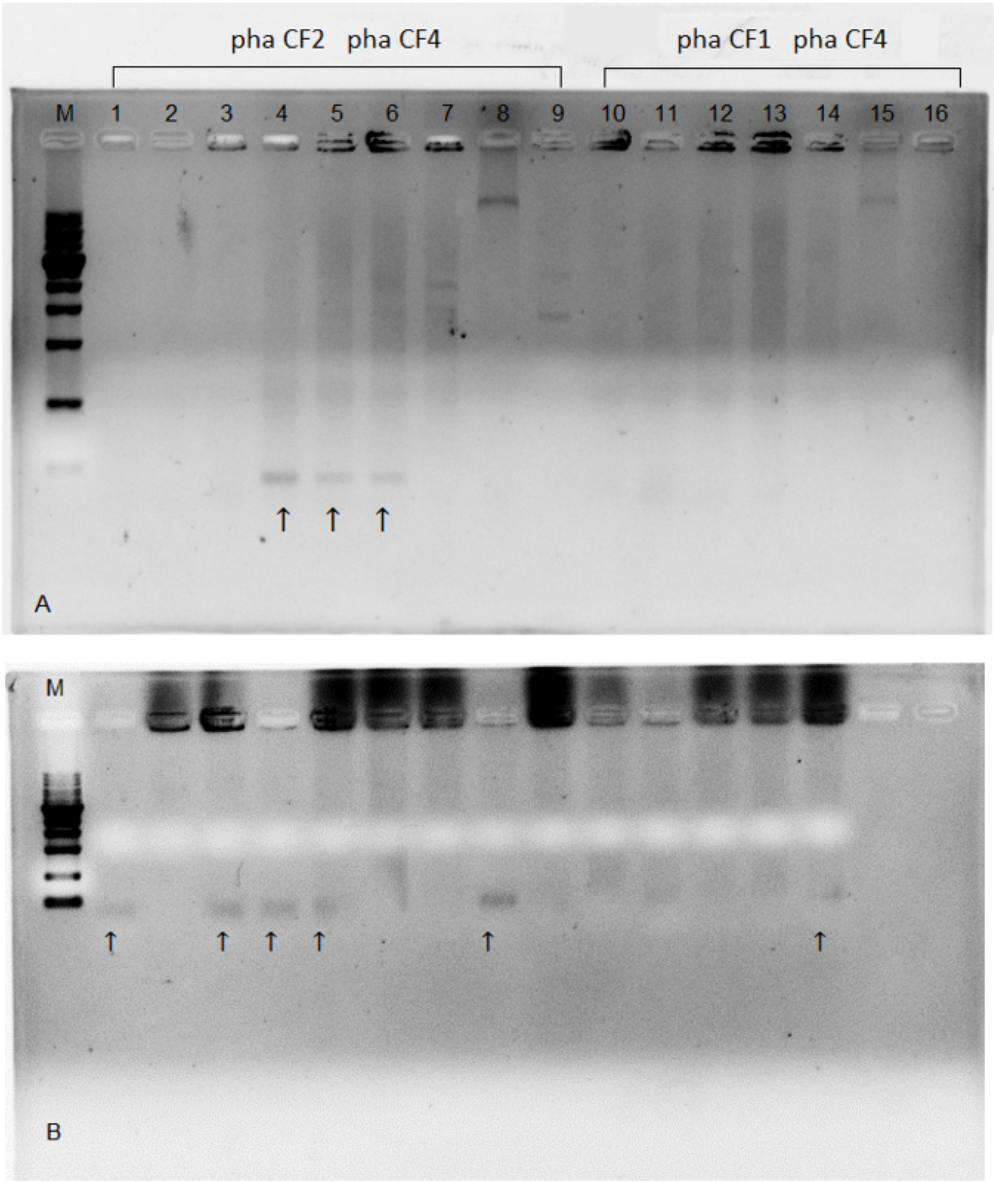

Figure 2

(A)The colony PCR result using different degenerate primers to detect PHAs positive and negative bacteria. M. Marker 0.2-14 kb 1. Saccharomyces cerevisiae, 2. Escherichia coli DH5α; 3. Pseudomonas putida□4. Bacillus megaterium 5. Cupriavidus necator BCRC 13036; 6. Cupriavidus necator BCRC17389; 7. Cupriavidus necator BCRC 148738. Haloferax mediterranei, 9. Gordonia phthalatica,10. Bacillus megaterium; 11. Pseudomonas putida;12. Cupriavidus necator BCRC 13036; 13. Cupriavidus necator BCRC17389; 14. Cupriavidus necator BCRC 14873;15. Haloferax mediterranei;16. Gordonia phthalatica. The phaCF2 and phaCR4 primers are1-9, and phaCF1 and phaCR4 primers are 10-16. (B) The colony PCR result of using phaCF2 and phaCR4 primers to detect wild samples.

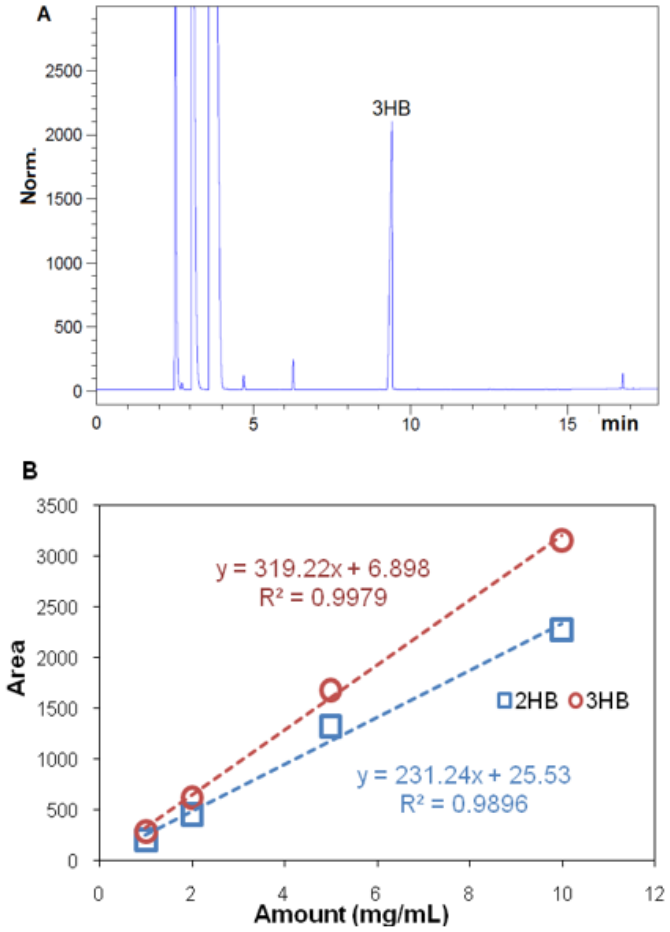

Figure 3

(A)The result of gas chromatography (GC) to detect the poly 3-hydroxybutyrate standard. (B) The standard curves of using GC to detect poly 3-hydroxybutyrate and poly 2-hydroxybutyrate standard.

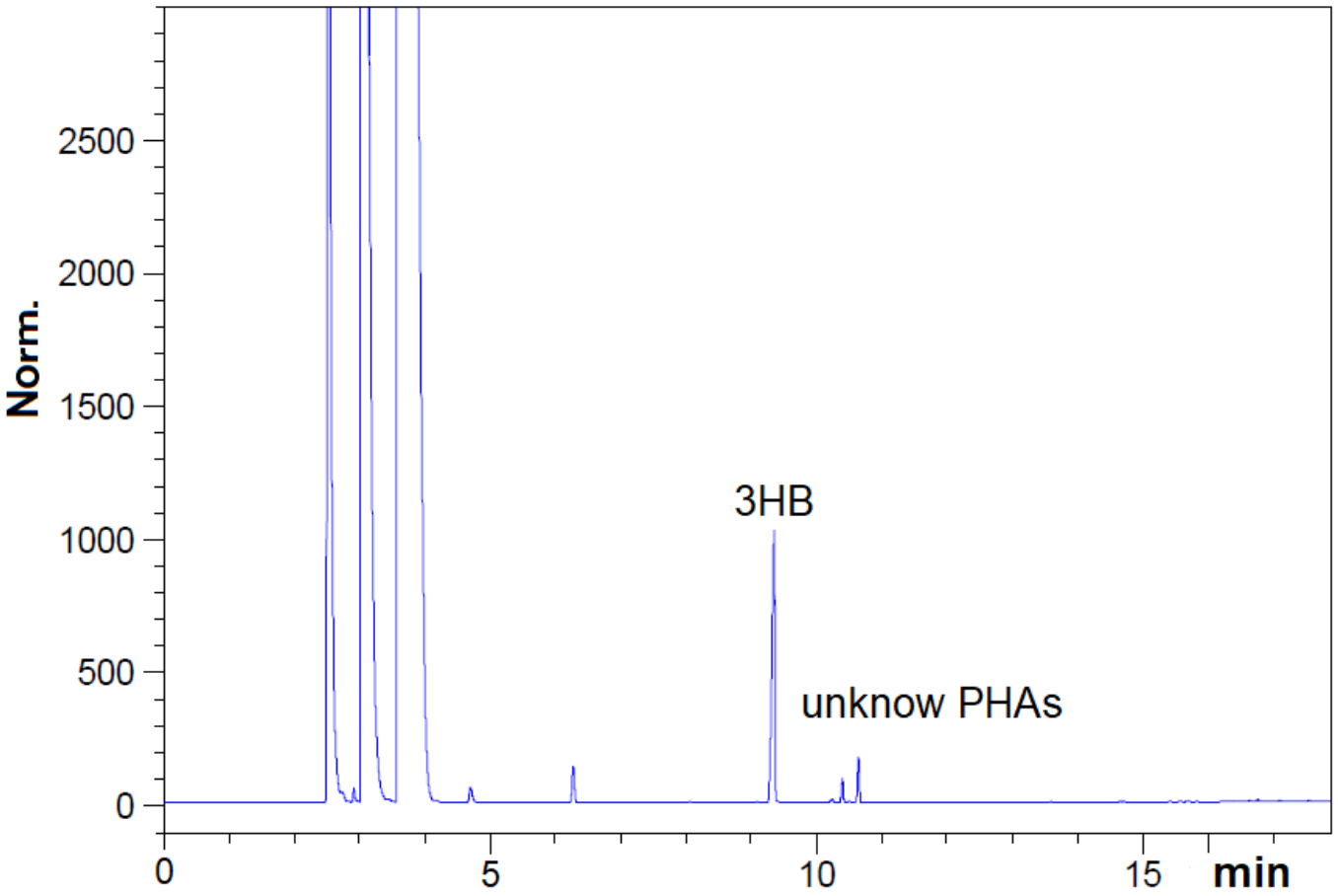

Figure 4

The gas chromatography result of to detect unknown wild samples after lyophilization and methylation.

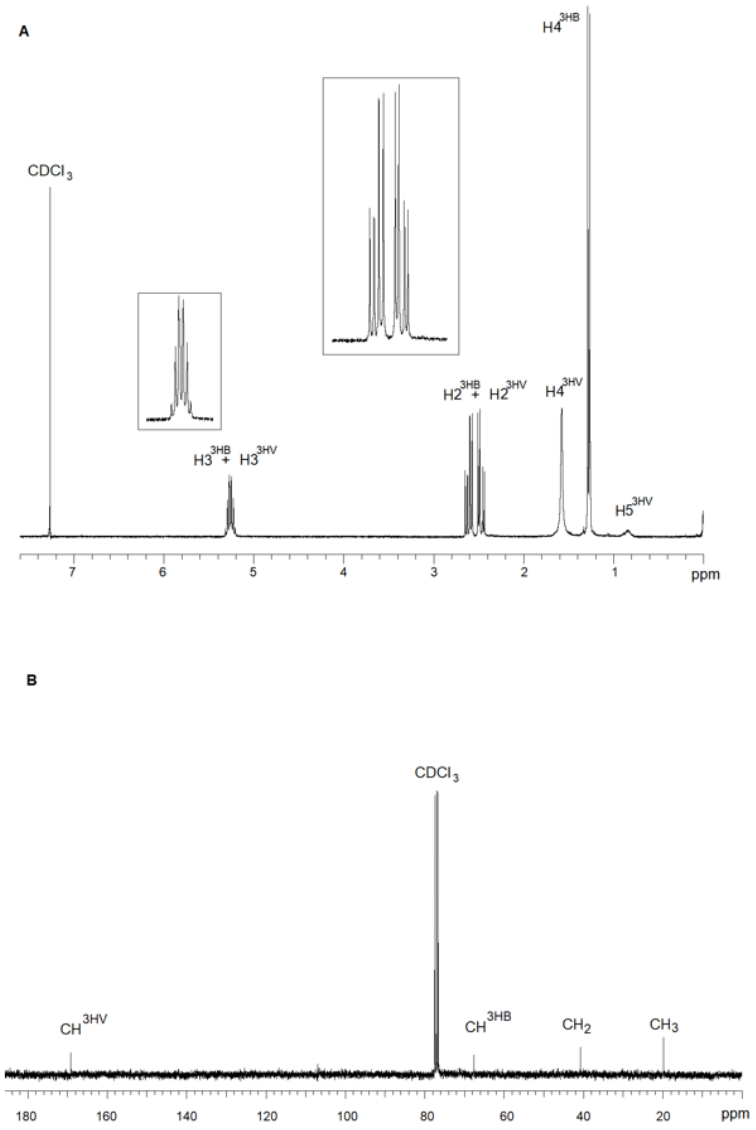

Figure 5

The (A) H1 and (B) C13 NMR spectra of the unknown PHAs polymer in CDCl3 solution from wild strain after gas chromatography analysis.

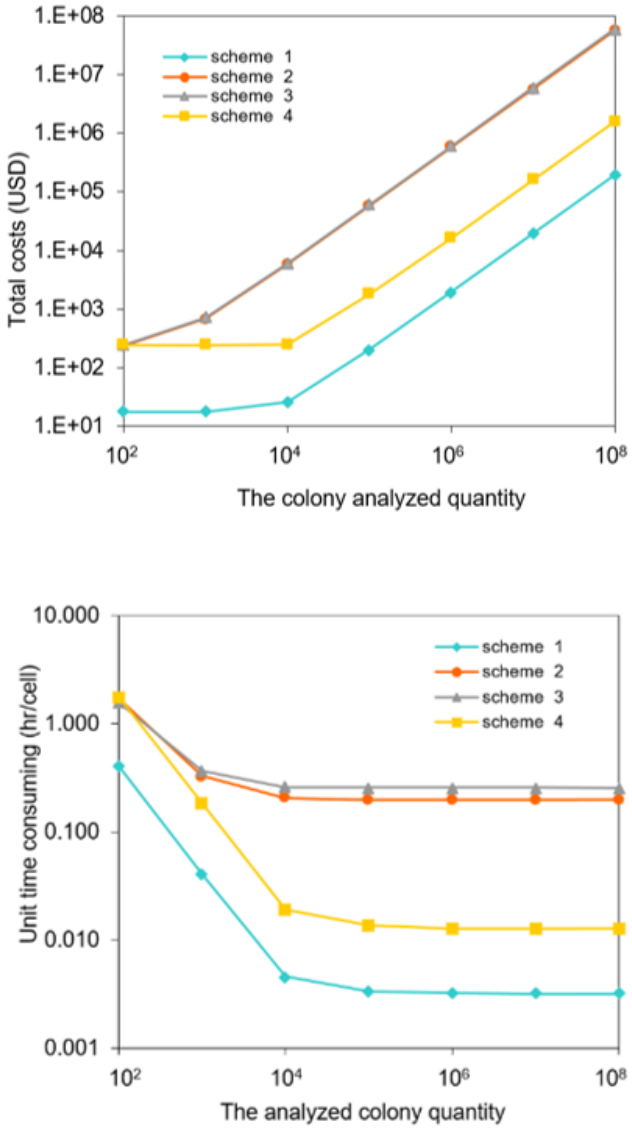

Figure 6

The total cost and unit of time consuming with the analyzed colony quantity of four detection permutation schemes.