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Development of a New *Pseudomonas* Agar Medium Containing Benzalkonium Chloride in Cetrimide Agar

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Abstract

Members of the *Pseudomonas* family are commonly found in nature, some species are pathogenic for humans, as well as being resistant to multiple disinfectants. Various studies have revealed that benzalkonium chloride (BKC) has an inhibitory effect on many bacteria but it has no significant effect on *Pseudomonas aeruginosa*. Cetrimide agar medium is recommended for the isolation and enumeration of *Ps. aeruginosa* in food and environmental samples. However, there are claims that for some food factories and in particular the bottled water industry, the selectivity of this medium is not sufficient. The aim of the current research is the creation of a more selective medium for *Ps. aeruginosa* with BKC. A total of 28 isolates were isolated with Cetrimide agar from raw water samples and identified using biochemical tests and commercial identification kits. All the bacteria were inoculated in Cetrimide agar plates containing 0 - 625 µg/mL BKC. The Petri dishes were incubated at 37°C and 42°C for 24 h. The results showed that 375 µg/mL BKC was sufficient to suppress *Burk. pseudomallei* at both incubation temperatures. *Ps. fluorescens*-35 could not grow at 42°C at any concentration, including the control, and was suppressed at 500 µg/mL BKC. All the *Ps. aeruginosa* isolates and control strain were grown at both incubation temperatures at 375 µg/mL BKC concentration. In conclusion, the analysis of *Ps. aeruginosa* showed that the growth of accompanying flora may be suppressed by adding 375-µg/mL BKC into Cetrimide agar and incubating at an elevated temperature of 42°C.

Keywords

Pseudomonas aeruginosa, Benzalkonium Chloride, Cetrimide Agar, Selective Medium, Membrane Filtration

1. Introduction

Pseudomonas is a genus within the family *Pseudomonadaceae*, and is classified

as Gram-negative aerobic psychrotrophic bacterial species [1] [2] [3]. Species within *Pseudomonas* have a great deal of metabolic diversity and are able to form colonies on a wide range of media. *Ps. aeruginosa* is an important opportunistic pathogen, which has been reported to be resistant to commonly used empirical antibiotics. Methods for the identification of *Ps. aeruginosa* are of great importance in the context of human health as the widespread occurrence of this pathogen in water [4] [5] [6].

The current identification of pathogens is routinely performed using conventional microbiological methods which require multiple cultivation steps and long periods of incubation. Research directed at increasing the selectivity of medium uses a conventional approach to improve the detection of *Ps. aeruginosa*. Although several selective media for the detection of *Pseudomonas* species have been commercially produced, a lack of specificity has been noted for these commercial products [7] [8]. Many selective agents, such as Cetrimide, cephaloridine, fucidin, nalidixic acid, phenanthroline, and irgasan, have been used to increase the selectivity for the isolation and identification of *Ps. aeruginosa*. Cetrimide agar as a newly developed alternative that is commonly used for the isolation and identification of *Ps. aeruginosa* from food and environmental samples; however, studies have indicated that certain strains of Gram-negative bacilli are capable of growing on the Cetrimide agar [9]. The absence of a medium with sufficient selectivity has compelled researchers to conduct long-term verification tests, which incur considerable expense for industrial applications, most importantly for the bottled water industry. Despite the ongoing research efforts to increase the selectivity, the current verification tests perpetuate the main disadvantage, which is the need for the extended duration of the cultivation of the samples. In the legislated European guidelines for drinking water, negative results are regarded as definitive whereas positive results are considered as presumptive and must be confirmed [10]. Therefore, without performing the verification tests using ultraviolet (UV) light it is not possible to distinguish fluorescent, non-pathogenic *Pseudomonas* spp., such as *Ps. fluorescens*, from the opportunistic pathogenic *Ps. aeruginosa* [11]. Hence, this situation brings about the unnecessary closure of water wells which have been drilled under difficult conditions and considerable expense.

The inhibitory effect of benzalkonium chloride (BKC; Zephiran) on various Gram-negative and Gram-positive bacteria has been reported; however, no suppressive effect on the growth of *Ps. aeruginosa* has been found in hospitals, even for a solution containing BKC [12]. It has been determined that *Ps. aeruginosa* is one of the most resistant microorganisms to BKC [13] [14]. Furthermore, antimicrobial products, such as BKC, that are frequently used as antibiotics can alter the environment and can trigger adaptive resistance mechanisms within pathogenic bacteria [15]. Studies have found that the mechanisms of action of BKC appear to be included in the class of cationic detergents that have a bactericidal effect against Gram-positive and Gram-negative bacteria. The bactericidal or bacteriostatic effect of BKC appears to depend on the treatment concentrations,

and the most BKC resistant Gram-negative bacteria occur within *Pseudomonas*.

Ps. aeruginosa is the most frequently isolated pathogenic bacteria species of the genus *Pseudomonas*. This species is resistant to adverse environmental conditions and disinfectants, and can even grow in iodized solutions. Due to the excess of *Pseudomonas* species, past classification had been conducted according to morphology, pigmentation structure, and metabolism [16]. *Ps. aeruginosa* has been shown to be: capable of growth in selective media containing Cetrimide, producing pyocyanin and fluorescence with exposure to a 360 ± 20 nm wavelength UV lamp, being oxidase positive, and producing ammonia from acetamide [10] [17]. *Ps. fluorescens* and *Ps. aeruginosa* are similar having many common features; however, unlike *Ps. fluorescens*, *Ps. aeruginosa* reduced methylene blue and prontosil. The temperature for the optimum growth of *Ps. aeruginosa* has been shown to be 30˚C - 37˚C, therefore, this aspect distinguishes *Ps. aeruginosa* from *Ps. fluorescens*, as the latter experiences optimum growth at 42˚C [1] [18]. Reproduction of *Burk. pseudomallei* occurs at 42˚C with no reproductive ability at 4˚C [1] [18]. It is resistant to low water activity but sensitive to heat and disinfectants. The differences of the pathogen *Ps. aeruginosa* from the accompanying flora were taken into account during the planning stages of the current study.

Considering all the factors given above, the design of a more selective medium to shorten the duration of the analysis of *Ps. aeruginosa* is essential. To our knowledge, only a limited number of studies have been conducted on *Pseudomonas* sp. grown on modified Cetrimide agar to investigate the suppressing effect of incubation at elevated temperatures. Thus, the current study aimed to determine whether BKC-Cetrimide agar coupled with elevated incubation at 42˚C could be used as an alternative analysis method to accurately distinguish between fluorescent and non-pathogenic species. In this context, different concentrations of BKC were added to Cetrimide agar, and 28 strains of bacteria were inoculated into modified Cetrimide agar and incubated at two different temperatures. Then the results were evaluated for the presence/absence of growth.

2. Material and Methods

2.1. Microorganisms and Culture Media

Ps. aeruginosa ATCC 27853 was used as a reference strain, which was procured from culture collection in the Department of Food Engineering of Ankara University. Twenty-eight isolates were obtained from raw water samples collected from several bottled water manufacturers in Turkey using the membrane filtration technique [10]. Cetrimide agar (Merck, Darmstadt, Germany) used for isolation was prepared from dehydrated powders according to the manufacturer's instructions. *Ps. aeruginosa*, *Burk. pseudomallei*, and *Ps. fluorescens*-35 were identified using the Microbact 24E Gram-negative Identification System (Oxoid, Cambridge, UK*). Ps. fluorescens*-25 was identified at the species level using 16S rRNA sequencing. The isolated and identified 28 bacterial strains were used in the experiments of the study. Stock cultures were maintained at −18˚C in Tryp-

tic Soy Broth containing 20% (v/v) glycerol (Merck, Darmstadt, Germany) for further use.

2.2. Isolation and Identification

For the isolation of *Pseudomonas* spp., nine water samples were used. Samples of 100 - 250 mL of water were transported to the laboratory at 4˚C and filtered through a sterile membrane filter (pore size, 0.45 μm; diameter, 47 mm; Sartorious, Germany) under vacuum and aseptic conditions. Using sterile forceps, the membrane filters were placed on the Cetrimide agar plates. The plates were incubated at 37˚C and 42˚C for 24 - 48 h. After incubation, the membranes were examined for colony development, the presence or absence of colonies with blue-green pigmentation (pyocyanin) and fluorescence.

2.3. Bacterial Growth in Cetrimide Agar Containing Different Concentrations of BKC

BKC was used as an inhibitory agent in this experiment. Cetrimide agar plates containing 0 μg/mL (control); 375 μg/mL; 437.5 μg/mL; 500 μg/mL; 562.5 μg/mL, and 625-μg/mL BKC were prepared based on a similar range of concentrations obtained from previous studies [19]. Point inoculation was performed on the Petri dishes. Following incubation at 37˚C and 42˚C for 24 - 48 h, the Cetrimide agar + BKC plates were evaluated for the presence/absence of bacterial growth. The concentrations of BKC in agar were carried out in triplicate.

2.4. Verification Tests

Ps. aeruginosa can grow in selective media containing Cetrimide, has been shown to produce pyocyanin as well as fluorescence under UV light of a wavelength of 360 ± 20 nm, and is oxidase positive. In addition, *Ps. aeruginosa* can produce ammonia from acetamide. A wide range of different methods have been reported to successfully distinguish between the strains of *Pseudomonas* species, such as oxidase test (Bactident Oxidase, Merck, Darmstadt, Germany), detection of growth at 42˚C, the methylene blue reduction test, and measuring the production of ammonia from acetamide. In the current study, for the verification of fluorescence, a portion of a typical colony was harvested and inoculated in general-purpose Tryptic soy broth at 42˚C for 24 - 48 h. The turbidity of the samples in the test tubes was used as an indication of the development of bacteria. In the methylene blue reduction test, typical colonies of *Pseudomonas* spp. were inoculated into 10% skim milk medium containing 50 ppm methylene blue. After incubation for 18 - 24 h, decolorization was considered a positive result for *Pseudomonas* spp.

3. Results

A total of 28 isolates (three *Burk. pseudomallei*, six *Ps. fluorescens*-35, eighteen *Ps. aeruginosa*, and one *Ps. fluorescens*-25) were obtained from Cetrimide agar and identified using the Microbact 24E kit. The tests performed on this kit pro-

vided adequate identification of all *Pseudomonas* spp. isolates, except for *Ps. fluorescens*-25. When the procedures described in TS EN ISO 16266 were performed, the *Ps. fluorescens*-25 strain gave a positive result for *Ps. aeruginosa* as shown in Table 1 and Table 2. Due to the inconsistency of these two different results, a 16S rRNA sequencing analysis was performed to confirm that this strain was *Ps. aeruginosa*. The 16S rRNA gene was amplified from the extracted DNA using the universal bacterial primers Bact-27F (5'-AGAGTTTGATCC TGGCTCAG-3') and Bact-1492R (5'-GGTTACCTTGTTACGACTT-3') [20].

Isolates of *Burk. pseudomallei* (three), *Ps. aeruginosa* (19, including one reference strain), *Ps. fluorescens*-35 (six), and *Ps. fluorescens*-25 (one) were identified by assays, including the TS EN ISO 16266; production of pyocyanin, pyoverdin, or pyorubin on Cetrimide agar; production of ammonium from acetamide; a positive oxidase reaction; and growth in Tryptic soy broth at 42 ˚C. The results are summarized in Table 2. All *Ps. aeruginosa* isolates produced ammonium from acetamide and reduced methylene blue. The methylene blue reduction test gave a result at 37˚C in 18 h. This test is a useful tool and provides results in a short time. In the current study, it was demonstrated that the methylene blue reduction test and acetamide test can be used interchangeably. Unlike *Ps. fluorescens*-35, *Ps. aeruginosa* developed at 42˚C and reduced methylene blue. In other words, *Ps. fluorescens*-35 displayed typical characteristics and was easily distinguished from *Ps. aeruginosa* by growth at 42˚C or the methylene blue reduction test.

Plates with different concentrations of BKC were evaluated for the presence/absence of bacterial growth after incubation as shown in Table 1. According to the results, the growth of all isolates occurred at 0-μg/mL BKC concentration at 37˚C, and 375 μg/mL BKC was sufficient for suppressing *Burk. pseudomallei* at 37˚C and 42˚C incubation temperatures. Based on this result, it can be concluded that *Burk. pseudomallei* is entirely susceptible to BKC. Most *Ps. aeruginosa* strains (except for three) were affected by the concentration of 437.5 μg/mL BKC but no difference was observed when they were compared in terms of incubation temperatures. As you could see on Table 1 the growth of *Ps. aeruginosa* occurred at all concentrations at 42˚C, whereas *Ps. fluorescens*-35 showed no growth at 42˚C for any concentration, even in the control strain, and was suppressed by 500 μg/mL BKC at 42˚C. Although some of the *Ps. fluorescens*-35 strains were more resistant than *Ps. aeruginosa* strains, either a high concentration of BKC (>625 μg/mL) or incubation conditions at 42˚C were required to suppress this strain. Since separation was observed at the concentration of 375 μg/mL at 42˚C, the results obtained from the concentrations of 562.5 μg/mL and 625 μg/mL are not included in Table 1. Unlike *Ps. aeruginosa*, the strains of *Burk. pseudomallei* were suppressed at 37˚C and 42˚C incubation temperatures at a concentration of 375 μg/mL. At the same concentration, *Ps. fluorescens*-35, which was incubated at 42˚C, was suppressed, and therefore, was distinguished from *Ps. aeruginosa*. In conclusion, the analysis of *Ps. aeruginosa* showed that the growth of accompanying flora may be suppressed by the addition of 375

µg/mL BKC into Cetrimide agar and incubation at an elevated temperature of 42˚C.

Table 1. Results from Cetrimide agar containing different concentrations of BKC for *Burkholderia pseudomallei* (3), *Pseudomonas fluorescens* (6) and *Pseudomonas aeruginosa* (20)*.

Code	Name of microorganism	0 µg/mL BKC - 42˚C	375 µg/mL BKC - 37˚C	375 µg/mL BKC - 42˚C	437.5 µg/mL BKC - 37˚C	437.5 µg/mL BKC - 42˚C	500 µg/mL BKC - 37˚C	500 µg/mL BKC - 42˚C
1	*Burk. pseudomallei*	+	−	−	−	−	−	−
2	*Burk. pseudomallei*	+	−	−	−	−	−	−
3	*Burk. pseudomallei*	+	−	−	−	−	−	−
ATCC 27853	*Ps. aeruginosa*	+	+	+	−	−	−	−
4	*Ps. aeruginosa*	+	+	+	−	−	−	−
5	*Ps. aeruginosa*	+	+	+	+	+	+	+
6	*Ps. aeruginosa*	+	+	+	−	−	−	−
7	*Ps. aeruginosa*	+	+	+	−	−	−	−
8	*Ps. aeruginosa*	+	+	+	−	−	−	−
9	*Ps. aeruginosa*	+	+	+	+	+	+	+
10	*Ps. aeruginosa*	+	+	+	−	−	−	−
11	*Ps. aeruginosa*	+	+	+	−	−	−	−
12	*Ps. aeruginosa*	+	+	+	+	+	+	+
13	*Ps. aeruginosa*	+	+	+	−	−	−	−
14	*Ps. aeruginosa*	+	+	+	−	−	−	−
15	*Ps. aeruginosa*	+	+	+	−	−	−	−
16	*Ps. aeruginosa*	+	+	+	−	−	−	−
17	*Ps. aeruginosa*	+	+	+	−	−	−	−
18	*Ps. aeruginosa*	+	+	+	−	−	−	−
19	*Ps. aeruginosa*	+	+	+	−	−	−	−
20	*Ps. aeruginosa*	+	+	+	−	−	−	−
21	*Ps. aeruginosa*	+	+	+	−	−	−	−
22	*Ps. fluorescens*-25[**]	+	+	+	−	−	−	−
23	*Ps. fluorescens*-35	−	+	−	+	−	+	−
24	*Ps. fluorescens*-35	−	−	−	−	−	−	−
25	*Ps. fluorescens*-35	−	−	−	−	−	−	−
26	*Ps. fluorescens*-35	−	+	−	+	−	−	−
27	*Ps. fluorescens*-35	−	+	−	−	−	−	−
28	*Ps. fluorescens*-35	−	+	−	−	−	−	−

*All the isolates grew at 0 µg/mL BKC - 37˚C; **This was identified as *Ps. aeruginosa* in the biochemical test conducted according to TS EN ISO 16266 and 16S rRNA sequencing analysis; +/− means presence/absence of bacterial growth.

Table 2. Summary of the test results of *Ps. aeruginosa*, *Burk. pseudomallei*, *Ps. fluorescens*-35 and *Ps. fluorescens*-25.

Name of Microorganism	Acetamide Broth**	Growth at 42°C	Methylene blue reduction***	Fluorescence
Burk. pseudomallei (3)	−	+	−	−
Ps. aeruginosa (19)	+	+	+	+
Ps. fluorescens-25 (1)*	+	+	+	+
Ps. fluorescens-35 (6)	−	−	−	+

*This was identified as *Ps. aeruginosa* in the biochemical test conducted according to TS EN ISO 16266 and 16S rRNA sequencing analysis; **A positive reaction is shown by a color change from orange-red to purple-red. ***Decolorization of the medium from blue to white is a positive result. A negative reaction is no color change for both medium.

4. Discussion

Cetrimide agar is used as a selective solid medium for the isolation and pre-identification of *Ps. aeruginosa* in standard microbiological in vitro analyses [21] [22]. *Ps. aeruginosa* is resistant to Cetrimide in a medium at a concentration of up to 0.3 g/L, and pigment formation is not inhibited within this range. In the current study, after incubation for 24 - 48 h, *Ps. aeruginosa* formed blue-green pigmented colonies and emitted fluorescence under UV light of an extended wavelength (360 ± 20 nm). Additional tests are recommended for definitive identification [10]. The analysis, which already has a long incubation period, becomes more difficult with the inclusion of the verification tests and their long durations. There is a need for experienced staff for the evaluation of the results. In the current study, these time and cost constraints were overcome by designing a more selective medium containing BKC and reducing the number of steps involved in the analysis. In addition, a standard analysis without a verification test may result in some microorganisms being counted as *Ps. aeruginosa* because despite being non-pathogenic, they also emit fluorescence (*Ps. aeruginosa*, *Ps. putida*, *Ps. fluorescens*, *Ps. chlororaphis*, *Ps. syringae*, *Ps. cichorii* and *Ps. flavescens*) [1]. As one of these microorganisms with many features similar to *Ps. aeruginosa*, *Ps. fluorescens* was also the most frequent accompanying flora in the current analysis (Figure 1). This microorganism creates pyoverdin, a yellowish green pigment, and cannot grow at 42°C. Another major companion of *Ps. aeruginosa* is *Burk. pseudomallei*, which is pigmented green−brown and can grow at 42°C; however, it can easily be distinguished since it is non-fluorescent. The current study is important in terms of showing that the correct identification of *Pseudomonas* sp. is possible without conducting a verification test. Furthermore, the use of specific tests chosen from Bergey's manual of systematic bacteriology minimized the number of required tests. This manual [18] specifies that the members of the oxidase-positive group of saprophytic or opportunistic fluorescent positive *Pseudomonas* spp. produce fluorescence under UV light and display growth at 42°C. However, these conclusions, mainly based on large studies, may overemphasize the role of fluorescent strains in identifying *Pseudomonas*
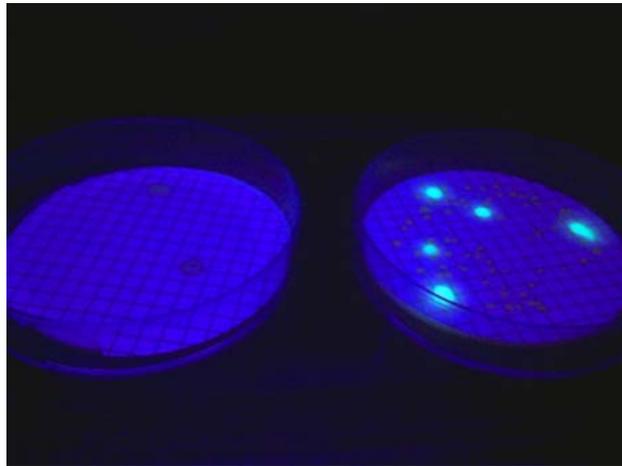
Ps. aeruginosa and *Ps. fluorescens* emit fluorescence.

Figure 1. Fluorescence under ultraviolet lamp (360 ± 20 nm wavelength).

spp. For a definitive identification, an incubation temperature of 42 ˚C and further tests are needed.

In the current study, the results of the *Ps. fluorescens*-25 were very similar to those obtained from *Ps. aeruginosa* in terms of development on Cetrimide agar + BKC, production of ammonium from acetamide, growth at 42˚C and the results of the methylene blue reduction test. Due to characteristics differing from those of other isolates of *Ps. fluorescens* and the *Ps. fluorescens*-25 strain, which was identified with the Oxoid Microbact 24E kit, the 16S rRNA sequencing analysis was performed and *Ps. aeruginosa* was confirmed. *Ps. fluorescens*-35 displays typical characteristics that can easily be distinguished from *Ps. aeruginosa* through the detection of growth at 42˚C and production of ammonium from acetamide. As stated in TS EN ISO 16266 and strongly recommended by Casanovas-Massana *et al.* (2010), unlike *Ps. fluorescens*, *Ps. aeruginosa* strains can grow at 42˚C. In other words, the growth of accompanying flora, in this case *Ps. fluorescens*, was suppressed at the elevated incubation temperature, and fluorescent strains were distinguished from each other. Many commercially available identification kits and automatic systems are based on probabilistic techniques, including the Oxi/Ferm system, the API and ATB system, the Minitek system, the Sceptor system, and Biologik-b. Such systems include codebooks or software that provide answers on the basis of the most probable taxa for the data obtained from the tests of the kits. Many of these commercial systems are aimed at shortening identification procedures in clinical laboratories and are, in fact, very useful for the identification of many pathogenic and opportunistic bacteria in biological specimens [23] [24]. Unfortunately, they are limited when used in food and environmental microbiology because of their inability to identify certain species and biovars, particularly those of environmental rather than clinical origin. In addition, many commercial identification systems have not kept pace with changes in bacterial taxonomy. For these reasons, computer-assisted techniques need to be developed as routine methods, which can identify taxa not in-

cluded in or well distinguished by commercial kits or, in some cases, a lower cost alternative. Since 89.83% of the strains obtained from food and environmental samples were successfully identified, the method employed in our study is considered satisfactory. Only *Ps. fluorescens*-25 was initially misidentified and required a further analysis.

BKC is a nitrogen-based quaternary ammonium compound (QAC) demonstrating broad-spectrum antimicrobial activity [25]. *Ps. aeruginosa* may adapt to high concentrations of QACs. The resistance mechanisms involved in the adaptation process are not as yet fully understood [26]. However, there are many studies showing that *Ps. aeruginosa* is resistant to BKC. Abraham *et al.* (2009) found that *Ps. aeruginosa* was resistant to certain antimicrobial agents. Bridier *et al.* (2001) used one standard test method (NF EN 1040) to evaluate the bactericidal activity of three different disinfectants commonly used in industrial or medical environments (peracetic acid, BKC, and ortho-phthalaldehyde) against 77 bacterial strains of different origins, and found that Gram-positive strains generally appeared to be more resistant than Gram-negative strains [27]. The results of the current study showed that *Ps. aeruginosa* is more resistant to BKC than *Ps. fluorescens* strains and *Burk. pseudomallei*.

Akoglu *et al.* (2012) investigated 55 raw milk isolates, and successfully suppressed the accompanying flora, in particular *Ps. fluorescens*, by modified Cetrimide agar containing 200 μg/mL BKC. The researchers determined minimum inhibitory concentrations (MICs) for BKC using the agar dilution method and tested concentrations in the range of 50 - 600 μg/ml. Similarly, in the current study, we assessed different concentrations within the same range. High BKC concentrations were required to suppress the accompanying flora since the bacteria in the current study were water-based and more resistant compared to those used by Akoglu *et al.*

In another study [28], a bacterium isolated as the contaminant of a batch of commercial BKC solution (10% (w/v)) stored in a loosely capped bottle was identified as *Ps. fluorescens*. The strain was highly resistant to BKC and the lowest concentration of BKC that inhibited visible growth of the strain measured on nutrient agar plates was ≥5000 μg/ml. The major contaminants were *Burk. cepacia*, *Ps. aeruginosa*, *Xanthomonas maltophilia* and *Ps. fluorescens* [13] [28]. The researchers' choice of a higher MIC can be attributed to the use of nutrient agar, which is a general non-selective medium and has a synergistic effect with BKC containing Cetrimide.

Selective media, including nutrient agar (supplemented with antibiotics), Cetrimide agar, *Pseudomonas* isolation agar and growth media (supplemented with C-390 or phenanthroline), have been used for the isolation of *Ps. aeruginosa*. However, none of these supplements proved to be a perfect selective agent. Some selective agents suppressed the growth of Gram-negative bacteria in addition to the growth of *Ps. aeruginosa* [9]. *Pseudomonas* spp. may adapt to survive against higher concentrations of QACs; however, these resistant strains can be eliminated with some disinfectants [14].

5. Conclusion

As a conclusion, using Cetrimide agar with BKC, *Ps. fluorescens*-35 and *Burk. pseudomallei* were both suppressed by incubation at 42˚C and 375 μg/mL concentration, *Ps. aeruginosa* continues growth at this concentration and temperature. Consequently, the most common accompanying flora would be suppressed when 375 μg/mL BKC + Cetrimide agar was applied at 42˚C for 24 h. The entire colony, which showed fluorescence under UV light, can be considered as verified *Ps. aeruginosa* for the current study. Since there is no need for an extra verification test, and resources in terms of time and money can be conserved. In the current study, an alternative *Ps. aeruginosa* analysis method for membrane filtration of water samples is proposed, which increases the selectivity of the medium and uses an elevated incubation temperature. In future studies, the proposed method can be tested against a wide range of concentrations and different companion flora.

Acknowledgements

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