Analytica Chimica Acta 1128 (2020) 80–89

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Preparation of natural indicator incorporated media and its logical use as a colorimetric biosensor for rapid and sensitive detection of Methicillin-resistant *Staphylococcus aureus*

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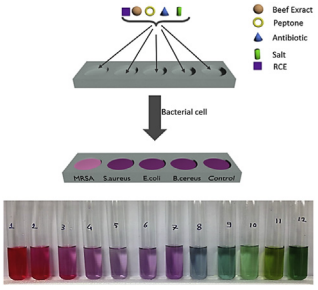
HIGHLIGHTS

- Rational design in preparation of natural indicator incorporated media.
- Using the culture media as a colorimetric biosensor for rapid detection of Methicillin-resistant *Staphylococcus aureus*..
- Use of cefoxitin as a broad-spectrum antibiotic for bacterial pathogens.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 March 2020
Received in revised form
28 May 2020
Accepted 1 June 2020
Available online 14 July 2020

Keywords:
Methicillin-resistant *Staphylococcus aureus*
Colorimetric assay
Antibiotic
Anthocyanins
Red cabbage (*Brassica oleracea*) extract

ABSTRACT

Herein, we report a rational design in preparation of natural indicator incorporated media used as a rapid, selective, sensitive and economic colorimetric biosensor for detection of Methicillin-resistant *Staphylococcus aureus* (MRSA). Anthocyanins obtained from red cabbage (*Brassica oleracea*) extract (RCE) are major components and acted as pH responsive indicators in the RCE media. The RCE media was tested for eight gram-positive, four gram-negative bacterial and one model fungal pathogens. We experimentally revealed how salt concentration and antibiotic (cefoxitin) in the RCE media influence the growth of pathogens and especially MRSA. Benefiting pH dependent color change function of anthocyanins is indispensable point of the RCE media in sensing of MRSA growth. The potential MRSA colorimetric sensing mechanism of anthocyanins relies on both protonation and decrease in electron density on structures of anthocyanins by acidic organic volatile compounds produced during growth of MRSA. The protonated anthocyanins with low electron density changed original purple color of the RCE media to pink color. We demonstrated that detection of MRSA was achieved by spectrophotometrically (one single cell) and visually with naked eyes (100 cells) in 2nd hrs and 6th hrs of incubation, respectively. We optimized concentrations of cefoxitin (from 1 μg/mL to 20 μg/mL) against MRSA and Methicillin-sensitive *Staphylococcus aureus* (MSSA) bacterial cell (3 McFarland) suspensions, then growth of MRSA was visually and clearly detected in the presence of 4 μg/mL cefoxitin between 90 min and 3 h. We persistently offer

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https://doi.org/10.1016/j.aca.2020.06.005

that the proposed RCE media can be a well alternative to currently used phenotypic methods in clinics owing to its much rapid, sensitive, selective and economic properties.

1. Introduction

The infectious diseases caused by bacteria are one of the human health threatening problems by leading to several millions of deaths worldwide each year [1,2]. Even recent studies reported that bacterial infectious highly increased the mortality compared to HIV/AIDS-related diseases in the United States [3,4]. The pathogens are dispersed in a wide range of areas such as soil, sea, faeces, contaminated water and foods [5–7]. Among bacterial microorganisms, *Staphylococcus aureus* (*S. aureus*), a member of the *micrococcacea* family, is considered as a one of the most dangerous pathogens. Although Methicillin-sensitive *Staphylococcus aureus* (MSSA) harmlessly hosts in nose, throat mucosa, or skin (such as hands, feet and face) of many people, however it may produce enterotoxins in polypeptide structure containing a much high toxin content, then life-threatening infections including adverse effects on joint rheumatism, meningitis, septicemia, cardiac inflammation and inflammatory wounds can be seen [8–10]. While MSSA was effectively treated with penicillin since its discovery by Alexander Fleming in 1928, however, intensive use of antibiotics led to the emergence of antibiotics resistant MSSA strain [11,12]. Despite the development of methicillin, as a new generation of antibiotics, to fight against *S. aureus*, Methicillin-resistant *S. aureus* (MRSA) strains as often called “superbug” shown resistance to beta-lactam group antibiotics and cephalosporins were seen in England in 1961 and in France in 1962 [13,14]. In addition to the damages caused by MRSA-related infections, today, up to 74% of worldwide *S. aureus* infections are caused by MRSA [13,14].

Unfortunately, broad-spectrum antibiotics are immediately prescribed until diagnosis of infection in today. For instance, if the patient has MRSA infection, it cannot be treated properly, and the wrong antibiotic treatment is applied until the diagnosis is confirmed. As a result, misuse of antibiotics harms paint health and economy and leads to the development of resistance [13–16].

Up to date, various phenotypic and/or genotypic methods have been developed and in use for the diagnosis of hospital-acquired infection pathogens, especially for MRSA owing to some vital reasons: i) treatment difficulty of MRSA, ii) very serious infections caused by MRSA and iii) its survival under wide and harsh range of conditions [17,18]. For instance, culture and colony counting methods; the immunological approach related to modern methods like polymerase chain reaction (PCR) and reverse transcriptase (RT-PCR), flow cytometry, mass spectrometry, microarrays, fluorescence and electrochemical based methods are currently used for MRSA identification. Unfortunately, these methods have serious disadvantages such as time-consuming, complex, laborious, requiring complicated tools and specialized personnel, expensive and not suitable for field work (lack of portability), and then these limit their widespread use [19–24]. The genotypic methods often result in 3 hrs and rarely cause positive errors, requiring high-cost devices, consumables, trained personnel and limited availability of genotypic devices in every hospital are major drawbacks of genotypic methods. Although the phenotypic methods are commonly used, results are usually obtained between 24 hrs and 48 hrs and but even additional 24 hrs can be needed for enrichment of culture. It is worth to mention that ingestion, inhalation, skin contact of consumables used in phenotype methods can be dangerous since most of them are animal origin and may contain animal-borne pathogen [25–27].

Over the past decade, several organic and biological targeting agents have been synthesized and integrated to various nanoparticles for designing of novel and sensitive fluorescent and colorimetric nano-biosensors [28–32]. For instance, various ligands including fluorescent anionic, cationic and amphiphilic based polymers and disulfide phenylboronic acid functionalized gold (Au) NPs were utilized for detection of target pathogens [28,33–35]. In terms of NPs incorporated sensors, Au NPs have been most widely used owing to easy and one pot synthesis, stable structure (can be stored for a long time at room temperature), high surface area, facile surface functionalization and intrinsic optical properties [36,37]. Li et al., very recently functionalized Au NPs with a series of molecules (mercaptopropionic acid), mercaptosuccinic acid, cysteamine and cetyltrimethylammonium bromide to manipulate their surface charges for developing colorimetric sensors which were used for rapid pathogen identification [38]. Furthermore, quantitative analysis for colorimetric sensors has been performed using basic optical technologies such as digital cameras and office scanners combined with image visualization, analysis, and processing software to achieve differentiation of color, hue or intensity measurements [39,40]. Although Au NPs based sensor arrays hold promising sensitivity and ligands dependent selectivity, complicated and multi steps ligands modification to NPs, need of trained personnel, low reproducibility and lack of practical use still remain unsolved problems.

Despite of currently used several phenotypic and/or genotypic methods and effort on designing NPs integrated nano-biosensors, there is still high demand to develop new methods for rapidly, sensitively and economically detection of MRSA. Herein, we developed a novel, smart and natural pH indicator-incorporated media called “RCE media” and rationally used it as a rapid, sensitive, selective and economic colorimetric biosensor for detection of Methicillin-resistant *Staphylococcus aureus* (MRSA) in clinics. The RCE media is environmentally friendly and causes no chemical waste and no environmental pollution. The RCE media contains four essential components such as RCE (natural pH indicator), salt, broth and antibiotic (cefoxitin). The anthocyanins obtained from red cabbage (*Brassica oleracea*) acts as natural pigment molecules for coloring plants, fruits and vegetables. The anthocyanins predominantly existed in cyanidin-3-O-diglucoside-5-O-glucoside form in the red cabbage extract (RCE) were benefited as natural pH indicators in RCE media. They are quite stable in aqueous solutions and give various colors based upon protonation and deprotonation on their structure at varying pH values (pHs) [38,41,42]. Various pathogenic bacteria, including MRSA, release acidic organic volatile compounds, which protonates anthocyanins and turn their color from purple to pink [43–47]. While peptone and beef extract were used in bacterial culture as broth for bacterial growth, sodium chloride (NaCl), a source of salt, has been used to meet nutritional requirements of microorganisms (7.5% sodium chloride allows the growth of salt tolerant staphylococci). Cefoxitin was used as a model and wide spectrum antibiotic added to bacterial culture to suppress pathogens except MRSA.

2. Experimental section

2.1. Materials and instrumentations

Nutrient broth (Merck, Germany), triptic soy agar (Merck, Germany), MRSA ID (bioMérieux, France), CHROMagar (bioMérieux, France), skimmed milk medium (Difco,USA), meat extract (Acumedia,U.K), peptone (Mast Diagnostic, U.K), NaCl (Isolab, Turkey), and cefoxitin (Merck, Germany) were purchased from the company stated.

Microorganisms: Bacterial pathogens: MRSA standard strain 43300, Methicillin-sensitive *Staphylococcus aureus* ATCC 25923 (MSSA), *Enterococcus faecium* ATCC 8459, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 9334, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhi* ATCC 14028, *Bacillus cereus* ATCC 14579, Vancomycin-resistant *Enterococcus faecalis* (VRE) ATCC 49532 and fungal pathogen, *Candida albicans* ATCC 90028 were obtained from Erciyes University, Faculty of Pharmacy, Pharmaceutical Microbiology research laboratory ATCC culture collection. All pathogens were stored in skim milk medium at −20 °C and regenerated prior to the experiments. The optical density was determined by spectrophotometer (Azure Ao, Azure Biosystems, Inc.).

Clinical MRSA and MSSA strains: The PCR which gold standard method for the detection of methicillin resistance on *Staphylococcus aureus* mecA gene, was used to included criteria of the study. Totally a hundred *S. aureus* (50 MRSA and 50 MSSA) isolates from hospitalized patients in a university hospital that were identified in southwestern of Turkey were used in this study. All strains of *S. aureus* identified by Gram stain, catalase, mannitol fermentation, and coagulase positivity also by Vitek2 automated system (bioMérieux, France). Methicillin susceptibility was determined by oxacillin and cefoxitin disc diffusion and the absence of specific colonies on a chromogenic medium MRSA ID and CHROMagar. Quality control testing was performed on each test solution and agar plate by inoculating *S.aureus* ATCC 29213 as a negative control and *S.aureus* ATCC 43300 as a positive control.

2.2. Red cabbage (Brassica oleracea)

Red cabbage was purchased from province Kayseri of Turkey. Red cabbage leaves were cut into small pieces by passing through a shredder. The weighed 100 g of material was placed in a 500 mL beaker containing 100 ml of distilled water. The mixture was exposed to extraction process by boiling it for 30 min. The resulting red cabbage leaves extract was filtered and stored at −20 °C for further use.

2.3. Preparation of natural indicator incorporated media

To prepare the natural indicator incorporated media, 10 g peptone, 1 g beef extract, 75 g/L NaCl and 250 ml 25% red cabbage extract were added to 1 L of distilled water. In the first step, peptone, beef extract and NaCl were added to 750 ml of distilled water and sterilized in autoclave at 121 °C for 15 minutes (min). 250 ml of red cabbage extract was added to the resulting medium by filtration through a membrane filter with a pore size of 0.45 μm. In the last step, 4 μg/ml cefoxitin was added to the final mixture. The prepared RCE medium is purple color.

2.4. Color image processing

For digital image analysis, wells which contain RCE media were placed on a white background. The colorimetric biosensor images were recorded using a camera and saved in JPEG format. RCE media images were analyzed by ImageJ software (National Institutes of Health) for quantification of obtained colorimetric responses. For RGB (Red Green Blue) analysis mean values were calculated in ImageJ software. Delta-E (ΔE) parameter was also calculated according to CIE76 algorithm [48].

3. Results and discussion

The anthocyanins are major phenolic compounds existing in the RCE. Both biocompatibilities approved by FDA and color change properties of anthocyanins depending of pHs inspired us to design a media to logically use it as a new generation, sensitive and selective colorimetric nano-sensor for MRSA [49]. The anthocyanin plays a vitally critical role as a sensing element in RCE media owing to its pH indicator property. For instance, when it is protonated by acidic organic volatile compounds produced by MRSA [42], original purple color of the RCE media is turned to pink and then presence and/or growth of MRSA in the RCE media can be confirmed.

Fig. 1 illustrates the preparation of RCE (anthocyanins)-incorporated media, colorimetric detection of MRSA compared to other pathogens, stability of RCE media itself and pH dependent color spectrum of anthocyanins. When the RCE media was incubated with some model bacteria or fungus, purple color generated from anthocyanin was not changed owing to their salt and/or antibiotic intolerance properties. Additionally, no change in purple color of the RCE media without including bacteria was observed, which can be considered as an indication of anthocyanin stability. The color spectrum of anthocyanin solutions shows that anthocyanin solutions in acidic environment (pH $\leq$ 3) give pink color due to their protonation. The anthocyanin solutions prepared in between pH 4 and pH 7 produce its original purple color. In contrast, when pH of anthocyanin solutions become alkaline (between pH 8 and pH 12), blue and various shades of green colors are witnessed.

The protonation of anthocyanin owing to presence of acidic environment is occurred as shown in Fig. 2. The carbon atom next to oxygen of center ring is protonated and double bond is disappeared, both of which caused low electron density, then color change from purple to pink is carried out. This reaction was successfully implemented to MRSA detection. As we mentioned above, the organic volatile compounds generated by MRSA made reaction environment acidic and anthocyanin molecules were protonated, then purple color of RCE media was converted to pink. As is seen in Fig. 2 above that pyrylium ion in the structure A (at top right in Fig. 2) is a strong electron-withdrawing group due to the conjugation and positively charged oxygen atom. Therefore, phenolic −OH protons at the flavylium ion (A) becomes more acidic and it is obvious that acidity of this phenolic −OH proton is more than phenol itself. Due to the higher acidity, quinonoid structure B (at left in Fig. 2) can be formed even at pH 4–7 since it is a neutral and more basic structure compared to the structure A [50].

In order to optimize the RCE incorporated media, it initially was prepared using % 25 w/w RCE, 10 g/L peptone, 1 g/L beef extract and 50 mg/mL NaCl. The 100 μL of MRSA and MSSA bacterial cell (0.5 McFarland) suspensions were added into the 100 μL RCE media and incubated for 4 hrs to demonstrate the color change based upon bacterial growth. Fig. 3 indicates that the growth of MRSA rapidly converts color of the media to clear pink (left) and its pH value was measured to be ~2.2. The faint pink color in the purple media slightly appeared when MSSA was cultured (pH ~3.57) in the RCE media as presented in Fig. 3 (middle). In order to prove the stability of the RCE media, it was left overnight at room temperature (RT: 20 °C), no color change or loss was observed on its original purple color (pH ~6.87) (at right in Fig. 3). For quantitatively supporting colorimetric response of RCE media, we performed a detailed RGB

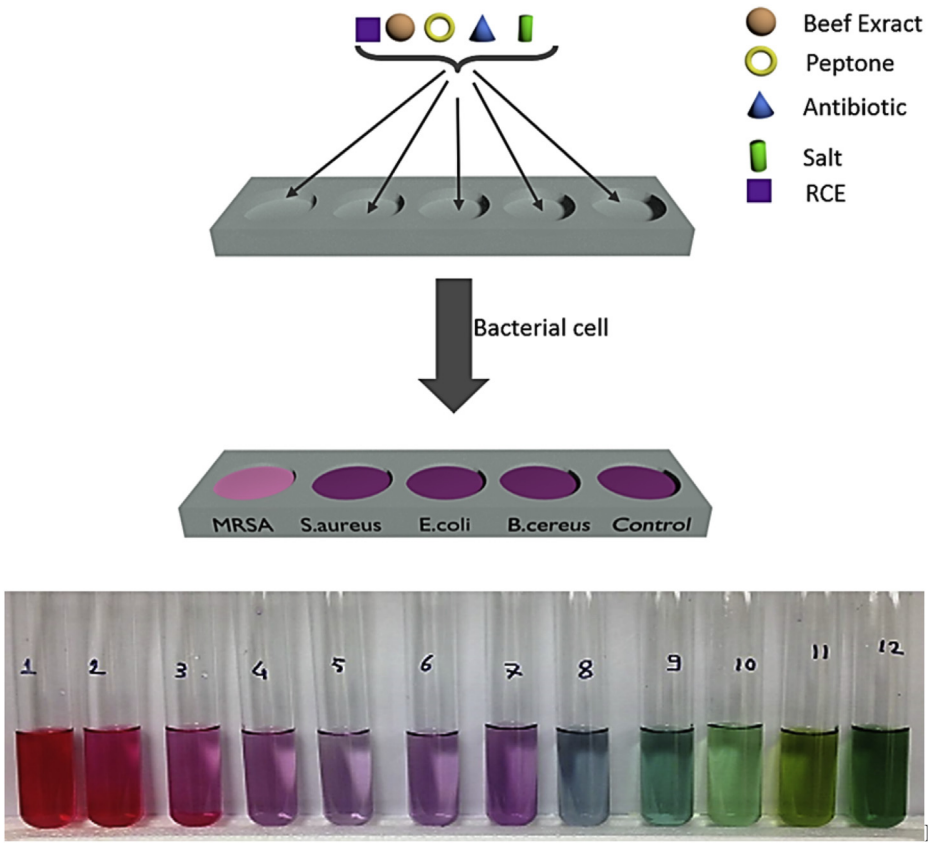

Fig. 1. Schematic illustration represents preparation of the RCE (anthocyanins) incorporated media called "RCE media", colorimetric bacterial detection and colors of anthocyanin solutions under various pH values. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

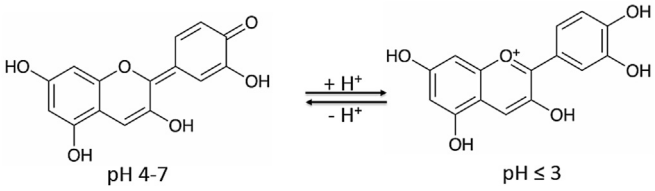

Fig. 2. The reversible protonation and deprotonation reaction of anthocyanin at varying pHs.

and Delta-E (ΔE) analysis. The ΔE value of 2 is a cutoff point of indicator and the human vision system can differentiate two colors above that value [51]. Therefore, larger differences in ΔE values enable more accurate detection of MRSA. Fig. 3B demonstrates differences in ΔE values for the samples containing MRSA and MSSA, as compared to control.

We interpret that MRSA substantially grew in 50 mg/mL NaCl concentration owing to its strong salt tolerance [52]. Although the complete inhibition for MSSA was predicted at that NaCl concentration, faint pink color in the RCE media was sign of partial growth of MSSA. NaCl concentration was increased to 75 mg/mL and 4 μg/ml cefoxitin was added into the RCE media for three reasons: i) to show the tolerance of MRSA to high salt concentrations and antibiotics, ii) to increase the selectivity of the RCE media and iii) to completely inhibit natural flora and potential antibiotic resistant microorganisms.

To test the performance of the high concentration NaCl and cefoxitin added RCE media, the 100 μL bacterial cell (0.5 McFarland) suspensions (MRSA, MSSA and *E.coli*) were added into the 100 μL

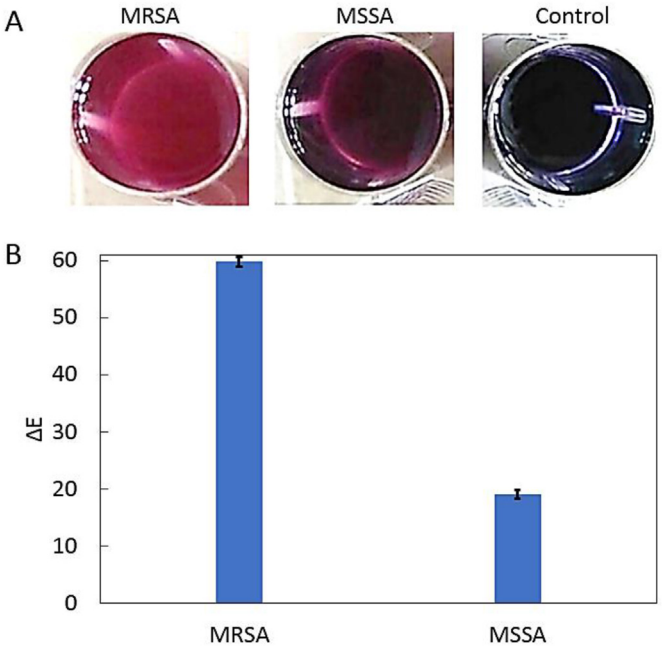

Fig. 3. A) Photograph of color change of RCE incorporated media (containing 50 mg/mL NaCl) with addition of MRSA (left), MSSA (middle) and the RCE media itself without any bacteria (right). B) ΔE analysis that shows color difference of MRSA and MSSA. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

RCE media and incubated for 4 h. The increase in absorbance (blue line) (Fig. 4A) and occurrence of pink color in the RCE media with pH ~2.22 (Fig. 4B) shows only growth of MRSA continued as presented in Fig. 4. The growth of MSSA and *E.coli* was completely inhibited, and then the sharp purple color of RCE media remains unchanged (pH ~6.50). Additionally, the increase in salt concentration and the addition of antibiotics did not cause a change in the original purple color of the RCE media (pH ~6.87 of RCE media as a control in Fig. 4). While ΔE values of MRSA and MSSA in Fig. 3C are 59.8 and 19.1, respectively, they were accordingly calculated as 60.1 and 7.24 in Fig. 4C. In addition to the visible distinctive color difference, the suppression of MSSA growth is clearly demonstrated by ΔE values.

The time dependent detection of MRSA based on color change of the RCE media was systematically employed using eight different gram-positive bacteria (MRSA, MSSA, *E. faecium, B. subtilis, S. pyogenes, B. cereus, E. faecalis,* Vancomycin-resistant *Enterococcus faecalis (*VRE), 4 gram-negative bacteria (*E. coli, H. influenzae, K. pneumoniae, S. typhi*) and one model fungus (*C. albicans*) as presented in Fig. 5A. Quantitative results of the differential color differences are also shown in Fig. 5B. While the pH of RCE media where MRSA grown is ~2.22, the pH of environment containing all other microorganisms ranging from ~6.50 to ~6.87. We applied standard protocol aforementioned above, 100 μL of each microorganism cell (0.5 McFarland) suspensions was added into 100 μL RCE media and the resulting mixture was left for incubation. We realized that the occurrence of pink color in the RCE media was observed in less that 4th hrs of incubation, which is an indication of growth of MRSA. It is worthy to mention that the growth of MRSA was not inhibited in the RCE media and exhibited great tolerance towards high salt concentration and antibiotic. Other thing is that amount of MRSA grown, or amount of acidic organic volatile compounds produced by MRSA in between 4th and 6th hrs of incubation are enough for protonation of anthocyanins and to convert the purple RCE media to pink color. In contrast to that, no growth for other microorganisms was observed in 4th hrs and even in 24th hrs due to presence of high salt concentration and antibiotic and then original purple color of the RCE media has remained.

For further study on selectively and sensitively detection of MRSA, various amount of CFU/mL (colony-forming units per milliliter) and number of bacterial cells of MRSA were mixed with the RCE media and each mixture was time dependent incubated. The MRSA growth was determined based upon the color changes visualized with naked eyes, the changes in absorbance values using a UV–Vis spectrophotometer, and ΔE analysis as shown in Fig. 6. The pH values of the RCE medias lowered from ~2.57 to ~2.22 when MRSA concentrations (CFU/mL) increased as seen in Fig. 6A. 100 μL of MRSA suspensions with 1 CFU/mL (dark blue line), 5 CFU/mL (orange line), 10 CFU/mL (gray line), 100 CFU/mL (yellow line) and 1000 CFU/mL (light blue line) were added into the 100 μL RCE media and each mixture was allowed for incubation. The changes in absorbance based upon bacterial growth were recorded at every 2 hrs intervals. The RCE media (green line) without MRSA was also used as a control media. The rapid and slight increases in growth of MRSA were measured at 2nd hrs of incubation in RCE media containing both 1000 CFU/mL (light blue line) and 100 CFU/mL (yellow line). As expected, the growth of 1000 CFU/mL and 100 CFU/mL reached to saturation point at 2nd hrs of incubation due to limited bacterial broth in the RCE media. 10 CFU/mL (gray line), 5 CFU/mL (orange line) and 1 CFU/mL (dark blue line) of MRSA started growth at first 4 hrs of incubation. While 10 CFU/mL and 5 CFU/mL of MRSA grew till 10th, 1 CFU/mL MRSA showed growth for during 18 hrs. For all colonies, the pink color was captured in 4th -6th hrs of incubation. We claim that the RCE media is greatly sensitive even for colorimetrically and spectrophotometrically detection of growth 1 CFU/mL MRSA in 4 hrs. In order to show the reproducible of the measurements, intra and inter assay coefficient of variation (CV) from 1 to 1000 CFU/mL were calculated. Results of intra-assay CV (%) from 1 to 1000 CFU/mL calculated as 2,11; 5,49; 5,80; 1,14; 2,64, respectively. Also result of inter-assay CV (%) is around 3,43. We believe that this RCE incorporated media is one of most promising and encouraging breakthroughs for clinical uses.

In addition to CFU based detection, we also examined the sensitivity performance of the RCE media for number of 10^3 (blue line), 10^2 (orange line), 10^1 (gray line) and 1 (yellow line) of single MRSA and pH values were found to be ~2.22, ~3.57, ~4, 97 and ~5.92, respectively as shown in Fig. 6B. Typically, 100 μL of MRSA suspension of each concentration was added into the 100 μL RCE media. Then, the changes on color of RCE media and in absorbance are proportional to bacterial growth and were recorded at every 2 hrs intervals. The results showed that although bacterial growth for each number of MRSA even for single cell were spectrophotometrically observed, only 1000 and 100 MRSA cells resulted in pink color in RCE media visually seen with naked eyes at 4th hrs. The RCE media (brown color) again showed no changes on its original purple color. In addition, the color difference in the wells is shown as ΔE analysis. Despite the difference in concentration in the analysis of Fig. 6A, ΔE values are relatively close to each other as all samples turn pink as a result of incubation, but some increase in ΔE values was seen depending on concentration. In the ΔE analysis of

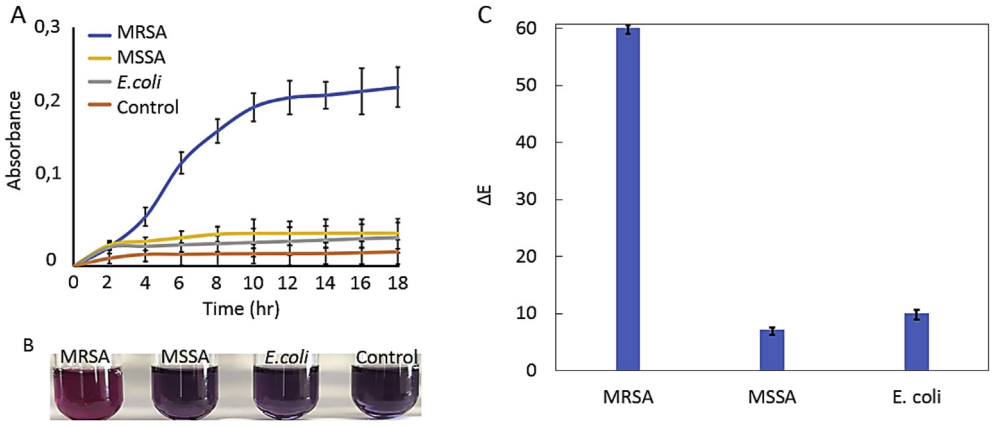

Fig. 4. A) Role of NaCl (75 mg/mL) concentration on specific detection of MRSA showing with absorbance spectrum, B) colorimetric result and C) ΔE values. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3).

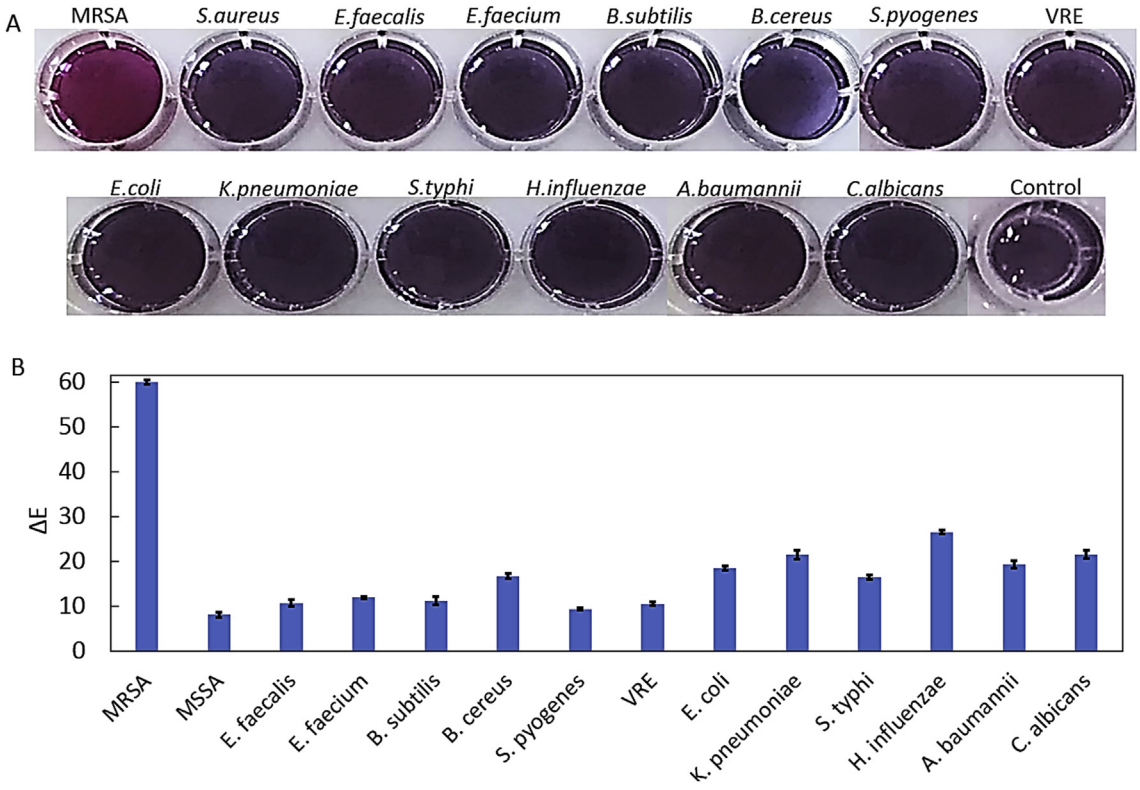

Fig. 5. A) Time dependent the selectivity of MRSA detection towards various gram-positive and gram-negative bacterial cells and fungus as well. B) ΔE analysis. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3).

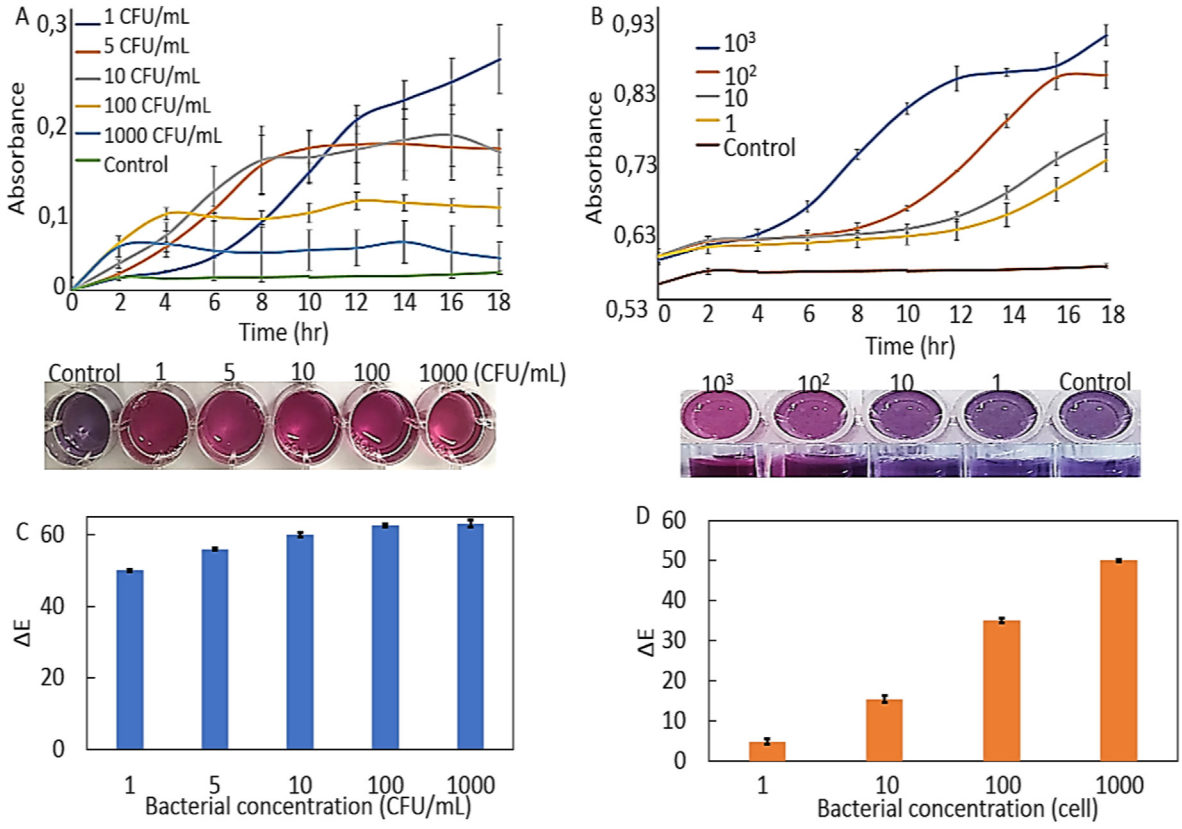

Fig. 6. Sensitivity studies based the concentration dependent of MRSA detection. A) in CFU/mL, B) cell number. ΔE analysis for C) in CFU/mL and D) cell number. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3).

Fig. 6B, color of all wells was not in similar tones but were seen in more distinctive colors owing to bringing down to lower concentrations and ΔE values also increased gradually. Also, the conversion from purple to pink was confirmed by RGB analysis as shown in supplementary material (Fig. SI1). when the wells containing control and MRSA were compared, it was observed that R/B values were more suitable than R/G, B/G values. The increase in R/B values supports the formation of pink color.

In order to visually detect the growth of MRSA in a short time, we examine the concentration of cefoxitin in the presence of MRSA and MSSA bacterial cell (3 McFarland) suspensions. We proposed that MRSA with high concentration (3 McFarland) in the corresponding cefoxitin concentration produced quite much acidic organic volatile compounds, then protonation and color change time of anthocyanin was shorted. Fig. 7 shows that two clinics MRSA (R) strains, one clinic MSSA (S) and blue RCE media (pH ~8.71) without any bacteria were studied based upon various cefoxitin concentrations. Although color of the RCE media was adjusted to purple color as initial point of media, we arranged the color of the RCE media to blue for observing clear and distinct color change from blue to pink. Thus, the 3 McFarland MRSA and MSSA cell suspensions were added into the blue colored RCE media including 1 μg/mL, 2 μg/mL, 4 μg/mL and 8 μg/mL of cefoxitin representing in first, second, third and fourth raw of microplates, no change on original blue color of the RCE media was observed until 60 min of incubation (Fig. 7A). While slight color change with pH ~5.70 was appeared in second MRSA of all raws in 90. min, quite clear pink colors (pH ~2.22were seen in where both MRSA strains added after 2 hrs incubation (Fig. 7A). Unfortunately, after 3 hrs of incubation, MSSA turned the color of the medium into a pinkish color (pH ~3.57) with partial growth when 1 μg/mL and 2 μg/mL of cefoxitin used. As we proved that before, 4 μg/mL and 8 μg/mL of cefoxitin completely inhibited growth of MSSA and pink colors were seen only where MRSA strains grown.

As a further study on concentration of cefoxitin, 12 μg/mL, 16 μg/mL and 20 μg/mL concentrations of cefoxitin were used, no growth was observed in MSSA solution as expected (Fig. 7B). However, pinkish (pH ~3.57) and sharp pink color (pH ~2.22) were observed in 2 hrs and 3 hrs of incubation in all cefoxitin concentrations, respectively. Interestingly, high concentration of antibiotic is used in Fig. 7B may lead to some inhibition even on growth of MRSA, then it can be reason not to see quite clearly pink color in 90 min and 2 hrs incubations. Additionally, the RCE media itself was left incubation for 6 hrs as control group, it preserved the original blue color, which is indication of much high stability of the RCE media. In order to support visual results with quantitative analysis, ΔE values were in Fig. 7. The color differences of 1 resistant (R) and 1 sensitive (S) *S. aureus* strains with 3 McFarland concentration incubated 3 hrs were drawn depending on the control (Fig. 7C). After 3 hrs incubation, as seen in the visual results, the colors did not change, and the calculated color differences were not added to the chart because they were repetitive. In addition, ΔE analysis of samples containing 4 μg/mL cefoxitin is shown in supplementary material (Fig. SI2).

In terms of elucidating the effect of bacteria concentration, two different MRSA strains (R) and one MSSA (S) bacterial cells suspensions (0.5 McFarland) were added to each blue RCE media (pH ~8.71) including 1 μg/mL (first raw), 2 μg/mL (second raw), 4 μg/mL (third raw), 8 μg/mL (fourth raw) and 12 μg/mL (fifth raw) of cefoxitin, then color change based upon bacterial growth at varying incubation time was observed (Fig. 8A). No color change was witnessed in where MRSA strains and MSSA strain were injected in 3 hrs of incubation. It refers two potential reasons: i) both growth of 0.5 McFarland MRSA strains and MSSA strain were inhibited in all cefoxitin concentrations or ii) the amount of acidic organic volatile compounds produced especially by MRSA strains was not enough to make the RCE media acidic and to protonate anthocyanins for color change. As expected, while MRSA strains were able to slightly

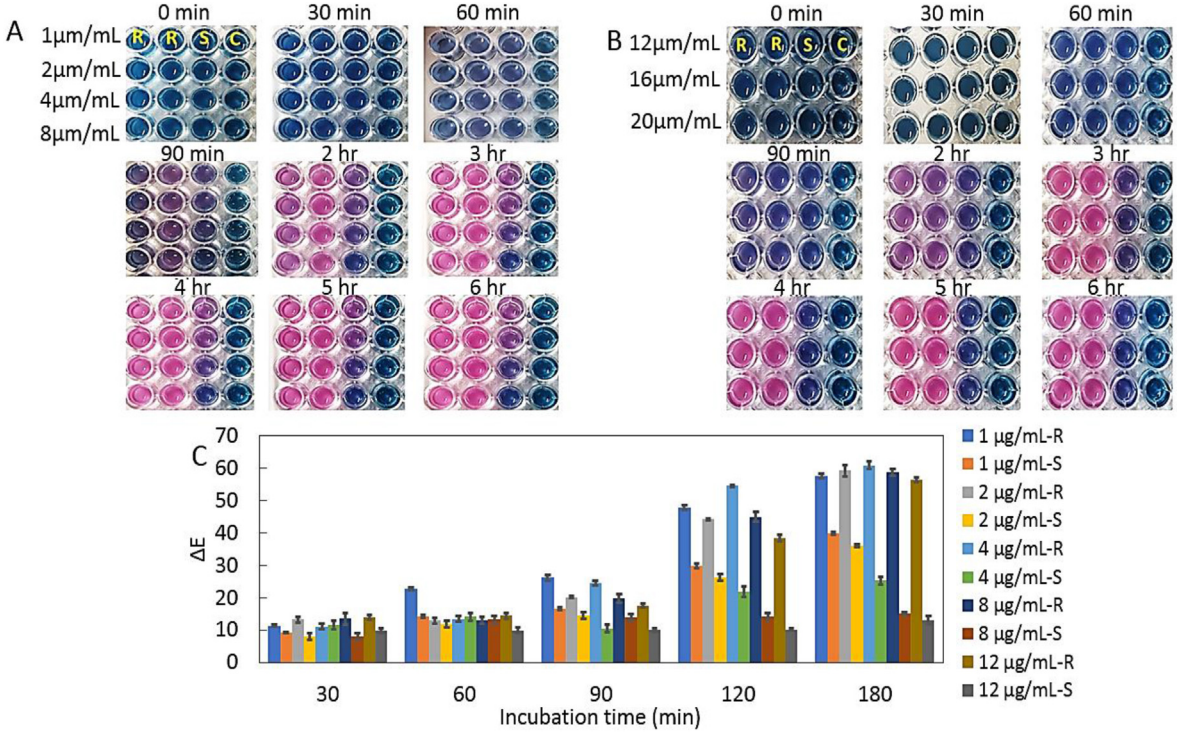

Fig. 7. Cefoxitin concentration dependent studies towards MRSA and MSSA (3 McFarland). Cefoxitin used between A) 1 μg/mL and 8 μg/mL, B) 12 μg/mL and 20 μg/mL and C) ΔE analysis. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3).

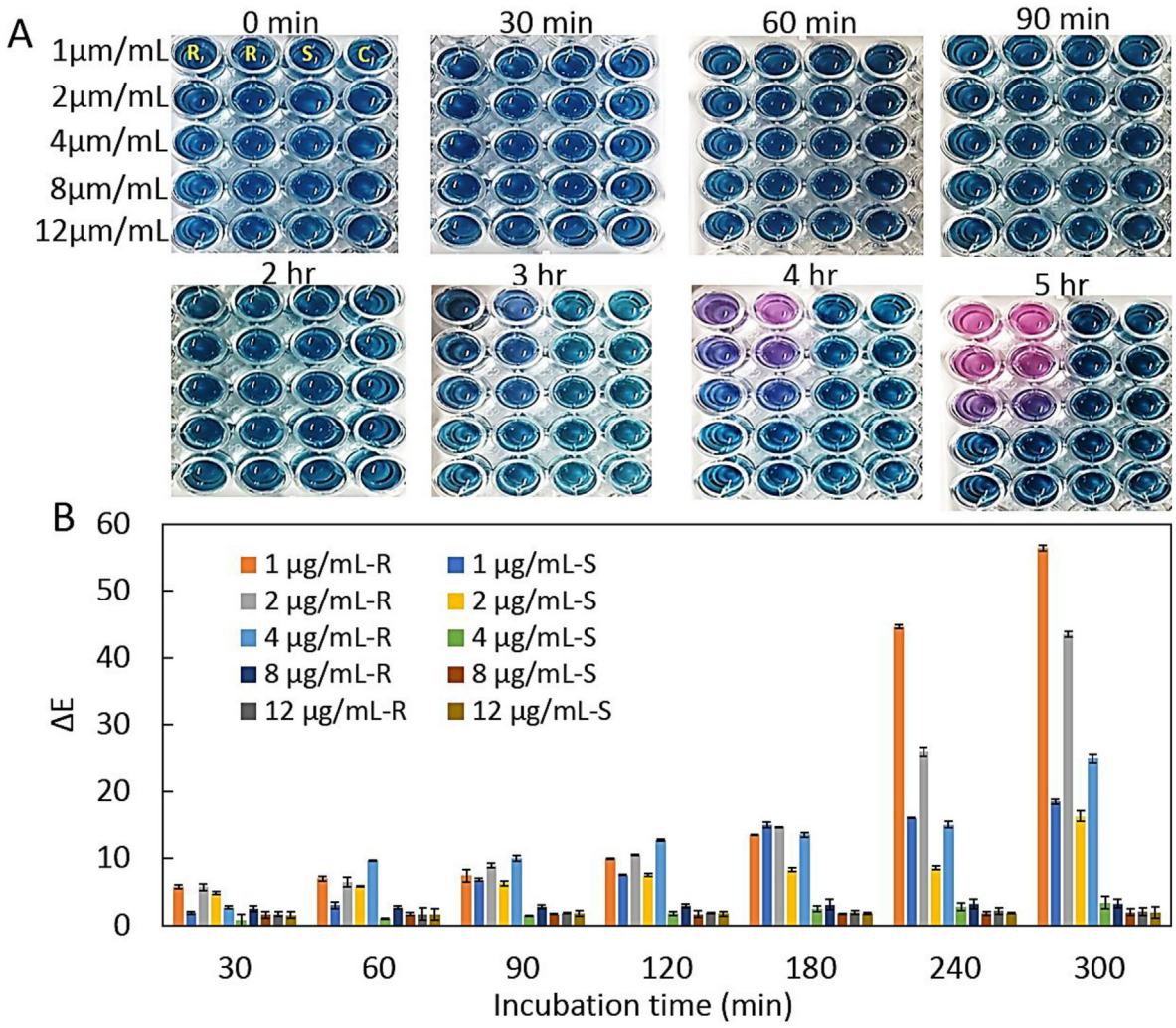

Fig. 8. A) Photography of cefoxitin concentration dependent study towards MRSA and MSSA (0.5 McFarland). B) ΔE analysis. The error bars demonstrate one standard deviation (SD) obtained from three independent measurements (n = 3).

change color of the RCE media including 1 μg/mL of cefoxitin from blue to purplish (pH ~5.92) and pinkish (pH ~3.57) in 4 hrs of incubation, however, clear pink color of RCE media with pH ~2.22 (media including 1 μg/mL and 2 μg/mL of cefoxitin) and purplish color of RCE media (media including 4 μg/mL of cefoxitin) were observed in 5 hrs of incubation. In all cefoxitin concentration and during the 5 hrs incubation, original blue color of the RCE media remained unchanged when 0.5 McFarland MSSA strain used. Color differences are clearly shown in Fig. 8B compared to control.

We demonstrated that this rationally prepared RCE media gave quite promising results for detection of MRSA. The growth of MRSA strains can be spectrophotometrically or visually with naked eye detected between 2 hrs and 3 hrs incubation by manipulating concentration of bacterial strains and antibiotic. The major advantages of the RCE media can be listed as selectivity, sensitivity, biocompatibility and quite affordability. It is worthy to mention that 100 different clinical strains were tested using standardized RCE media. Then, the sensitivity and specificity of RCE media were determined to be 100% and 98.04%, respectively within 4–6 hrs incubation. If incubation time increases, specificity may reach to 100% accordingly owing to time dependent growth of low concentration bacterial cell. When 100 clinical strains were tested with other commercial phenotypic methods, while CHROMagar gave 99% sensitivity and 99% specificity, sensitivity of MRSA ID was around 99% and no applicable specificity (NA) was obtained after 18–24 hrs incubation. It is noticed that both CHROMagar and MRSA ID require at least 18–24 hrs incubation time to give a response. It is a great advantage that the RCE media needs only a few hrs for harvesting the results compared to its closest competitor tests. Since this may lead to identification of bacteria in mixed cultures about a day or even 2 days ahead, excessive and unnecessary or even wrong use of antibiotics can be prevented.

4. Conclusions

Herein, we present rational preparation of natural indicator incorporated media and used as a rapid, selective, sensitive and economic colorimetric biosensor for detection of Methicillin-resistant *Staphylococcus aureus* (MRSA). The RCE media contains four essential components including peptone and beef extract as broths, salt (NaCl), antibiotic (cefoxitin) and anthocyanins from red cabbage (*Brassica oleracea*) extract (RCE). Peptone and beef extract are broth needed for bacterial growth. The NaCl and cefoxitin suppressed growth of all microorganism used in the RCE media except MRSA and provided the selectivity to the RCE media. We experimentally demonstrated that anthocyanins functioned as key

elements in the RCE media for detection of MRSA owing to their pH dependent color change properties. The anthocyanins were rapidly protonated from acidic organic volatile compounds generated during the growth of MRSA, then protonated anthocyanins converted original purple color of the RCE media to pink. The RCE media was employed as a colorimetric sensor towards series of total of 13 microorganisms, then only growth of MRSA was rapidly, selectively and sensitively detected. We achieved detection of 1 cell and 100 cells of MRSA via a UV–vis spectrophotometer in 2nd hrs of incubation and via naked eyes in between 90 min and 4 hrs of incubation based upon bacteria concentration, respectively. The results are presented both visually and by RGB-based quantitative analysis. We believe that the RCE media with the unique properties aforementioned above and very low cost would be actively used in clinics for MRSA detection in near future.

Author contributions

C.C. and N.I. contributed equally. The project was conveyed and designed by I.O., N.I. and M.Z.K. C.C, N.I and M.Z.K run all the experiments. A.B.K. made contributions on experimental section. N.I. and I.O. guided in all the experiments. C.C., N.I. and I.O. wrote the manuscript.

CRediT authorship contribution statement

Cagla Celik: Writing - original draft. **Nilay Ildiz:** Writing - original draft. **Melih Zeki Kaya:** Investigation, Methodology. **Ayse Baldemir Kilic:** Investigation, Methodology. **Ismail Ocsoy:** Project administration, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is supported by grants awarded by the Erciyes University Scientific Research Office (TCD-2019-8016) and Turkish Academy of Sciences. This study was partially presented as an oral presentation at the International Erasmus Symposium, 2019 Turkey.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.06.005.

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