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Characterization and Evaluation of Biofilm Formation by *Klebsiella pneumonia* MBB9 Isolated from Epilithic Biofilms of the Porter Brook River, Sheffield

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Abstract

Microbes generally attach to surfaces and produce an Extracellular Polymeric Substance (EPS) matrix. The exopolysaccharide production plays a role in biofilm protection against environmental stress factors. Biofilm-forming bacteria exhibit different physiological properties in their response to environmental influences compared with their planktonic counterparts. This study aimed to investigate the impact of changing the concentrations of glucose, peptone, and yeast extract and environmental parameters, such as temperature, pH, anaerobic conditions, osmotic stresses, and growth media on biofilm formation by *K. pneumonia* MBB9 recovered from river-stones collected from the Porter Brook, Sheffield using crystal violet and resazurin assays in microtiter plates. The different concentrations of glucose (0.25, 0.5 and 1 g L^{-1}), peptone (0.25, 0.5 and 1 g L^{-1}) and yeast extract (0.25, 0.5 and 1 g L^{-1}) as carbon and nitrogen sources found to have an impact on biofilm formation by *K. pneumonia* MBB9. The greatest biomass level being at 0.25 g L^{-1} for glucose whereas the density of biofilm increased significantly with increasing the concentration of peptone and yeast extract until 1 g L^{-1} of peptone and yeast extract, suggesting that higher levels of peptone and yeast extract can be beneficial for biofilm formation by *K. pneumonia* MBB9 in microtiter plates. The amount of biofilm was high at pH 4.5 and 0.6% NaCl; however, the significant reduction at pH 10.5 and 10.6% NaCl could be as a result of the slow growth under higher NaCl concentrations and highly alkaline condition. High-density biofilm produced at 40 °C; however, a temperature of 50 °C reduced the amount of biofilm by *K. pneumonia* MBB9, suggesting that more extreme temperatures might affect the formation of biofilm by inhibiting growth. Besides, biofilm production under anaerobic conditions was significantly lower (83% less) than under aerobic environments. *Klebsiella pneumonia* MBB9 possessed a high capacity to form biofilms on the surface of glass slide coupons.

Keywords: Biofilm Formation, *Klebsiella pneumoniae*, Microtiter Plate, Quorum Sensing, Environmental Factors, Alamar Blue, Resazurin.

Introduction

Bacterial biofilms can be identified as microbial communities adhering to abiotic and biotic surfaces and forming a secure mode of growth of the extracellular matrix [1-3]. The matrix of biofilm typically guards microbes against external conditions, such as pH variations, high salinity, pressure, depletion of nutrients, oxygen radicals, antibiotics and disinfectants [4,5]. Biofilm formation on different surfaces can be through different steps, including the free floating planktonic cells adhesion, maturation, and attached cell dispersion [6,7].

Establishing and developing bacterial biofilm is considered a dynamic and complex process controlled by inherent biological characteristics and also by several environmental factors as variations in the environment usually trigger the biofilm formation [8,9].The environmental factors controlling biofilm attachment and formation can also affect bacteria's ability to grow and survive [10]. Many factors, such as incubation time, nutrient concentrations, pH, temperature, and ionic strength can influence the formation of bacterial biofilms; however, bacterial cell surface appendages and surface properties are also required for this process [11].

Surface attachment biofilm formation is governed primarily through electrostatic, hydrophobic, van der Waals, and contact communications [12,13]. Hydrophobic interactions play a role in bacterial adhesion to different surfaces and promote biofilm formation. Surface hydrophobicity can affect microbial material colonization since bacteria have developed different ways to adhere to the substrate using the hydrophobic force. However, the bacterial physiological state can change the hydrophobicity of the cell surface [14-16].

Bacteria form biofilms have many physiological properties compared to their counterparts in suspensions in their response to environmental impacts; thus, change in necessary nutrients availability can affect microbial physiology [17]. Temperature plays an important role in regulating the bacterial activity rate and the proliferation of biofilm and

organism settlements in aquatic environments [18]. However, the biofilm accumulation rate can be increased by increasing carbon levels [19]. An increase in water temperature, nutrients levels and flow velocity may enhance the rate of bacterial attachment, provided that the critical values of these factors are not exceeded [20].

The present study aimed to examine the impact of changing the levels of glucose, peptone and yeast extract and environmental parameters, such as pH, temperature, osmotic stresses, temperature and growth media on *K. pneumoniae* MBB9 biofilm formation using crystal violet and resazurin (also known as the Alamar Blue) assays in microtiter plates.

Material and Methods

Source and Sampling

The same day (March 2015), river-stones (thick, light brown, sticky growth) on the upper surfaces were collected in a sterile plastic container from Porter Brook in Sheffield, United Kingdom, and were stored until analysis in the cool icebox.

Isolation of bacteria from environmental biofilms

Epilithic biofilms on the stones were scraped and suspensions were serially diluted 1:100 in physiological saline (0.85%) using an aseptic technique [21]. Different selective media: R2A agar, Eosin-Methylene Blue (EMB) agar, MacConkey agar, Xylose Lysine Deoxycholate (XLD) agar, nutrient agar, and Violet Red Bile (VRB) agar were used to inoculate the suspension. The inoculated plates were incubated aerobically at 37ºC for 24-72 h. To obtain pure cultures of the bacterial isolates, colonies with various colors and morphologies were streaked again on freshly agar plates.

Bacterial morphological and biochemical characterization

Isolated colonies from the agar plates were selected and characterized for preliminary identification using various morphological and biochemical properties [22]. Morphological parameters, such as colony form, elevation, margin, surface, optical features, consistency and color are used along with biochemical tests, including catalase and oxidase activities.

Molecular identification of bacteria via 16S rRNA gene sequencing and phylogenetic analysis

GenElute™ Bacterial Genomic DNA Kit was used according to the manufacturer's instructions to extract bacterial genomic DNA from all isolated bacteria. DNA preparations purity was assessed spectrophotometrically using a Nano drop 1000 (A260/280) (Nano Drop Technologies, Wilmington, DE, USA). PCR was used to amplify 16S rRNA genes of the bacterial isolates using forward (27F) 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse (1492R) 5'-GGTTACCTTGTTACGACTT-3' primers (universal, 16S rDNA gene) to amplify the V1-V9 region (1500 bp) of the16S rRNA gene. In brief, a master mix of 25 µl total volume was prepared as follows: 12 µl of 2X master mix (BioLabs, England), 2 µl of each oligonucleotide primer (10 µM), 7 µl of Molecular Grade Water and 2 µl of template DNA. All reactions were run on a LabCycler (SensQuest, Germany) under the following conditions: initial denaturation at 98 °C for 30 s, 35 cycles of 95 °C for 1 min, 58 °C for 30 s, 72 °C for 5 min and a final extension at 72 °C for 5 min followed by a hold at 4 °C. The amplified DNA fragments were separated on a 1% (w/v) agarose gel electrophoresis (BIO-RAD, USA).

SYBR Safe® (Invitrogen) was used to stain the gels. A UVI tech photo documentation system was used to visualize DNA bands for viewing the DNA fragments. Following the sequencing, each DNA sequence chromatogram was examined using the bioinformatics tool Finch TV software to evaluate its quality; however, sequences with low quality were trimmed from both ends and moved the remaining good-quality sequences into a new file. Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) were used to produce taxonomic information about the source species. The neighbor-joining method implemented in the program MEGA software was used for construction of phylogenetic tree.

Assessment of biofilm formation by the isolated bacteria using microtiter plate assay

The microtiter plate method of O'Toole and Kolter (1998) [23] was applied with a few adjustments. Briefly, bacterial isolates were grown overnight in nutrient broth at 37 °C. The OD_{600} of the bacterial suspensions was adjusted to 0.5 McFarland standards (approximately 10^8 CFU/ml). A flat-bottomed polystyrene 96-well microtiter plate (Costar; Corning Incorporated., USA) was used to inoculate aliquots (200 µl). As plate sterility controls, the sterile nutrient broth was used and plates were covered and incubated at 37 °C for 24 h [24].

Planktonic cells in the fluid were then removed by inverting the plate and decanting the contents, followed by thoroughly rinsing three times with 200 µl of sterile deionised water (dH_2O) to remove any remaining unattached planktonic cells. The microtiter plates were dried by air at 37 °C, and adherent bacteria were stained with 200 µl of 1% (w/v) crystal violet solution (crystal violet; Merck, Germany) for 25 min [25,26]. The supernatant was discarded after the staining step and the wells were rinsed with repeated washing with sterile deionized water (dH_2O) for any excess stain removal. Any biofilm-integrated CV was solubilized by adding 250 µl of 30% glacial acetic acid. A multi-well plate reader (BioTek FLx800, UK) at the absorbance of light at 595 nm was used to assess the CV liberated from the attached material and control wells [27].

Determination of cell activity of biofilm of *Klebsiella pneumoniae* MBB9 using resazurin

The metabolic activity of adherent cells was determined using resazurin (7-hydroxy-3H-phenoxazine-3-on-10-oxide). The microtiter plate method of O'Toole and Kolter (1998) was applied with a few adjustments. Briefly, *Klebsiella pneumoniae* MBB9 was grown overnight in nutrient broth at 37 °C. The optical density of the bacterial suspensions at 600 nm was adjusted to 0.5, equivalent to 10^8 $CFUml^{-1}$ [27]. Aliquots (200 µl) were then inoculated into wells of a flat-bottomed polystyrene 96-well microtiter plate (Costar; Corning Incorporated., USA). Plates were covered and incubated at 37 °C for 24h. Following the incubation, cells in planktonic forms in the fluid were discharged by inverting the plate and thoroughly rinsed three times with 200 µl of sterile deionized water (dH_2O) to remove unattached bacteria. The wells were then loaded with 180 µl of sterile nutrient broth and 20 µl of resazurin solution (Invitrogen™). The plates were immediately covered with aluminium foil and incubated at 37 °C in the dark for 4 hours according to the manufacturer's instructions. Using the microplate reader (BioTek FLx800, UK), fluorescence signals (λexcitation: 540 nm and λemission: 580 nm) were measured. All experiments performed in triplicates and the values of fluorescence were corrected by subtracting the readings from the control.

Effect of glucose, peptone and yeast extract on biofilm formation by *Klebsiella pneumoniae* MBB9

Various levels of glucose, peptone and yeast extract were individually changed to assess the impact of carbon and nitrogen sources on biofilm formation by *K. pneumoniae* MBB9 in microplates using crystal violet and resazurin assays.

Effect of environmental factors on biofilm formation by *Klebsiella pneumoniae* MBB9

Effect of pH on biofilm formation: Nutrient broth medium adjusted to various pH values 4.5, 5.5, 6.5, 7.5, 8.5 and 10.5 was used to test the impact of pH on biofilm formation by *K. pneumoniae* MBB9 in microplates using crystal violet and resazurin assays.

Effect of temperature on biofilm formation: The impact of temperature on biofilm formation was assessed through incubating the inoculated 96-well plates under static conditions at 25, 37, 40 and 50 °C for 24 h using crystal violet and resazurin assays.

Effect of anaerobic conditions on biofilm formation: To evaluate the effect of anaerobic conditions on biofilm formation by *K. pneumoniae* MBB9, microtiter plate cultures were incubated anaerobically at 37 °C under anaerobic conditions for 24 h using crystal violet and resazurin assays.

Effect of osmotic stress on biofilm formation: Nutrient broth medium adjusted to various NaCl values was used to test the impact of pH on biofilm formation by *K. pneumoniae* MBB9 in microplates using crystal violet and resazurin assays.

Effect of different growth media on biofilm formation: Nutrient Broth (NB), Lysogeny Broth (LB) and Tryptic Soy Broth (TSB) were used to assess the effect of growth media on biofilm formation by *K. pneumoniae* MBB9 using crystal violet and resazurin assays.

Assessment of biofilms formed by *K. pneumoniae* MBB9 on glass surfaces

Biofilm formation on glass coupons: The method of Adetunji and Isola (2011) [28] was used with a few alterations to investigate the impact of substratum type on biofilms formation by *Klebsiella pneumoniae* MBB9. Briefly, an overnight bacterial culture was grown under aerobic conditions to the mid-log phase in nutrient broth at 37 °C with shaking. The OD_{600} of the bacterial suspensions was then adjusted to 0.5 McFarland standards (approximately 10^8 CFU/ml) in a fresh nutrient broth medium. The glass slide coupons (2.5 cm × 2 cm) were soaked in detergent, washed with sterile deionized water (dH_2O) and dried by air before being autoclaved at 121 °C for 15 min. Every plastic beaker holding sterilized glass coupon immersed in 9,000 µl sterilized nutrient broth was then inoculated with 1,000 µl of the bacterial suspension [29]. As sterility control, the sterile nutrient broth was used, and then containers were incubated under static (without agitation) and shaking conditions with a low shear fluid for 12, 24, and 48 hours.

Quantification of biofilms: At the end of each incubation period, a set of glass chips were aseptically removed from the inoculated broth culture and rinsed three times with sterile deionized water (dH_2O) to remove unattached bacterial cells. Coupons were then put into a test-tube containing 40 ml of sterile deionized water (dH_2O) and sonicated at 20 Hz for 3 minutes. This mixture was homogenized by vortexing in order to disperse the sessile cells from the chips' surfaces. Quantification of biofilm cells was determined by direct count pour plate; suspensions were serially diluted in sterile saline 0.85% w/v of normal saline, and the plates of nutrient agar were inoculated with an aliquot (100 µl) of each dilution and incubated at 37 °C for 24-48h. The number of cells was then expressed as CFU per cm^2 [30-32].

Fluorescent staining and microscopical examination of live/dead cells: A Live/Dead BacLight Bacterial Viability Kit (Eugene, Oregon, USA) was used according to the manufacturer's instructions to assess the biofilm cells on the glass slide coupons. This set consists of a combination of SYTO® 9; the green fluorescent nucleic acid stain and propidium iodide; the red-fluorescent nucleic acid stain that can mark both live and dead bacteria [33,34]. In brief, 20 µl of fluorescent stain was prepared as a working solution by incorporating 2 µl of SYTO®9 stain with 498 µl of sterile deionized water (dH_2O). The glass slide coupons were flooded with 200 µl of the working solution, covered with aluminum foil and incubated at room temperature for 15 minutes in the dark according to the instructions of the manufacturer. Fluorescence microscopy was used to examine the glass chips and to detect the fluorescence from SYTO®9 using a filter with an excitation wavelength of 485 nm and an emission filter of 498 nm under 100X oil immersion fluorescence objective. Fluorescence microscopy images were analyzed using ImageJ software.

Statistical analysis

The results were used to calculate the mean and Standard Deviations (SD). In the analysis of data, one-way ANOVA was used. Statistically significant p values < 0.05 were considered. The error bars represent the default deviation.

Results

As described in my previous research, 22 different bacterial strains were isolated and identified from biofilms formed on stones recovered from the Porter Brook, Sheffield. Of the 22 isolates, ten gram-negative potential pathogens were selected and screened for biofilm production. The modified microtiter-plate test, as a quantitative assay, showed that all tested strains produced biofilms as the mass of the retained crystal violet stain on the test plate indicated the biofilms presence. *Klebsiella pneumoniae* MBB9 were among these isolates that showed the highest biofilm production in the CV microtiter plate assay [35].

Effect of glucose, peptone and yeast extract

As shown in **Figure 1**, the maximum amount of biofilm was at 0.25 g L^{-1} glucose and then the number of biofilm significantly (p <0.05) diminished with increasing the levels in the medium to 0.5 g L^{-1} and remained relatively consistent for up to 1 g L^{-1} glucose. The biofilm mass significantly (p <0.05) increased with increasing the concentrations of peptone and yeast extract (**Figure 2 and Figure 3**).

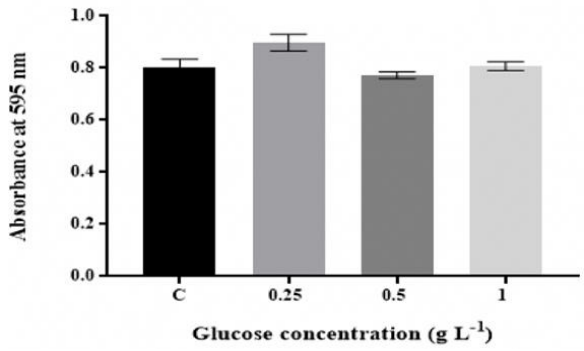

Figure 1: Effect of D-glucose concentration on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Cultures were grown in the basic medium without glucose (0.25 g L^{-1} peptone, 0.125 g L^{-1} yeast extract and phosphate buffer (0.188 g L^{-1} KH_2PO_4 and 0.26 g L^{-1} Na_2HPO_4) and in three media with different glucose concentrations 0.25, 0.5 and 1 g L^{-1} and were incubated for 24 h under static conditions. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

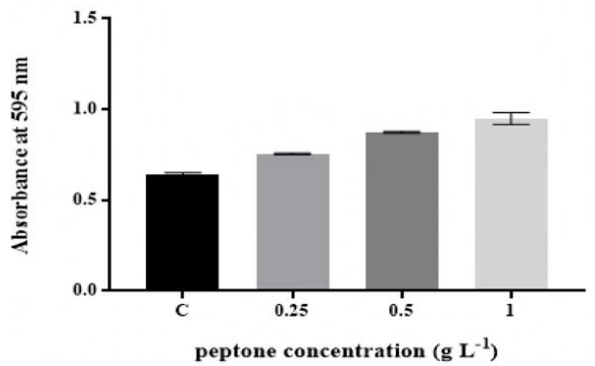

Figure 2: Effect of changing peptone concentration on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Cultures were grown in the basic medium without peptone (0.55 g L^{-1} glucose, 0.125 g L^{-1} yeast extract and phosphate buffer (0.188 g L^{-1} KH_2PO_4 and 0.26 g L^{-1} Na_2HPO_4) and in three media with different peptone concentrations 0.25, 0.5 and 1 g L^{-1} and were incubated for 24 h under static conditions. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

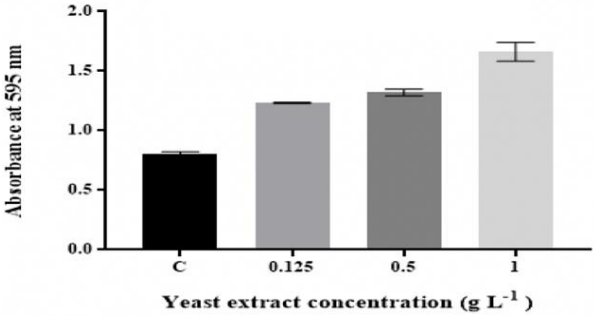

Figure 3: Effect of changing yeast extract concentration on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Cultures were grown in the basic medium without yeast extract (0.55 g L^{-1} glucose, 0.25 g L^{-1} peptone and phosphate buffer (0.188 g L^{-1} KH_2PO_4 and 0.26 g L^{-1} Na_2HPO_4) and in three media with different yeast extract concentrations 0.125, 0.5 and 1 g L^{-1} and were incubated for 24 h under static conditions. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Effect of pH

The findings showed that *K. pneumoniae* MBB9 were capable to form biofilm under acidic pH conditions and the quantity of biofilm reduced significantly (p <0.05) at higher pH values (**Figure 4**).

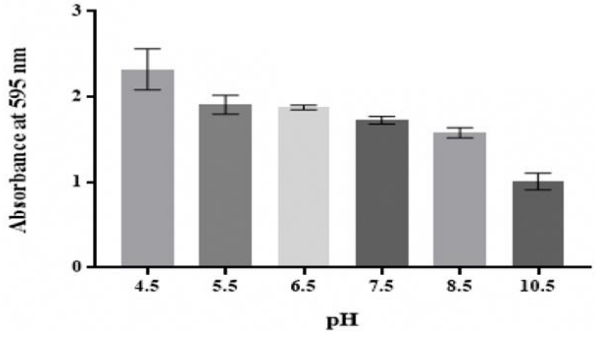

Figure 4: Effect of pH on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Polystyrene microtiter plates containing nutrient broth at indicated pH values were inoculated and incubated at 37 °C for 24 h. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Effect of temperature

The production of biofilm was maximal at 40 °C, followed by 37 °C, whereas the biofilm thickness was significantly (p <0.05) reduced at 25 °C and 50 °C (**Figure 5**).

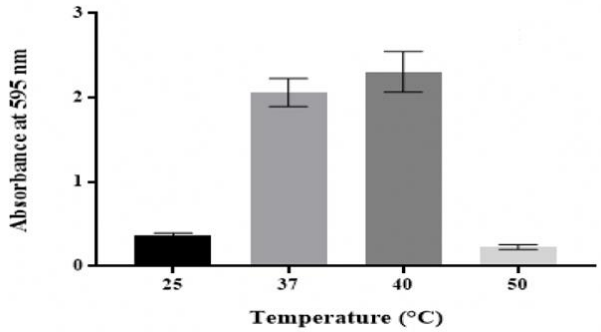

Figure 5: Effect of temperature on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Biofilm was developed in nutrient broth medium at different temperatures: 25, 37, 40 and 50 °C in 96- well plates under static conditions. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Effect of anaerobic conditions

From the results, *K. pneumoniae* MBB9 have an ability to form biofilm under anaerobic conditions, albeit less than under aerobic conditions (**Figure 6**).

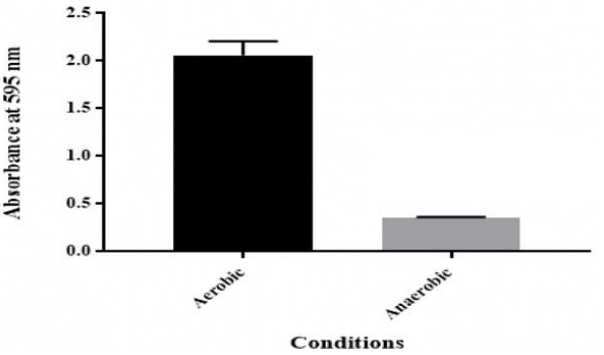

Figure 6: Effect of anaerobic conditions on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Microtiter plates were incubated at 37 °C under aerobic and anaerobic conditions for 24 h. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Effect of osmotic stress

The findings showed that the number of biofilm by *K. pneumoniae* MBB9 decreased significantly (p <0.05) with increasing levels of NaCl, with maximum biofilm formation being seen in the unadjusted nutrient broth (C: 0.6% NaCl) (**Figure 7**).

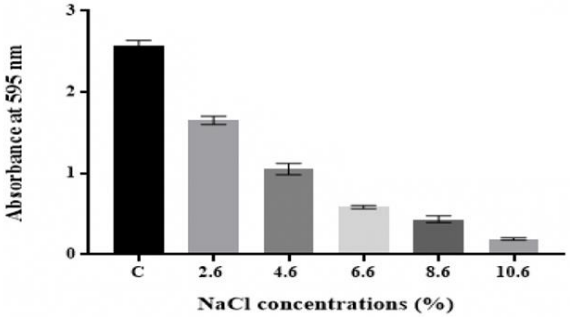

Figure 7: Effect of osmotic stress on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Biofilm was developed in nutrient broth medium (C: 0.6% NaCl) and in medium amended to the indicated NaCl concentrations by incubation at 37 °C for 24 h. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Effect of different growth media

From the results, the quantity of biofilm formed in NB was considerably (p <0.05) higher compared to other media, approaching a maximum in NB followed by LB and subsequently by TSB (**Figure 8**).

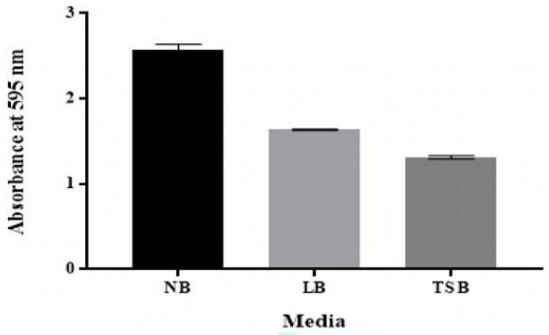

Figure 8: Effect of different growth media on *in vitro* biofilm formation by *K. pneumoniae* MBB9. Inoculated Nutrient Broth (NB), Lysogeny Broth (LB) and Tryptic Soy Broth (TSB) media were incubated at 37 °C for 24 h. Data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

Assessment of biofilms formed by *K. pneumoniae* MBB9 on glass surfaces

The findings showed that *K. pneumoniae* MBB9 were capable to form biofilm on the glass slide coupons surfaces. The number of biofilm by *K. pneumoniae* MBB9 after 12 hours of incubation under static conditions was statistically (p <0.05) more compared with shaking conditions with values of 6.60×10^4 and 5.50×10^4 CFU per cm^2, respectively (**Table 1**). In both conditions, the quantity of biofilms formed tended to decrease significantly (p <0.05) over time **(Table 1)**.

	Bacterial count CFU per cm^2		
	12 h	**24 h**	**48 h**
Static	6.60×10^4	1.96×10^4	1.20×10^4
Shaking	5.50×10^4	3.00×10^4	2.45×10^4

*Glass slide coupons measuring (2.5 cm × 2 cm) submerged in sterilized nutrient broth in each plastic beaker.

Table 1: Effect of substratum surfaces and time on bacterial adhesion at 37 °C.

Bacterial viability results showed that under both conditions bacteria in a biofilm were higher after 12 hours compared to those with damaged membranes. Under shaking conditions, cells with intact membranes were a few higher with the same order of magnitude; however, dead cell numbers increased with the increase of incubation time from 12 to 24 to 48 hours (**Figures 9-11**).

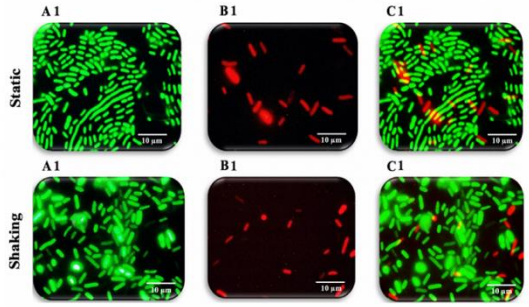

Figure 9: Fluorescence microscopy images of *K. pneumoniae* MBB9 biofilms after 12 hours of incubation. Adherent cells were stained with LIVE/DEAD cell viability fluorophores; SYTO9 and propidium iodide on glass slide coupons under both conditions and showed differential staining patterns of live and dead cells. (A1) Live bacteria; (B1) Dead bacteria; (C1) Live/Dead bacteria. Scale bar of all microscopic images:10 µm and images were analyzed using ImageJ software.

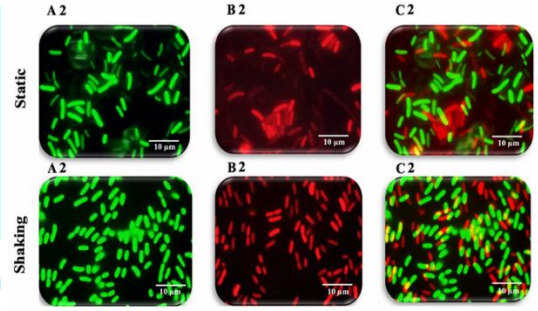

Figure 10: Fluorescence microscopy images of *K. pneumoniae* MBB9 biofilms after 24 hours of incubation. Adherent cells were stained with LIVE/DEAD cell viability fluorophores; SYTO9 and propidium iodide on glass slide coupons under both conditions and showed differential staining patterns of live and dead cells. (A2) Live bacteria; (B2) Dead bacteria; (C2) Live/Dead bacteria. Scale bar of all microscopic images:10 µm and images were analyzed using ImageJ software.

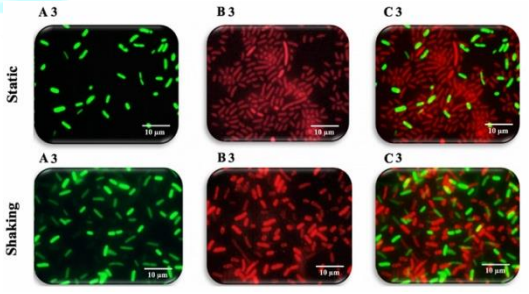

Figure 11: Fluorescence microscopy images of *K. pneumoniae* MBB9 biofilms after 48 hours of incubation. Adherent cells were stained with LIVE/DEAD cell viability fluorophores; SYTO9 and propidium iodide on glass slide coupons under both conditions and showed differential staining patterns of live and dead cells. (A3) Live bacteria; (B3) Dead bacteria; (C3) Live/Dead bacteria. Scale bar of all microscopic images: 10 µm and images were analyzed using ImageJ software.

Discussion

The capacity of biofilm formation of 10 gram-negative potentially pathogenic bacteria showed that *K. pneumoniae* MBB9 had the greatest biofilm capability to form biofilm in the microtiter plate assay [35]. Therefore, *K. pneumoniae* MBB9 were chosen to assess the impact of different factors on biofilm formation using the crystal violet and resazurin assays.

Biofilm formation is known to be powerfully affected by the nutritional environment and the rate and amount of biofilm accumulation increased with increasing carbon levels [19,36]. Biofilm in wells with 0.25 g L^{-1} glucose showed high growth in comparison to the biofilm formed in the remaining media, implying that 0.25 g L^{-1} glucose can be beneficial for *K. pneumoniae* MBB9 biofilm (Figure 1). Ninety-three percent of *Listeria monocytogenes* isolates, as previously demonstrated by Pan *et al*. (2010) [37], formed higher biofilm in the presence of glucose than glucose-free medium. Bühler *et al*. (1998) [17] have shown that glucose can enhance the quantity of biofilm formed by *E. coli* and *Burkholderia cepacia.* For the same cultivation conditions, the amount of biofilm significantly decreased with increasing glucose concentrations in the medium to 0.5 g L^{-1}, suggesting that glucose might be useful for biofilm formation up to a certain limit.

Hence, glucose metabolism might be a key factor in the process of biofilm formation by *K. pneumoniae* MBB9, but increased concentrations of glucose might lead cells to cell detachment from the biofilm. Similarly, the density of biofilm by *P. putida* improved when increasing glucose levels up to a limit (0.5 g L^{-1}), whereas high glucose level (1 g L^{-1}) was found to decrease the rate of biofilm accumulation [19]. This was interpreted as suggesting that biofilm formation can be regulated by the processes of detachment in response to nutrient availability. The production of biofilm by *E. coli* was found to raise linearly with rising glucose levels up to 10 mmol L^{-1}, implying that the total yield of biofilm could be controlled by the medium substrate levels [17]. Jackson *et al.* (2002) [38] have shown that the addition of glucose to different media might have an effect on biofilm formation in different species of *Enterobacteriaceae* family, such as *K. pneumoniae*, *Citrobacter freundii*, *E. coli* and *Salmonella enterica* due to the catabolite repression system, implying that catabolite repression of the development of biofilm is a prevalent theme in this family of bacteria. Carbon Catabolite Repression (CCR) which is usually regulated by the second messenger cyclic AMP (cAMP) controls glucose uptake, and thus the presence of glucose in the growth medium has been proved to repress the production of cAMP in several bacteria [39].

In classical catabolite repression, glucose transport may lead to dephosphorylation of enzyme IIAGlc of the bacterial phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system that restricts this protein from stimulating membrane-bound adenylate cyclase (Cya); thus, leads to a reduction in the concentration of the intracellular cAMP [38]. The decline in *K. pneumoniae* MBB9 biofilm at 0.5 g L^{-1} glucose level might due to the active detachment that requires enzymatic cleavage of matrix polymers or phenotypic adaptation of the attached cells which is controlled by various physiological mechanisms, such as quorum sensing and catabolic repression signals in response to the availability of nutrients [19]. After dispersion, microbial cells begin to accustom and form biofilm again, and the biofilm mass at 1 g L^{-1} glucose was comparable to that produced in the glucose-free medium, suggesting that high glucose level has no impact on *K. pneumoniae* MBB9 biofilm.

There is some information about the influence of glucose concentrations on the production of biofilm, but little is known about the effect of changing nitrogen levels, particularly peptone and yeast extract, in the same process. Meat peptone, a protein from animal sources that broke down into amino acids and peptides, is an important nitrogen source because its nitrogen content exceeds 13% of its total content. Yeast extract is a multiple, ill-defined blend of natural sources and is known to be rich in nitrogen, amino acids, vitamins, and carbon needed for the growth of microbes as its nitrogen content exceed 10% of the total content [9].

The quantity of *K. pneumoniae* MBB9 biofilm was considerably enhanced with increased concentrations of peptone and yeast extract, indicating that higher levels of peptone and yeast extract might be useful for the formation of *K. pneumoniae* MBB9 in microtiter plates (Figure 2 and 3). Peptone has enhanced, as previously demonstrated, the development of biofilm by *E. coli*, *Lactobacillus rhamnosus* and *Mycobacterium avium* [24,40,41]. In media with a yeast extract, biofilm thickness was higher than that in media with different peptone levels. This might be due to the availability of nutrients as peptone consists only of protein hydrolysis products, while yeast extract includes different kinds of nutrients, such as trace metals and vitamins [42].

Klebsiella pneumoniae MBB9 biofilm mass tended to decline considerably with rising pH (Figure 4). The highest biofilm formation was observed at pH 4.5, indicating that the possibility of this microbe to form biofilm under acidic conditions. It has been found that biofilms can form under pH stress-induced conditions. Biofilm formation has been observed to develop under pH stress-induced conditions [43].

The formation of biofilm can help bacteria to withstand highly acidic environments as their gel-like compositions can lessen the diffusion of ions and support the development of a pH gradient within the extracellular matrix [44]. The growth at varying pH values might imply that biofilm can also be affected by environmental conditions at the sites of colonization. Nicolau *et al*. (2013) [44] have revealed that *K. pneumoniae* MBB9 isolates were able to form biofilm on various materials, such as glass, polyester strip, and polystyrene under acidic environments pH 4.5 and neutral pH 7. There was no significant difference between values at pH 5.5, 6.5, 7.5 and 8.5, whereas the biofilm mass by *K. pneumoniae* MBB9 under alkaline states (pH 10.5) was considerably decreased (approximately 60% mass reduction).

A previous study of 23 *Staphylococcus epidermidis* clinical isolates showed that for most of the tested strains, the biofilm mass decreased under highly alkaline conditions than those at pH 7 in polystyrene microtiter plates [45]. Similarly, Zmantar *et al*. (2010) [43] have also demonstrated that alkaline conditions have an inhibitory impact on *S. aureus* biofilm on 96-well culture plates. This was interpreted as suggesting that the biofilm production might depend on the pH content of the medium due to the effect on initial bacterial attachment. In the same vein, Nostro *et al*. (2012) [46] have also shown that alkaline solution might hinder bacterial colonization and hence could be used to control the environmental risks associated with biofilm. The notable inhibitory action of alkaline environments on the biofilm formation observed here could suggest that bacterial cells failed to adhere to the microtiter plate wells surface. It could also be due to the slower growth under highly alkaline conditions.

The media characteristics, such as pH may affect the adherence by *K. pneumoniae* MBB9 as the physicochemical characteristics of the surface of a bacterial cell can be modified by pH, and therefore the microbial adhesion to the substrate [47]. In addition, alkaline pH could also impair the normal development of biofilm that held at the microcolony stage [46]. Also, the reduction in biofilm at alkaline pH might be due to the weak electrostatic repulsion between the cells and the substrate as the amount of adhering cells was found to be enhanced when the pH of the medium was near to the isoelectric point of the substrate surface, thus the electrostatic properties of polystyrene

surfaces may be influenced by pH value that might lead to a weaker interaction with surfaces and thereby compromise the bacterial adhesion [43].

Temperature is one of the important factors that affect bacterial growth and biofilm development, and the majority of pathogens are mesophiles that grow well at optimum temperatures between 25 °C and 40 °C [18]. Results showed that *K. pneumoniae* MBB9 biofilm density was highest at 40 °C (Figure 5). Similarly, *K. pneumoniae* MBB9 were shown to form more biofilm on glass and polystyrene surfaces at 40 $^{\circ C}$ than at 35 $^{\circ C}$ [44]. It has also been reported that *Pseudomonas putida* tolerate temperatures of 40 °C compared with 30 °C by enhanced the biofilm production, suggesting a complex multilevel regulatory process in which many different genes are involved [48]. Maximum production for biofilms at 40 °C might suggest that 40 °C is the ideal temperature for biofilm development (Figure 5). Bonaventura *et al*. (2007) [49] have shown that *Stenotrophomonas maltophilia* have the capability of forming biofilm at room temperature, 32 °C and 37 °C. This was interpreted as suggesting that slower bacterial growth might lead to the lower absorbance obtained at room temperature. Similarly, the decrease in *K. pneumoniae* MBB9 biofilm at 32 °C compared to 37 °C was also interpreted as being as a result of the slowly growing bacteria at 32 °C [50].

The reduction in biofilm by *K. pneumoniae* MBB9 at 25 °C might be due to the slower growth of bacteria. More extreme temperatures (50 °C) might negatively affect the formation of biofilm by inhibiting growth. A study of the temperature on the polymeric and mechanical properties of *Staphylococcus epidermidis* biofilm has shown that morphology, cell viability, and mechanical characteristics of bacterial biofilm can be affected by higher temperatures that can generate a remarkable decrease in the yield stress and the biofilm small strain elastic modulus, suggesting that high temperature can reduce the integrity of the biofilm as increasing temperature close to the bacterium optimum growth temperature has been found to improve the bacterial biofilm elasticity [8,51].

The biofilm formation under aerobic conditions has been well investigated, but little is known about biofilm formation under anaerobic environments. Under anaerobic conditions, *Klebsiella pneumoniae* MBB9 were able to form biofilm. However, biofilm production was lower (83% less) compared to that achieved under aerobic conditions. Similarly, Worlitzsch *et al.* (2002) [52] noted that *P. aeruginosa* produced further biofilm when grown anaerobically. *Pseudomonas aeruginosa* can enter into the adherent mucus and grow in hypoxic/anaerobic slime, and the potential of these bacteria to proliferate in this area would generate fully hypoxic (anaerobic) environments as the enhanced formation of alginate may serve as a stress response to hypoxia that is part of the process that forms biofilm-like microcolonies. Similarly, some *E. coli* clinical strains were able to yield biofilms under anaerobic conditions, suggesting that the ability of biofilm formation is strain-dependent [10].

The biofilm formation ability under anaerobic conditions seen here may suggest that the facultative anaerobe *K. pneumoniae* MBB9 can produce biofilm in anoxic environments using available alternative electron acceptors. Moreover, facultative anaerobes' growth may play a role in the formation of biofilm in the environment by anaerobes. For example, in dental plaque biofilm formation, many strict anaerobes, such as *Bacteroides forsythus* and *Fusobacterium nucleatum* need the former attachment of an aerobe or a facultative microbe to start the biofilm formation [36].

Sodium chloride has been tested as an osmotic agent to study its effect on microbial biofilm formation and development [2,53]. Results showed that biofilm formed by *K. pneumoniae* MBB9 decreased with increasing NaCl concentrations in the medium from 0.6% to 10.6% with the highest biofilm occurring at 0.6% (Figure 7). The biofilm decline might suggest that higher concentrations of NaCl negatively affect the ability of *K. pneumoniae* MBB9 to produce biofilm through an osmotic impact or might due to weak growth of cells under high levels of NaCl. These findings are in accordance with an earlier study revealing that raises in NaCl levels from 0.5 to 10.5% suppress the formation of biofilm by *Salmonella* in 96-well polystyrene microtiter plates [2]. Similarly, a study by Rinaudi *et al*. (2006) [54] has shown that high osmotic potential limits the biofilm establishment produced by *Sinorhizobium meliloti*, suggesting that NaCl negatively affects the formation of biofilm by an osmotic force. Consequently, the inhibitory influence of NaCl on biofilm formed by *K. pneumoniae* MBB9 could be a result of an osmotic impact or could be due to a particular ion effect [54].

The growth media structure has been reported to affect the ability of bacteria to form biofilm *in vitro* [55]. LB and TSB broths were both supported the biofilm development of *K. pneumoniae* MBB9; however, higher biofilm was yielded significantly in NB (Figure 8). This indicates that nutrient provision might influence the ability of biofilm formation by *K. pneumoniae* MBB9. However, compared to the biofilm formed in the TSB medium, the quantity of biofilm was higher in the LB medium. The reduction of biofilm could presumably be a result of the glucose that present in the TSB medium (2.5 g L^{-1} (0.25%)) compared to the LB medium that lacks glucose.

The concentration of 0.5 g L^{-1} glucose in this study was found to reduce the biofilm produced by *K. pneumoniae* MBB9 on polystyrene plates (Figure 1). Jackson *et al.* (2002) [38] have noted that glucose addition to different culture media inhibited the biofilm formation in several species of *Enterobacteriaceae* family, among them *K. pneumoniae*, due to catabolite repression of the biofilm development. Besides, the medium that facilitates the high production of microbial biofilm might vary for each microbe [56]. Stepanovic *et al*. (2004) [57] has been shown that *Salmonella* spp. formed more further biofilm in nutrient-poor media, while *L. monocytogenes* were observed to produce more biofilm in nutrient-rich media, suggesting that the structure of medium nutrients influenced the biofilm mass in various ways. In the same vein, a study by Hood and Zottola (1997) [58] on five different bacterial isolates has shown that the medium allowing a high level of adherent cells can be varied for each microbe. So from the results, it can be concluded that *K. pneumoniae* MBB9 biofilm can be cultivated in LB and TSB broths, but NB medium might be more suitable since more biofilm was yielded in such medium.

Crystal violet provides a good biofilm mass measurement but does not estimate the biofilm viability as it quantifies the matrix of biofilm, including viable and dead bacterial cells [59,60]. Accordingly, the resazurin assay is used to determine the active biofilm cells as it only quantifies the viable bacterial cells within the formed biofilm [60]. An increase in metabolic activity leads to increase the resazurin conversion (oxidized) to fluorescent resorufin (reduced) since a higher rate of resazurin uptake by bacterial cells could cause a higher fluorescence reading [61]. Although the resazurin assay showed the activity of *K. pneumoniae* MBB9 biofilm cells, some of the obtained crystal violet assay results were not in accordance with those from the resazurin assay (Figures S1-S8 in supplementary material). The culture medium has presumably influenced the values of fluorescence and thus resazurin assay outcome [62,63]. However, decreased pH can lead to a reduction in the fluorescence intensity of the resazurin [64]. From the results, highly acidic conditions had no effect on the viable bacterial cell numbers in biofilm; however, the trend was comparable to that obtained using the crystal violet assay (Figure S4 in supplementary material).

In vitro, biofilms formation by *K. pneumoniae* MBB9 was evaluated using the glass coupons incubated under both static and shaking

conditions at 37 °C for 12, 24, and 48 hours. The number of cells attached to surfaces or associated with biofilms was quantified using the plate count technique. Microbial adhesion to surfaces is the first step in colonization, invasion, and biofilm formation [65,66]. Most bacteria have the ability to form biofilms on abiotic and biotic surfaces [67]. The attachment of biofilms can occur readily on surfaces that are rough, hydrophobic, non-polar and coated by surface conditioning films [66]. The presence of extracellular appendages, including flagella, pili, fimbriae and exopolysaccharide, and the adhesion properties of surfaces, such as roughness, are the most important factors that can affect the ability of cells to adhere to the substratum [68]. Since the ability of microbes to form biofilms may differ depending on the substrate, the adherence by *K. pneumoniae* MBB9 to abiotic surfaces, such as hydrophilic glass was tested, and the bacterial adhesion was expressed as CFU per cm^2.

It is generally accepted that hydrophobic materials can provide a greater bacterial adherence [69,70]. It has been found that *Listeria monocytogenes* can adhere in higher numbers to materials that are more hydrophobic [71]. Karunasagar *et al*. (1996) [72] have been reported that the variation in the density of biofilms depends on the surface's properties. Results revealed that *K. pneumoniae* MBB9 possesses a high capacity to form biofilms on the surface of glass slide coupons. Similarly, it has been reported that *K. pneumoniae* species isolated from rivers and drinking water distribution systems can adhere to glass, plastic (polycarbonate, chlorinated polyvinyl chloride), and carbon steel [44,73,74]. Microbial load of biofilms by *K. pneumoniae* MBB9 after 12 hours of incubation under static conditions was statistically more compared with shaking conditions with values of 6.60×10^4 and 5.50×10^4 CFU per cm^2, respectively (Table 1).

In this study, 12 h incubation was found to be most efficient in biofilms formation by *K. pneumoniae* MBB9 on the glass surface under both conditions. The duration of the incubation period can considerably influence the production of biofilms as the amount of biofilms increases with the incubation period [11]. Although the density of biofilms was higher during the initial phase, the amount of biofilms formed tended to decrease significantly (p <0.05) for both conditions over time. Significant differences (p <0.05) were found between the biofilms formed by *K. pneumoniae* MBB9 under both conditions between the control and the different incubation periods and between each incubation time. It is possible that some biofilm-associated cells composing the outermost layers of the biofilm changed to the free-floating state, while others may have died as a consequence of nutrient depletion and exposure to accumulated toxic metabolic wastes generated by the biofilm after 24 hours of incubation. The incubation period can be defined as the contact time between the surface and the bacterial cells [75].

More biofilms were obtained during shaking conditions compared with static conditions for the 24 and 48 hours of incubation with values of 3.00×10^4, 2.45×10^4, 1.96×10^4 and 1.20×10^4 CFU per cm^2, respectively. Significant differences (p <0.05) were found between the biofilms formed by *K. pneumoniae* MBB9 under static and shaking conditions for 12, 24 and 48 hours. It is evident that shaking conditions appear to enhance the cell adhesion to the glass surface as a better degree of mixing can provide higher nutrient levels to *K. pneumoniae* MBB9 cells, increase the oxygen transfer rates, and thus promoted the bacterial growth within the biofilm. It is known that shear forces may have an impact on the structure, production of exopolysaccharide, mass transfer, and metabolic/genetic behaviors of biofilms [76]. Zhang *et al*. (2011) [77] have been stated that high hydrodynamic shear may lead to the detachment of *Pseudomonas aeruginosa* from the surface. The multiple effects of hydrodynamic and nutrients were probably enhanced the bacterial growth as there were no shear forces promoting cell detachment under these low shaking conditions. It has been stated that shear stress is a dominant factor that can influence the biofilm formation strongly when the fluid flows at a higher velocity over the biofilm surface [76,78]. Some materials, such as glass, stainless steel, and mica are hydrophilic and negatively charged, while the plastic surface is more suitable for bacterial attachment due to its hydrophobic nonpolar nature with little or no surface charge [69,71].

To obtain a more definite result, a Live/Dead BacLight Bacterial Viability Kit was used to monitor the viability of bacterial populations. This kit is widely used for the enumeration of bacteria [79,80]. It consists of two nucleic acid stains: SYTO9, which penetrates cell membranes more easily, and propidium iodide, which is a highly charged stain that cannot permeate cells but can penetrate damaged membranes [81]. Application of both dyes results in intact cell membranes stain fluorescent green, whereas dead cells, because of a compromised membrane, show intense red fluorescence [82]. Biofilms on each glass chips were stained and incubated at room temperature for 15 minutes before fluorescence microscopy examination. Images of the single focal plane or vertical section of the stained biofilms were captured using a fluorescence microscopy system.

As can be seen in Figure 9A1 and 9B1, the ratio between living and dead cells. Although the viable bacteria in a biofilm were higher compared to those with damaged membranes after 12 hours under both conditions, cells with intact membranes under shaking conditions were a little higher compared to their counterparts under static conditions with the same order of magnitude. The number of dead cells increased with the extension of incubation time from 12 to 24 to 48 hours as a result of the depletion of nutrients and exposure to metabolic byproducts (Figures 9B1 static, 9B1 shaking, 10B2 static, 10B2 shaking, 11B3 static and 11B3 shaking). It is concluded that *K. pneumoniae* MBB9 strains study are capable of adhering, colonizing, and forming biofilms on glass surfaces.

Conclusion

The effect of environmental conditions on bacterial behaviour has been extensively studied on planktonic cells, but more information about how these factors modulate biofilm formation is required. In the present investigation, some conclusions can be drawn:

1. The biofilm in 0.25 g L^{-1} of glucose exhibited greater growth in comparison to the biofilm formed in the remaining media.
2. The *K. pneumoniae* MBB9 biofilm profiles for the different peptone and yeast extract concentrations were similar where the biofilm increased with increasing the level of peptone and yeast extract and the highest absorbance values were obtained for peptone and yeast extract concentration of 1 g L^{-1}.
3. The peak of *K. pneumoniae* MBB9 biofilm was at pH 4.5 and a significant decrease in the absorbance values occurred with further pH increasing until (pH 10.5).
4. *Klebsiella pneumoniae* MBB9 biofilm increased up to 40 °C before decreasing with more extreme temperatures (50 °C).
5. The amount of biofilm was maximal in the control media and tended to decrease with increasing NaCl concentrations until (10.6%).
6. Under anaerobic condition, *K. pneumoniae* MBB9 biofilm was lower (83% less) than that obtained under aerobic conditions.
7. *Klebsiella pneumoniae* MBB9 have the capacity to form biofilms on the surface of glass slide coupons.
8. The amount of biofilms at 12 hours of incubation under static conditions was statistically higher compared with shaking conditions with values of 6.60×10^4 and 5.50×10^4 CFU per cm^2, respectively.
9. The number of biofilms formed under both static and shaking conditions tended to significantly decrease over time.

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Supplementary material

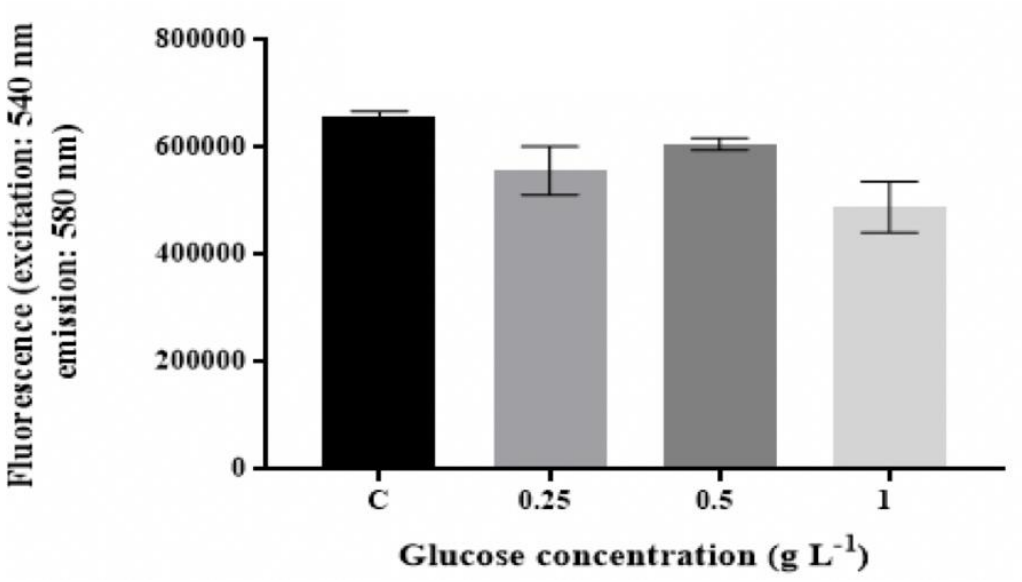

Figure S1: The metabolic activity of adherent cells of *K. pneumoniae* at different glucose concentrations. Biofilm was developed under static conditions. Cultures were grown as described in Section 2.5 and in the legend to Figure 1. The microtiter plates were then washed, filled with 180 µl of sterile fresh media and 20 µl of resazurin solution (Invitrogen™), covered in aluminium foil, incubated at 37°C in the dark for 4 h and the fluorescence signals (λexcitation: 540 nm and λemission: 580 nm) were measured using the microplate reader (BioTek FLx800, UK). Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

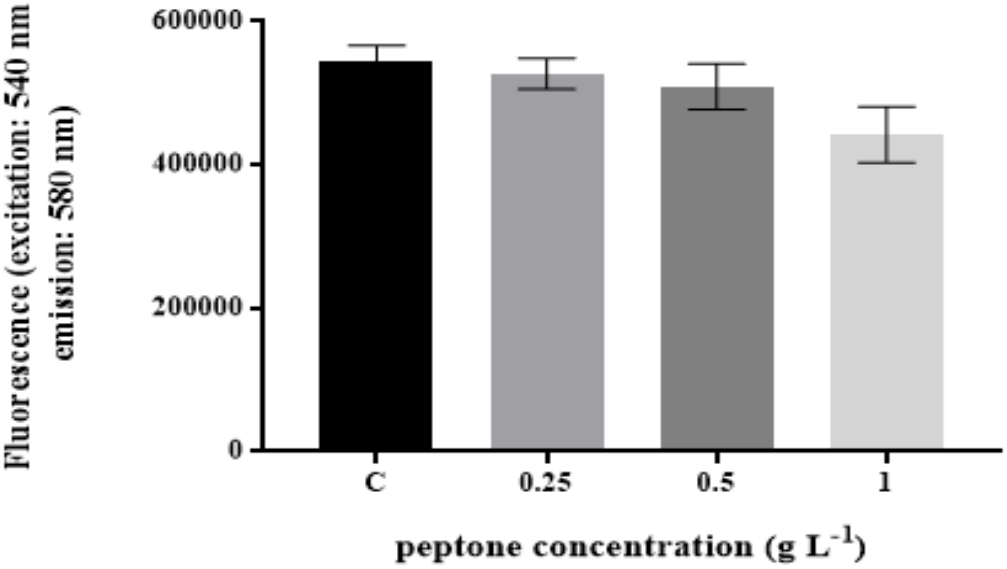

Figure S2: The metabolic activity of adherent cells of *K. pneumoniae* at different peptone concentrations. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figure S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

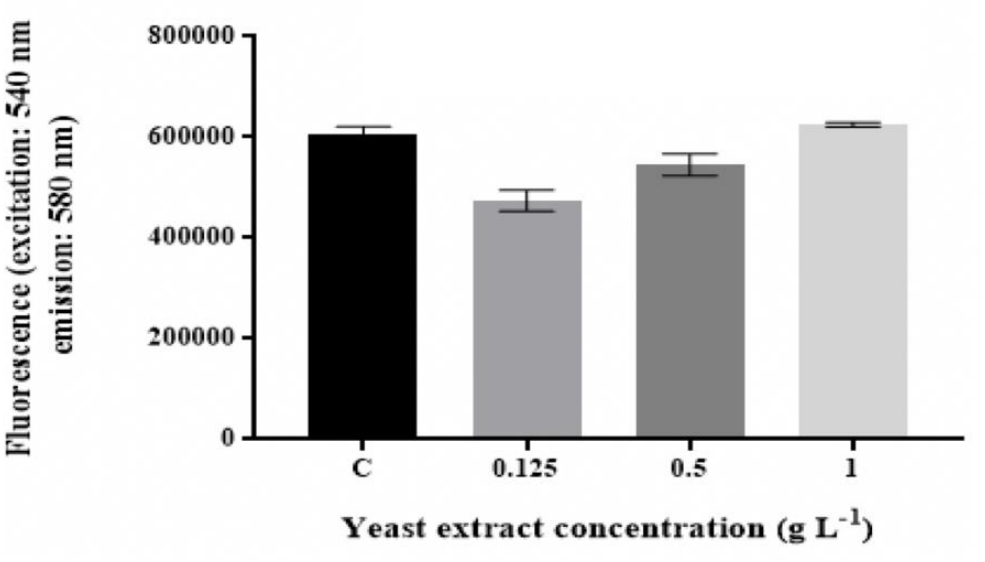

Figure S3: The metabolic activity of adherent cells of *K. pneumoniae* at different yeast extract concentrations. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figure S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

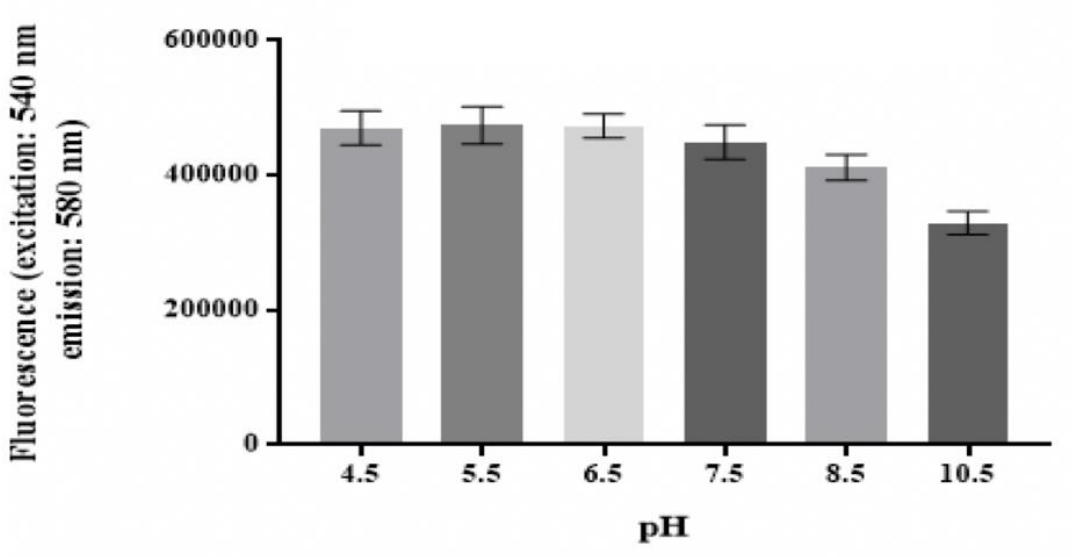

Figure S4: The metabolic activity of adherent cells of *K. pneumoniae* at different pH levels. Cultures were grown and analysed as described in Section 2.5 and in the legends to S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

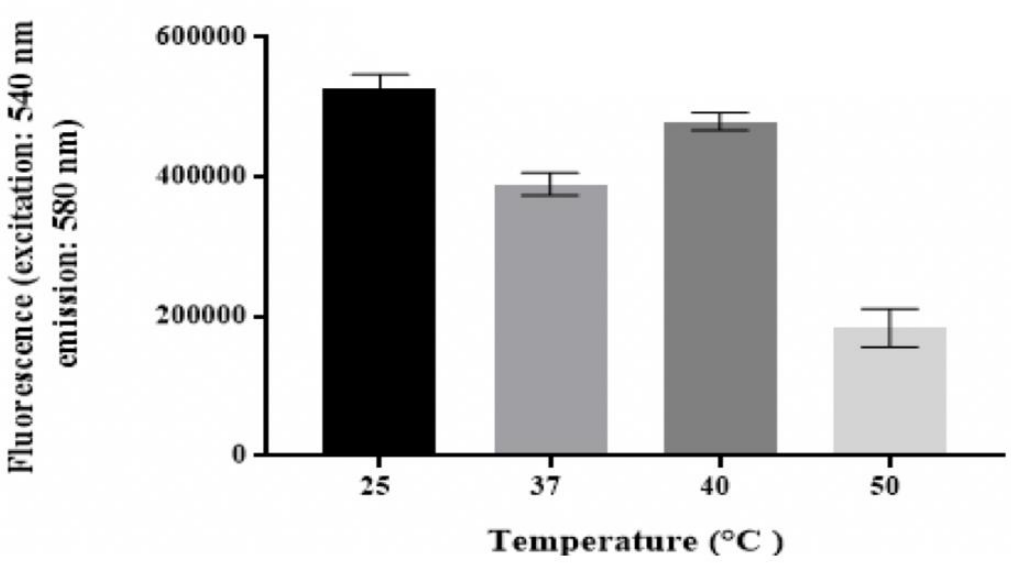

Figure S5: The metabolic activity of adherent cells of *K. pneumoniae* at different temperatures. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figure S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

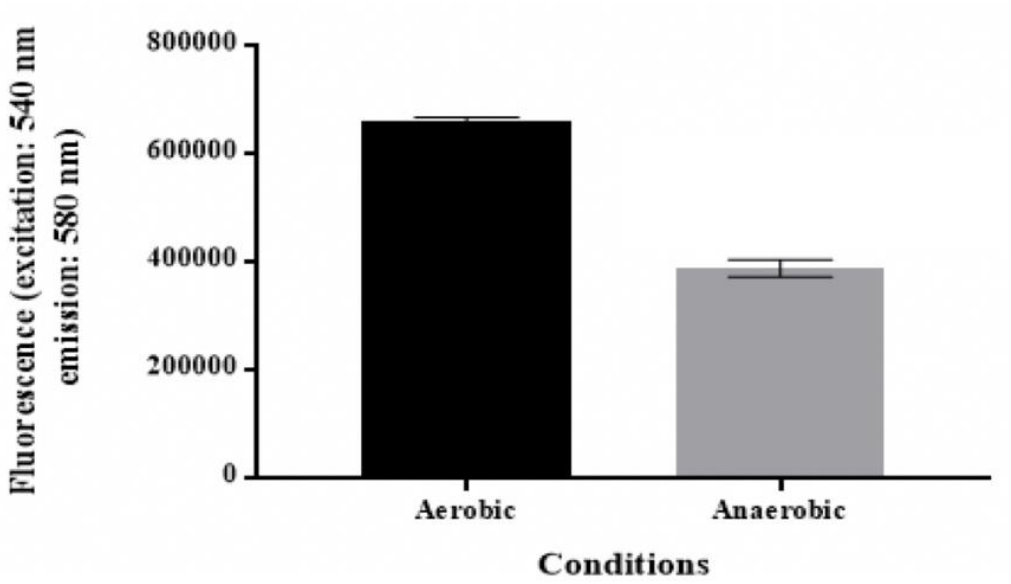

Figure S6: The metabolic activity of adherent cells of *K. pneumoniae* under aerobic and anaerobic conditions. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figure S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

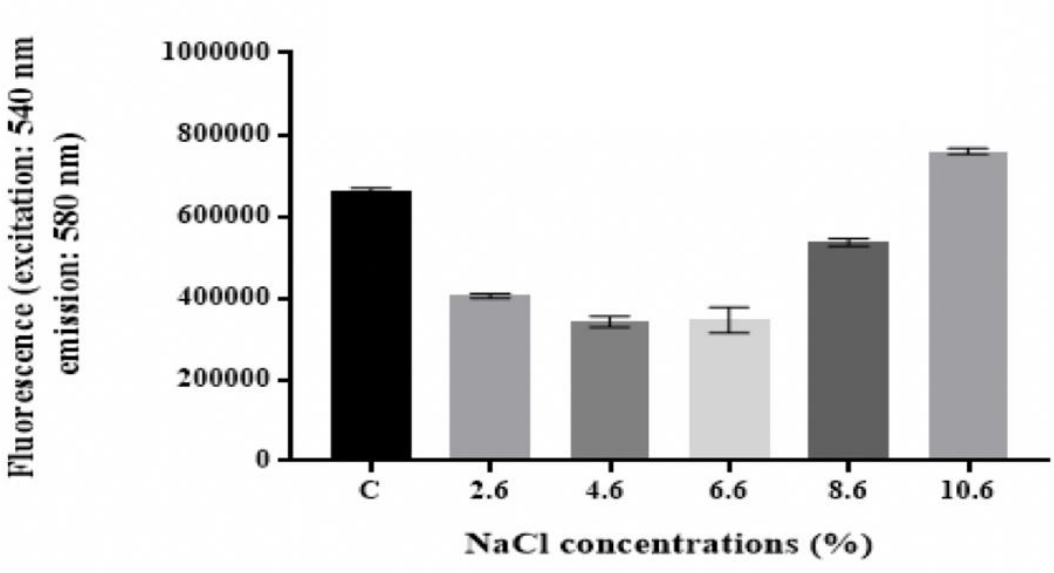

Figure S7: The metabolic activity of adherent cells of *K. pneumoniae* under different concentrations of NaCl. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figures S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.

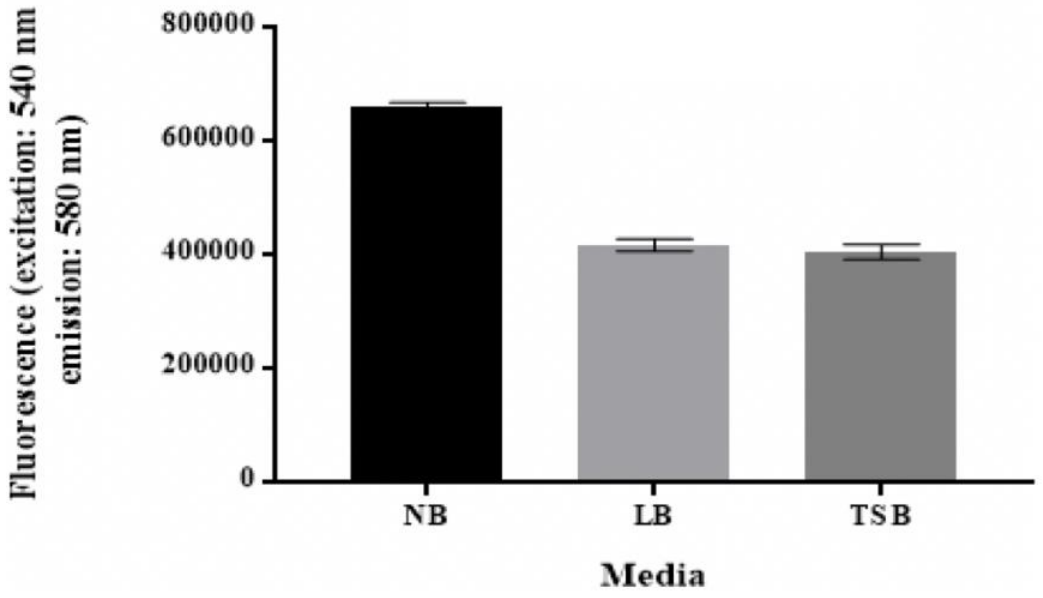

Figure S8: The metabolic activity of adherent cells of *K. pneumoniae* in different media. Cultures were grown and analysed as described in Section 2.5 and in the legends to Figures S2. Fluorescence intensity data shown are the mean values, error bars represent standard errors (n=3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test.