HETEROPOLYSACCHARIDE OF KGM-XANTHAN HYDROGELS FOR THE REMOVAL OF BACTERIA IN A WOUND MODEL

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ABSTRACT: This project focused on developing polysaccharides hydrogels from Konjac Glucomannan (KGM) and Xanthan gum in removing the bacterial load on the wound. This new therapy design is due to the traditional wound care methods using antibiotics and normal hydrogel, which cannot adequately treat biofilm infected woundsin the healing process. For the treatment of bacterial infected wound, bacterial removal need to be done physically and manually by debridement, which is very painful and require the use of general anesthesia. Therefore, to reduce the formation of slough and necrotic tissue by the formation of bacterial biofilm, there is a need for specific hydrogels that will be able to remove bacteria from the wounded site to prevent further infection and to enhance the healing process. This study focused on the design and optimization of polysaccharide based hydrogels for the wound treatment. Polysaccharide based hydrogels from Konjac Glucomannan (KGM) and Xanthan gum were developed at different ratios to optimize the physical and chemical properties of the hydrogel that relate to the effectiveness of bacterial removal. Film casting technique was used in the development of the hydrogel film. Konjac Glucomannan (KGM) and Xanthan gum were selected because of their natural polymer properties which are non–toxic, biocompatible and safe to be used. The hydrogels were tested in vitro for bacterial removal efficacy, using E.coli species colonies cultured on a gel inside a petri dish to represent the condition of bacteria infested wound bed. Hydrogel with 50 : 50 % composition blend of KGM-XG was found to be the most effective in the removal of E.coli colonies with $13x10^3$ CFU/mL at 95% water content. Morphological studies of hydrogels showed flat interfacial morphologies, except for the sample with 100% of Xanthan without KGM. The results present in this study suggested that the biological activities and adhesion of the hydrogels were not controlled only by the chemical structure but also by the composition of the compound and the amount of water present in the hydrogels.

KEY WORDS: *Hydrogels, Glucomannan, Xanthan, Bacteria Removal, Heteropolysaccharides And Biofilm*

1. INTRODUCTION

Bacterial biofilms, prominently *Pseudomonas aeruginosa* are being recognized as a major detrimental to chronic wound healing, especially in diabetic foot ulcers (DFU) [1] The gram-negative bacteria, *P. aeruginosa* can cause clinical problems and severe tissue damage in diabetic foot ulcers resulting from high resistance to antimicrobial agents [1]. The appearance of bacterial resistance to antibiotic and antimicrobial agents brings to the discovery

of a new therapeutic design of new material that able to remove bacteria physically from the wounded site.

In recent years, hydrogels have gained attention for its benefits in wound dressing. Generally, hydrogels are water-swollen, three-dimensional cross-linked polymer networks that can respond to the fluctuations of environmental stimuli [2]. Hydrogels contain 90% of water and help to regulate fluid exchange from the wound surface. The high water content of hydrogels will create a moist healing environment that promotes granulation, epitheliazation and autolytic debridement. Because of diabetic wound is complex and unpredictable, the therapy design should be accordingly to the conditions of the wound, where either biodegradable and non-biodegradable hydrogel can be used for the purpose [3]. In this study, the experiments will be focused on the design and optimization conditions for the effectiveness of polysaccharides hydrogels in removing bacterial load on agar, which represents an infected wound bed.

In particular, physicochemical properties of the polymer's surface are important factors such as hydrophobicity, hydrophilicity and roughness in defining the effect on the adhesion of bacteria [4]. Hydrophobicity is a crucial hydrogel's surface property. Microbial cells adhesion to surfaces can be categorized into two, which are passive and active [5]. Passive adhesion involves van der Waals forces and hydrophobic interactions forces between surfaces and cells. Van der Waals' interactions is when all molecules attract one another by a group of attractive forces collectively. In simplified definition, these forces include discussion as the attractive forces that are generated by polarizing molecular dipoles [6]. These kind of interactions can manipulate the physicochemical properties of the polymer surface by adding hydrophilic binding groups to the polymer [7].

In vitro, bacteria tend to adhere to the hydrophobic surface in greater numbers and form biofilms which will often cause an inflammatory response and infection in the human body, while the hydrophilic surface is harder for protein to attach since the hydrophilic brush could inhibit protein's adsorption. A previous study has identified that the water content inside the hydrogels will affect bacterial adhesion. Bacteria will adhere with higher affinity to low-energy,which is hydrophobic surfaces than to high-energy, hydrophilic surfaces [8]. Surface topography of biomaterial is another important factor in molecular scale which can affect bacterial to adhere to surface for initial adhesion [9]. The actual geometry of a surface can show a complex mix of features even the areas considered very smooth. In fact, the surface roughness of biomaterial was demonstrated as major factor of biofilm deposited on the contact lens study [10]. The irregularities behaviour of polymeric surfaces will promote bacterial accumulation and adhesion, due to the increased surface area and depression which provide additional and favourable sites for colonization [11]. However, a linear relation of bacterial adhesion with surface roughness is not always verified. The bacterial adhesion is significantly increased for a small increase in roughness but has no significant effect for larger increase [12].

2. MATERIALS AND METHODS

2.1 Preparation of Hydrogels

Materials were obtained from the following manufacturers: *A. konjac* Koch without any purification (99% GM content) from Health Plus Ltd, U.K and Xanthan gum powder from House of Ingredients Sdn Bhd, Selayang. Five sets of hydrogels with different compositions at different ratios of *konjac* powder and Xanthan gum powder were mixed with 100 mL of

distilled water. The powder was weighed in the plastic weighing boat by using an analytical balance and placed into 250 mL of cleaned beaker. 100 mL of distilled water was measured in a measuring cylinder and poured into the same beaker then mixed homogeneously for 30 minutes using a magnetic stirrer. the mixed solution was poured into a petri dish. The weight of hydrogels in petri dish before drying process was recorded. Then, the hydrogels were dried in a fume hood cabinet for 4 days to reduce the contamination. Every petri dish was labelled with the percent of KGM : XG represented the compositions of konjac and xanthan gum powder in the hydrogels. During the drying process, the cover of the petri dish was opened halfway and the lid was kept pointed down. After 4 days, the weight of dried hydrogels were recorded.

The formulation data for each hydrogel is provided in Table 1.

Table 1. Material compositions in hydrogels

No	KGM : XG (%)	KGM : XG (g)	Amount of distilled water (mL)
1	100 : 0	1.00 : 0	100
2	70 : 30	0.70 : 0.30	100
3	50 : 50	0.50 : 0.50	100
4	30 : 70	0.30 : 0.70	100
5	0 : 100	0 : 1.00	100

2.2 Characterization of the hydrogels

2.2.1 *Physical integrity of the hydrogels*

The physical appearance of hydrogels were observed before and after the drying process. The determination of stability structure was done by immersing the 2 x 2 cm of dried hydrogels samples into distilled water over 30 minutes. The state of physical changes of hydrogels was recorded after each 10 minutes period.

2.2.2 Analyzation of chemical structure of hydrogels using FTIR

Each dried hydrogel sample was cut into 1 x 1 cm to be characterized using FTIR. The FTIR spectrophotometer recorded the changes in confirmation of chemical structures of hydrogels that are sensitive to surrounding changes. The FTIR showed the spectra of polysaccharide hydrogels with their characteristics absorption and identified the band positions and the deformation/formation of hydroxyl groups in the hydrogels.

2.2.3 Hydrogels morphology studies using FESEM

Field emission scanning electron microscope (FESEM) was used to characterize the morphologies of hydrogels ' surface and cross-section. For surface analysis, the hydrogel was cut into 1 cm^2, sputter coated with gold and imaged using FESEM (Jeol JSM-6700F). For cross section analysis, the squared hydrogel was cut vertically at the centre, and sputter coated with gold. This was then imaged using the same FESEM model. Magnification of 250-1000x were utilized to observe the morphological differences between the hydrogels.

2.2.4 Hydrogels degradation study using DSC

The dry samples were weighed (approximately 5-15 mg) in aluminium DSC pans. The empty pan was used as a reference Analyses were started at 25 ºC and continued up to 250 ºC, with a linear increase of 10 ºC min^{-1}. Data were analyzed using STARe evaluation software (Mettler Toledo). The glass transition temperature (T_g) is normally based on the onset temperature or midpoint of the glass transition temperature range (Eliasson, 2006). The significant differences in glass transition temperature of sugars are due to the molecular structure of sugars and interactions between sugar molecules.

2.2.5 Determination of water content

The determination was done by immersing the 2 x 2 cm of dried hydrogel samples in distilled water for 10 minutes. The weight before and after the immersion was recorded for calculation. The amount of absorbed water is calculated as swelling ratio and being expressed in the Equation 1.

$$WC : [(W_s - W_d) / W_s] \times 100 \quad \text{(Eq 1)}$$

where W_s : the weight of swollen hydrogel

W_d : the weight of dried hydrogel

2.3 Bacterial culture

2.3.1 Media preparation

25 g of LB Broth (Luria – Bertani) powder was dissolved with 1 L distilled water in 1000 mL of Schott bottle. The broth was sterilized by autoclaving at 121ºC for 15 minutes. After the sterilization, the media was left cool down and covered with aluminium foil. The media was stored ina chiller at 4ºC until further used. For preparing agar plates, 37 g of LB agar powder (Luria – Bertani) was dissolved in 1 L of distilled water. The agar solution was sterilized by autoclaving at 121ºC for 15 minutes. After the sterilization run, the agar solution was left cool down until 55ºC – 60ºC to ensure that the bottle could be held in bare hand. The solution was ensured not to cold down too long; otherwise, the agar would start to be solidified. While the agar was cooling, the bench of the laminar flow hood was wiped down with 70% ethanol to sterile the working area in order to prevent contamination. Each petri dish was marked with a date and description of the media. When the agar bottle was cool enough to be held, the lid of the petri dish was lifted halfway only enough to pour the agar solution. After that, the agar solution was quickly poured into the petri dish. The plates were tilted in order to spread the agar and the lid was immediately replaced. The agar was allowed to be solidified for about 10 – 15 minutes. After they were totally solidified, the agar was stored in the chiller at 4ºC with an inverted position before used to avoid the dew droplets of condensation dropping onto the agar.

2.3.2 Preparing overnight culture

A sterilized 50 mL of Erlenmeyer flask was labelled with date, description of media and bacterial species. The mouth of LB bottle was flamed and 25 ml of LB Broth was pipetted into the flask. A well-defined colony about 1 to 4 mm diameter on a streaked plate was located.

The inoculating loop was flamed with a Bunsen burner until it showed a red hot glow. The loop tip was cool down by stabbing several times onto the agar plate and after that ,a visible cell mass from the selected bacterial colony *Escherichia coli* was scrapped up. The colony was transferred into the flask by immersing the loop into the broth and agitated to dislodge the cell mass. The mouth of the flask was flamed before and after being transferred to the colony. The flask was covered with aluminium foil and sealed with parafilm. The culture was incubated overnight, 12 until 18 hours in a shaking incubator at 37ºC, 170 rpm.

2.3.3 Plating culture

After overnight culture, 0.l mL of diluted bacterial suspensions was transferred onto the agar plate using 1000 µl of the micropipette. The mouth of the flask was flamed before transferred the inoculum. The L-shape spreader was wiped out with 70% ethanol and then flamed to sterilize it. The inoculum was spread evenly onto agar by a spreader. The petri dish was sealed with parafilm and then incubated in an inverted position in an incubator at 37ºC for 24 hours until a confluent growth of colonies was formed.

2.4 Bacterial removal test

The sample of hydrogels (70 : 30 %, 50 : 50 % and 30 : 70 % of KGM : XG) was cut off into 1 x 1 cm. The samples were soaked in distilled water for 2 minutes and then placed on top of confluent bacterial colonies on the agar plates for 30 minutes. After that, the hydrogels were removed and immersed in the 3 mL broth for another 30 minutes. The total viable bacterial population densities were determined by doing a serial dilution (10^1) and plating onto fresh nutrient agar. All the experiments were run in duplicate to get an average for a better result.

2.5 Validation

2.5.1 Colony forming unit (CFU)

CFU was used in the counting of viable bacterial or fungal cells. For the validation of this study, the total viable count of the bacterial population that attached to the hydrogel's surface was determined by using the colony forming unit technique. 1 mL of broth solution with the immersed hydrogel was pipetted into a tube containing 9 mL of another broth solution. This dilution was mixed well as labelled as 10^1 of serial dilution. The dilution (0.1 mL) was plated onto fresh agar and incubated overnight. The CFU was calculated by using the formula in Equation 2.

$$\text{CFU/ mL} = (\text{number of colonies x dilution factor}) / \text{volume of culture plate}$$

If the plate of the 10^1 dilutions produced 90 colonies, then the number of bacterial colonies in 1 mL of the original sample will be calculated as below ;

$$\text{Bacterial / ml} = (90 \times 10^1) / 0.1$$

Thus the total number of colony-forming units are : 9,000 CFU / ml. This calculation was repeated for every duplicated experiment of other samples to compare the total number of bacterial colonies that attach to hydrogels' surfaces.

2.5.2 Optical density (OD)

The equation relation of CFU / ml to OD can provide useful information in comparison of bacterial concentrations that are attached to hydrogels. Bacterial concentrations for the diluted cells were used for determination of the colony forming unit (CFU) by serial dilutions of bacterial culture and determined by UV-Visible spectrophotometer at 600 nm by measuring the absorbance of the bacterial solutions. A graph was plotted to show the relationship between hydrogels' compositions and CFU/mL and OD_{600nm} .

3. RESULTS AND DISCUSSION

3.1 Observation on the hydrogels' physical intergrities

The dried sample of hydrogels was transparent, very soft for 100% KGM and brittle for 100% Xanthan, and those with higher concentrations of xanthan appeared slightly yellowish. The physical integrity of hydrogels was observed over 30 minutes (Fig. 1). It was observed that the hydrogels with the single composition of KGM and Xanthan degraded faster compared to blend hydrogels. In this case, the hydrogels with 100% of KGM and 100% of Xanthan also appeared fragile and partially degraded after 10 minutes,and totally dissolved after 30 minutes. Meanwhile, blend KGM-Xanthan hydrogels appeared with a rigid and stable structure over 30 minutes. The observations on the hydrogel‘s physical integrities are summarized in Table 2.

Table 2 Qualitative observations of the physical integrities of hydrogels

KGM : XG (%)	Time (minute) 0	10	20	30
100 : 0	Solid	Start to break	90 % degraded	Dissolved
70 : 30	Solid	Solid	Solid	Solid
50 : 50	Solid	Solid	Solid	Solid
30 : 70	Solid	Solid	Solid	Solid
0 : 100	Solid	Start to break	70 % degraded	Dissolved

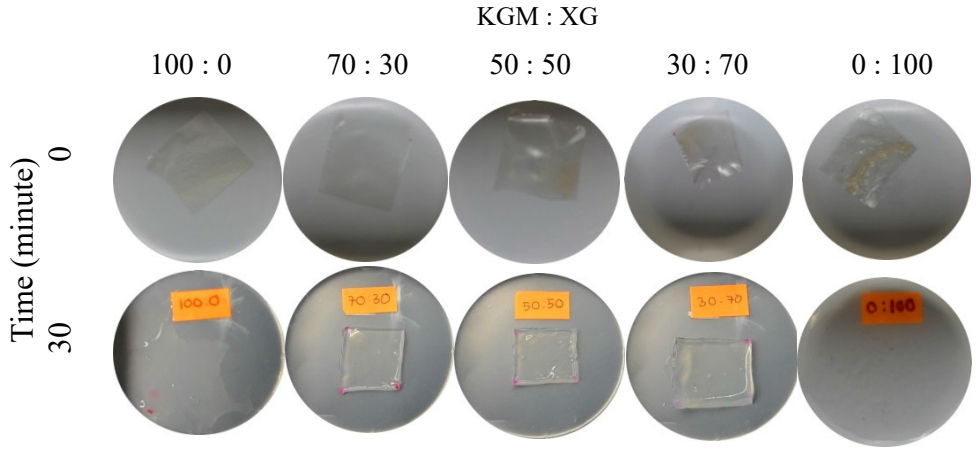

Fig. 1 Pictures of hydrogels swelling and breakdown after immersion in water for 30 minutes

3.2 Observation on the hydrogels' morphologies and surfaces by FESEM

Characteristic morphology of dried hydrogel samples were observed by FESEM. All hydrogels were opaque and possessed no particular texture or porosity on the surface (Fig. 2). Each sample of hydrogel with different concentrations of KGM and Xanthan was conducted at magnification 1000x. The surface of (A,F) 100% KGM without XG, (B,G) crosslinked 70% KGM with 30% XG, (C,H) crosslinked 50% KGM with 50% XG, and (D,I) crosslinked 30% KGM with 70% XG were showed flat and even surfaces, but uneven and brittle for (E,J) 100% XG without KGM.

Further analysis of the hydrogel's cross section (the hydrogels were cut vertically from the centre) showed intricated sponge-like morphologies embedded inside the hydrogel. It can be observed the blend KGM-Xanthan hydrogels had a much better polymeric network, where the composition of the helical structure of xanthan was evident. The cross-section of KGM-Xanthan containing hydrogels appeared to have regular and linear structures, while the single composition of KGM and Xanthan containing hydrogels appeared with irregular and brittle structures.

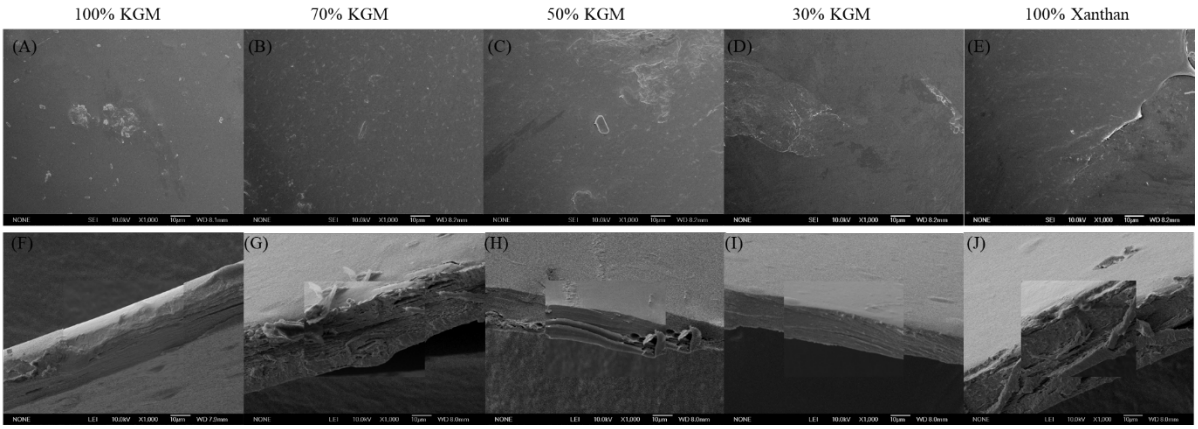

Fig. 2 The morphologies of the surface and cross sections of (A, F) 100% KGM without XG, (B,G) crosslinked 70% KGM with 30% XG, (C,H) crosslinked 50% KGM with 50% XG, (D,I) crosslinked 30% KGM with 70% XG and (E, J) 100% XG without KGM observed using FESEM

3.3 Observations of molecular interactions of KGM-Xanthan using FTIR

The molecular interactions of KGM and Xanthan is examined using Fourier Transform Infrared (FTIR) as shown in Fig. 3. All samples of hydrogels displayed the presence of –OH groups (hydroxyl) which gave a broad band between 3650 - 3000 cm^{-1}. Generally, the presence of hydrophilic and hydrophobic moieties in hydrogels verified by FTIR spectroscopy gives them a unique ability to be soluble in both organic and aqueous solvents. Hydrophobicity is a crucial hydrogel's surface property where hydrophobic molecules have to be conducted in living physiological conditions. C–H broad (alkyl) group can be observed at the stretching band (2850–3000 cm^{-1}). Also, band from C=O (carbonyl) group that is associated with the aldehyde group was identified at 1700 – 1720 cm^{-1}. The band positions of KGM and Xanthan were marked to be higher at 1370 cm^{-1} and 1371 cm^{-1} of KGM-Xanthan blend hydrogels, where the assignment shifted from 1369 cm^{-1} in 100% KGM and 1374 cm^{-1} for 100% Xanthan spectra. Carbon–single oxygen bonds displayed stretching bands in the region 1248 -1100 cm^{-1}. The assignments were observed to be lower as increasing of Xanthan concentration in hydrogels where the positions were shifted from 875 cm^{-1} in 100% KGM to 873 cm^{-1} in blend KGM-Xanthan, and 872 cm^{-1} in 100% Xanthan.

3.4 Attachment of bacterial colonies onto KGM-xanthan blend hydrogels

Up to this experiment, only KGM-Xanthan blend hydrogels were used to examine the bacterial adhesion onto hydrogel since their physical properties which are remained stable after being immersed in water. Otherwise, the result might not be correct because both 100% KGM and 100% Xanthan hydrogels started to degrade faster when contacting with water. Since the optical density of a bacterial solution can be easily determined using a spectrophotometer, the equation relating to OD to CFU/ml would provide useful information on total biofilm viable count which is essential for the expression of bacterial concentrations\. Based on these two relationships, a relation was derived between CFU/mL and bacterial concentrations. In general, the spectrophotometer was set at a wavelength of 600 nm. Total biofilm viable count was calculated as colony forming unit (CFU/mL). The measured data on the relationship between blend KGM : XG hydrogels ratio and CFU/ml and OD of E.coli is shown in Fig. 4. The bacterial

cells showed almost a linear relationship with a slope of 976.99 CFU/mL/OD, indicating that 1.0 OD corresponds to 976.99 CFU/mL. This relation was also represented by a linear regression line with Y= 976.99x + 12.439. From the graph, it can be seen that 50 : 50 % of blend KGM : XG hydrogel attracts a greater number of bacterial colonies from petri dish which 13 x 103 CFU/mL, then followed by 70 : 30 % (8.9 x 103 CFU/mL) and 30 : 70 % (4.3 x 103 CFU/mL) of blend KGM : XG hydrogels.

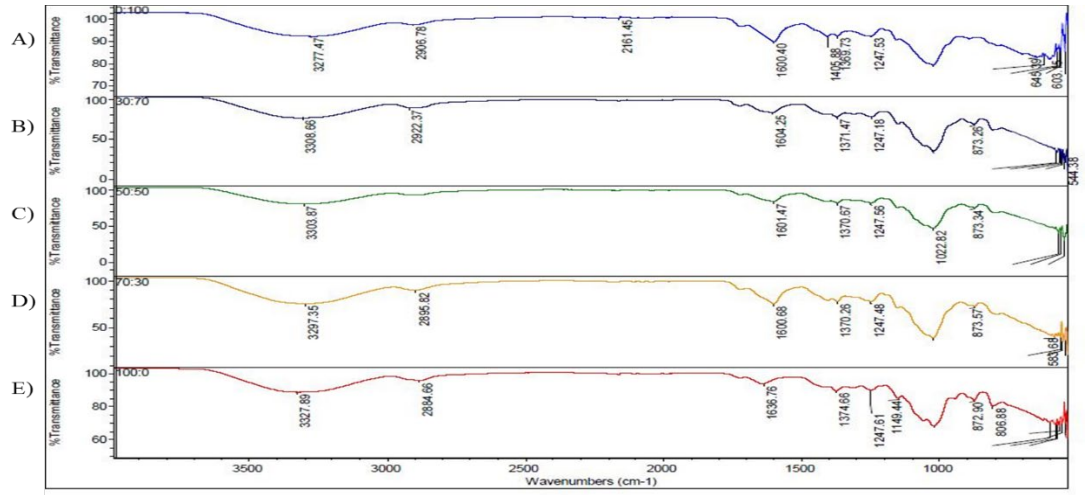

Fig. 3 FTIR spectra of (A) 100% KGM without XG, (B) crosslinked 70% KGM with 30% XG, (C) crosslinked 50% KGM with 50% XG, (D) crosslinked 30% KGM with 70% XG and (E) 100% XG without KGM.

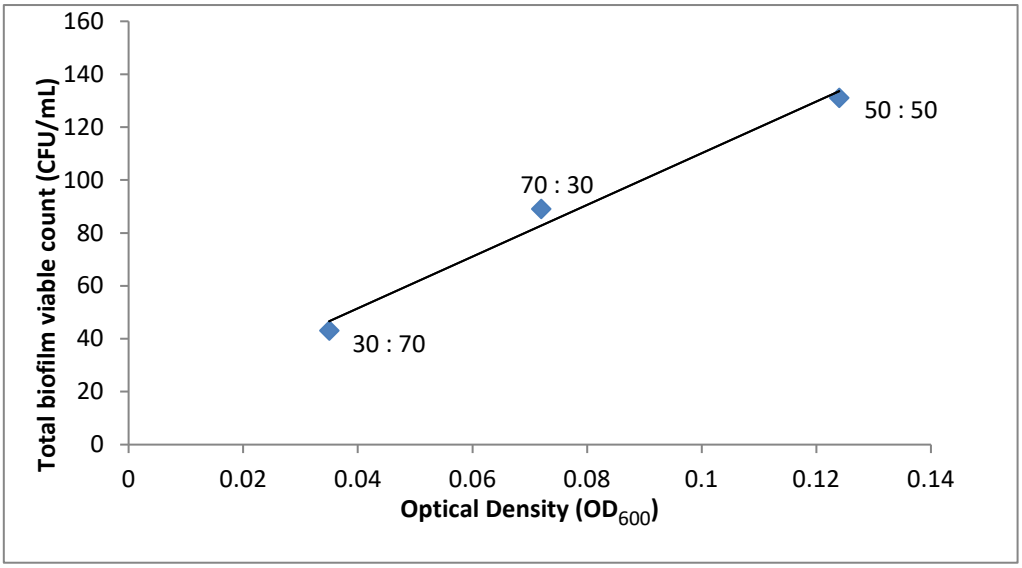

Fig. 4 Graph of total biofilm viable count (CFU/ml) adhered to 30:70, 70:30 and 50:30 KGM : XG hydrogels ratio.

4. CONCLUSION

In summary, this project explores the use of polysaccharide based hydrogels for medical applications. The aim of this project was to demonstrate the potential of Konjac Glucomannan and Xanthan gum in the development of hydrogels for bacterial removal activity during the wound healing process. The choice of polysaccharide as a base of hydrogels was because of its

properties consist of bacterial's envelope polysaccharides that will help the bacterial cell recognize the carbohydrate moities. To achieve this, a series of objectives were identified: 1) to produce polysaccharide based hydrogels, 2) to optimize the ratio of KGM : Xanthan gum in the production of hydrogels and 3) to understand the effect of hydrophobicity and hydrophilicity in the removal of bacteria from the petri dish. The hydrophilic interaction between hydrogels and bacterial species plays an important role in adhesion of bacterial colonies to the hydrogels' surface.

Characterizing analysis showed all hydrogels are biodegradable and biocompatible which non-toxic to be used. Initially the attempts were made to produce KGM, Xanthan and KGM-Xanthan hydrogels with crosslinked properties that would benefit wound healing. However, the nature of the single composition of KGM and Xanthan hydrogel that breaks easily when contacting with water limits their use in experimental bacterial removal tests, especially when placing the medium onto the agar plate, and can result in the pre-dissolution state. Therefore, the blend KGM-Xanthan hydrogels with greater strength and mechanical properties were conducted by placed onto the confluent bacterial colonies on the agar. These hydrogels have different biological and chemical properties, whereas the hydrogels contain lower water content had remarkable properties for bacteria to successfully attach to the hydrogel. The water content inside the hydrogels presented in this project is equally important for degradation profile and hydrophilicity-hydrophobicity determination.

Increasing the degree of crosslinking to the desired mechanical property of the hydrogel could be achieved. However, too high degree of crosslinking will decrease the % elongation of the hydrogels, creating a more brittle structure. Hence, it would be necessary to have an optimum degree of crosslinking to achieve a relatively strong yet elastic hydrogel [13]. The interpenetrating phases with different degradation profiles and swelling responses to physiological conditions provided multiple controls over bacterial adhesion onto the hydrogels. A detailed study on the characterizations of the hydrogels would also be advantageous to further understand the factors that influence bacterial - polymer interactions. Adhesive polysaccharide hydrogels will aid the healing process of wound by attaching onto the bacterial colonies and remove them physically from the wound, which that essential in the prevention of further infections. Due to the limitation of time for research and facilities, it would be necessary for the future to conduct more repeat experiments for better results. In brief, KGM-Xanthan based hydrogels showed great potential for biomedical applications of wound treatment especially for diabetic foot ulcer (DFU) where the hydrogels able to reduce bacterial infections as well as lower the use of antibiotics, which speed up the recovery time and reduce the cost in treatment.

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