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Development and evaluation of quercetin enriched bentonite-reinforced starch-gelatin based bioplastic with antimicrobial property

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ARTICLE INFO

Keywords:
Quercetin
Starch
Gelatin
Bentonite
Antimicrobial
Experimental design

ABSTRACT

Nowadays novel bio-based materials have been widely employed in food and pharmaceutical industry because of their wide acceptability by the consumers rather than the synthetic materials nevertheless, they possess poor mechanical properties. Reinforcement of biopolymers with intercalation of mineral clays can improve their physicochemical properties; so that such biocomposites possess superior barrier and mechanical properties as well as stability and drug loading efficacy. Thus, this research aimed at formulating quercetin loaded bentonite-reinforced starch-gelatin based novel bioplastic with diverse applicability. The methodology of the study included Box Behnken optimization as well as physical, structural, mechanical and antimicrobial properties evaluation of the proposed reinforced bioplastics. Amount of starch, bentonite and glycerin were the independent variables while the tensile strength, swelling index and elongation percentage were studied as dependent variables. The optimized bioplastic film showed excellent physicochemical and morphological characteristics and also for efficient percentage drug content. The antimicrobial activity showed the highest activity against Escherichia coli followed by Pseudomonas aeruginosa and Staphylococcus aureus. Scanning electron microscopy (SEM) revealed the non-homogenous nature of the film. Generally, the results revealed that quercetin loaded bentonite-reinforced starch-gelatin based could be used as ecological friendly active food packaging as well as pharmaceutical application with significant antimicrobial properties.

1. Introduction

The massive manufacture of conventional plastics and their use in various industrial applications creates a substantial threat to the fossil fuel resources as well as the environment (Emadian et al., 2017). Plastic is a rapidly growing segment of municipal solid waste (MSW). Single-use products like plastic bottles, bags and product wrappings presently include the major sector of plastic manufacture and also plastic waste. According to U.S. Environmental Protection Agency in 2018, the containers and packaging category which includes sacks, wraps, bags etc. contributed to the most plastic tonnage at more than 14.5 million tons (U.S. Environmental Protection Agency, n.d.). Synthetic plastics take many years to decompose and hence adversely affects the land, water ways as well as air.

As a result of environmental awareness and the execution of strict environmental regulations in current years, there has been enhanced effort to use bioplastics as packaging materials especially in food industry (Jariyasakoolroj et al., 2020). The biodegradability and reduced carbon dioxide productions are the major advantages of bioplastic as a packaging material. These biopolymers are considered to be superior not only due to their biodegradability and edibility, but also, they are obtained from natural renewable resources. In recent years, massive

Peer review under responsibility of King Saud University. Production and hosting by Elsevier.
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https://doi.org/10.1016/j.jsps.2023.101861
Received 6 August 2023; Accepted 1 November 2023
Available online 2 November 2023

studies have been published on the production of bioplastics from bio-resources (Ahari et al., 2022; Byun and Kim, 2014; Shamsuddin et al., 2017). Now a days novel bio-based materials have been widely employed in food and pharmaceutical industry because of their wide acceptability by the consumers rather than the synthetic materials. Fascinatingly, bioplastics have been proved valuable for their biomedical advantages. They are even investigated for use in wounds as well as surgical dressings (Galiano et al., 2018; F. Garavand et al., 2022; Heydari et al., 2018; Mitrus et al., 2009).

The major ingredients of bioplastics are renewable biomass sources, like a variety of proteins, polysaccharides, as well as lipids (Dilshad et al., 2021; Omrani-Fard et al., 2020; Shafqat et al., 2020). Starch is the utmost important polysaccharide, which is splendidly accessible in nature at minimal cost. Even though it is widely used for the manufacture of bioplastics its poor mechanical characteristics and fragile behavior limits its sole use (Anugrahwidya et al., 2021; Babaee et al., 2022; Y. Garavand et al., 2022).

Some studies proved that a combination if starch with protein create a powerful network of H-bonds and intermolecular interactions which can leads to formation of stable three-dimensional objects (Gómez-Heincke et al., 2017; Haghighi et al., 2019; Omrani-Fard et al., 2020; Pommet et al., 2003). So, these biocomposites are superior in barrier, mechanical properties as well as stability and drug loading efficacy. Proteins as one of the biological polymers possess strong molecular interactions in the form of H-bonds, ionic bonds and covalent bonds can generate an exclusive network in their structure. This is the reason for their better mechanical strength as compared to bioplastics generated from polysaccharides and lipids (Gómez-Heincke et al., 2017; Hanani et al., 2012). Recent years research showed that proteins from a variety of biological sources have been successfully utilized for the production of bioplastics (Araújo et al., 2018; Arfat et al., 2017; Felix et al., 2017; Flores et al., 2016; Hanani et al., 2012; Jones and Sharma, 2016; Miethke et al., 2021; Moosavi et al., 2020; Slayter and Slayter, 1992; Uranga et al., 2018). Even if proteins are renewable resources their per capita production is high. Combination of animal protein with starch can produce highly transparent bioplastic with acceptable mechanical properties. However processing situations have a high influence on tensile strength and viscoelastic properties of bioplastics (Gonzalez-Gutierrez et al., 2010).

Reinforcement of biopolymers with intercalation of mineral clays can improve their physicochemical properties. Bentonite provides mechanical strength to starch-based bioplastics also have proven antibacterial and anti-inflammatory activities. Bentonite is an inorganic natural clay, which contains a variety of mineral substances like montmorillonite, zeolite, quartz etc. The major element montmorillonite exists as lamellar sheet like structures with exchangeable cations in the layers. Bentonite has a high swelling and adsorption capability due to montmorillonite. Due to these properties it is widely used in food, cosmetic and pharmaceutical industries (Benucci et al., 2020; Cacciotti et al., 2019; Carretero and Pozo, 2010; Eisenhour and Brown, 2009; Park et al., 2016; Qureshi et al., 2021). Apart from this, bentonite can act as a filler to reinforce the thermal and mechanical properties of polymer nanocomposite systems (Devi and Dutta, 2017; Zhang et al., 2020). Bentonite can effectively interact with the carrier molecule and can sustain the drug release in controlled release delivery systems. In addition to this the bio-compatible nature makes this natural clay as an excellent excipient in pharmaceutical formulations (Park et al., 2016).

Quercetin is a flavonoid occurring abundantly in fruits, vegetables, grape wine and tea (Harwood et al., 2007). It is a penta-hydroxy flavone that possesses numerous beneficial biological effects like anti-inflammatory, antioxidant, antiaging, prebiotic and metabolomic modulatory activities. The special structure have abundant capability for scavenging free radical and can be easily oxidized (Afonso et al., 2007; Castangia et al., 2022). In this work we incorporated quercetin due to its anti-inflammatory, antioxidant, as well as antibacterial activities.

So here in our research, we aimed to prepare quercetin enriched bentonite reinforced starch-gelatin based bioplastic with diverse applicability. The formulation has been optimized using Box Behnken Design (BBD). Concentration of bentonite, starch and glycerin were considered as the process variables to optimize tensile strength, elongation percentage, moisture uptake of the bioplastic film. We incorporated quercetin as an antibacterial agent to make the film suitable for drug delivery as well as food packaging applications. Quercetin enriched film with antimicrobial properties using biobased materials starch and bentonite has not been reported so far. The use of experimental design for the perfect optimization is an added advantage of our study. Moreover, the formulation proved superior antimicrobial properties with purely natural and biodegradable ingredients. The optimized formulation was further evaluated for film thickness, weight variation, water vapor transmission rate, folding endurance, drug content, film morphology by scanning electron microscopy biodegradability and antimicrobial activity.

2. Materials and methods

2.1. Materials

Quercetin, (purity $\geq$ 95), Starch soluble synthetic grade purchased from scharlau, ChemSupply Australia) and gelatin (gel strength $\sim$ 300 g Bloom, Type A) purchased from Sigma-Aldrich (St. Louis, MI, USA). Bentonite (Sigma, USA), Glycerol and all other reagents used in the study were commercially available and of analytical grade.

2.2. Experimental design for formulation of quercetin loaded bentonite-reinforced starch-gelatin bioplastic films

To optimize the formulation factors and variables, a three level, three factor, Box Behnken statistical Design was used for the formulation of bioplastics (Design-Expert 13 Stat Ease Inc., Minneapolis, MN, USA) [12]. The independent variables were selected based on the preliminary trials. Concentration of bentonite, starch and glycerol were selected as the independent variables at three different levels. The dependent variables were swelling index, tensile strength, moisture uptake and elongation percentages. The combination of the factors' levels in each experimental run is presented in Table 1. Based on the best fitting polynomial model for each response (selected according to model fit statistics), the observed data were analyzed using Analysis of Variance to assess the effects of the variables and the possible interactions among them. The optimized formulation was selected utilizing numerical optimization and desirability approach. The goal of the study was to maximize the measured responses (Biscarat et al., 2015).

2.3. Preparation of antibacterial bioplastic film

In this experiment, simple solution-casting technique was used for the formulation of the novel antibacterial bioplastic film. The concentration of gelatin in all the film-forming solutions were 15 % (w/w). Before dissolution, gelatin was allowed to hydrate at room temperature for about 1hr. Gelatin was dissolved with the help of a magnetic stirrer at 50 ^{0}C for about an hour. After dissolution the plasticizer-glycerol was added as per the experimental design and 30 min time was allowed to complete the plasticization reaction. To this solution, the required amounts of soluble starch and bentonite as per the formulae generated

Table 1
Combination of independent variables levels for the prepared bioplastic runs and their corresponding responses*.

Run	A:Bentonite conc.	B:Starch conc.	C:Glycerol conc.	Percent moisture uptake (%)	percent elongation (%)	Tensile strength (N/m^2)	Swelling index
1	0.50	4.00	10	21.8	120	697.0	477.1
2	0.50	2.50	15	19.8	129	231.5	609.7
3	0.50	2.50	5	21.4	135	345.7	612.8
4	1.25	2.50	10	10.4	39	77.2	401.7
5	1.25	2.50	10	10.7	39	76.8	416.6
6	1.25	1.00	5	5.0	25	0.0	322.4
7	2.00	2.50	5	6.5	31	3.6	481.5
8	0.50	1.00	10	17.5	135	158.8	428.4
9	2.00	1.00	10	17.0	9	200.0	404.2
10	1.25	2.50	10	10.4	35	80.2	409.1
11	1.25	2.50	10	10.7	35	78.9	416.6
12	1.25	1.00	15	18.5	31	0.0	304.7
13	1.25	2.50	10	11.6	35	77.3	416.6
14	2.00	2.50	15	19.8	39	82.7	377.2
15	2.00	4.00	10	11.0	56	134.5	199.6
16	1.25	4.00	15	11.2	36	115.0	241.34
17	1.25	4.00	5	11.6	52	192.0	273.56

* The table include 17 experimental runs including factorial points in addition to five center points.

by experimental design was slowly added. The stirring continued under the same conditions until the whole ingredients were mixed well. Then the solution was kept in a bath sonicator to remove the entrapped air bubbles. The solution was poured into a mold with a diameter of 88 mm and dried at ambient temperature where the drug was incorporated to the solution just before casting to prevent any degradation in high temperature (Biscarat et al., 2015; Cao et al., 2009; Mroczkowska et al., 2021; Qureshi et al., 2021; Shikinaka et al., 2010). The addition of quercetin was accompanied by constant stirring to enable thorough mixing of the drug with the bioplastic. The finished whole bioplastic films were then peeled off and stored. The film samples were stored in a desiccator containing saturated solution of magnesium nitrate at 25 °C and 50 ± 3 % relative humidity preceding to analysis.

2.4. Characterization of quercetin loaded bentonite-reinforced starch-gelatin bioplastic films

2.4.1. Percentage moisture uptake

The bioplastic films were kept in a desiccator containing saturated solution of potassium chloride to produce a relative humidity of 84 % at room temperature for three days. The films were weighed again, and percentage moisture uptake was calculated according to the following equation (Joshi and Garud, 2021; Suksaeree et al., 2022).

$$\text{Percentage moisture uptake} = \frac{(Finalweight - initialweight)}{Initialweight} * 100$$

2.4.2. Tensile strength and percentage elongation

Tensile strength gives an understanding about the mechanical property as well as the strength of the film. It was established by a device set in the lab with slight modification as previously mentioned by Joshi et al. and Ahmed and El-say (Ahmed and El-Say, 2014; Joshi and Garud, 2021). Briefly, a strip of bioplastic film (1 cm wide and 4 cm long) was hung down between jaws of upper clips. A 2 cm distance was set between the upper and lower clips and the lower clip was tied to a pan so that weight can be gradually added. The weight on the pan was gradually added enough to break the film. Series of weights was gradually added on the pan. This weight was noted, and the tensile strength was calculated by the equation given below. The tests were repeated three times, and the average of the results were reported.

$$\text{Tensile strength} = \frac{F}{\left\{\left(a.b\right)\left(1 + \frac{L}{E}\right)\right\}}$$

Where, F is the break force, a is the width and b is the thickness and L is the length of the film and E is the elongation of the film.

$$\%\,\text{elongation} = \frac{\text{final length of bioplastic film just before breaking} - \text{initial length}}{\text{initial length}} * 100$$

2.4.3. Swelling index

The swelling behavior of bioplastic films were assessed by using phosphate buffer of pH 7.4. The films were cut into 10 x 10 mm square pieces and weighed and then 2 ml of buffer solution was poured and allowed to swell. The excess buffer solution was carefully removed and films were weighed again. The same volume of buffer was added again, and the procedure was continued until the weight of the film stayed constant (Akram et al., 2018; Shivalingam et al., 2021). The swelling index of the film was estimated using the equation.

$$\%\,\text{Swelling} = \frac{\text{Wt. of swollen film} - \text{initial wt. of film}}{\text{initial wt. of film}} * 100$$

2.5. Characterization of the optimized bioplastic film

The optimized bioplastic film was prepared and evaluated for percentage moisture uptake, percentage elongation, tensile strength, and swelling index as previously mentioned. In addition, the optimized film was evaluated for the following properties. The thickness of the optimized films was measured using a vernier caliper (Aerospace, Kovea Co. Ltd, Gyeonggi, South Kore) at six different places from three randomly selected patches to confirm the uniformity of thickness. The values were recorded as a mean of 3 determinations with standard deviations. For the weight variation the weight of quercetin enriched bentonite-reinforced starch-gelatin based bioplastic was estimated by randomly choosing 10 films from each batch and were independently weighed. After that the mean weight was measured and the difference in weight was recorded (Joshi and Garud, 2021). Water vapor Transmission rate test is done as follows. Cleaned and dried glass vials of equal diameter were used as transmitting cells for this study. Anhydrous calcium chloride (1 g) was placed in the vials and the bioplastic film were tightly fixed on the brim of the vials. The weight of these were noted and then kept in a desiccator containing potassium chloride to create relative humidity of 84 %. After 24 h the vials were weighed again. The volume of water vapor transmitted was calculated using the following formula (Joshi and Garud, 2021; Kusum Devi et al., 2003).

Water vapor transmission rate = [(final weight − initial weight) x time of exposure × area of the film] 100

The films were constantly folded at the same place until it is broken for measuring the Folding endurance. Folding endurance is the number of times the film can be folded at the same place without splitting (Joshi and Garud, 2021). Specified area (1 cm^2) of the quercetin enriched bentonite-reinforced starch-gelatin based bioplastic where cut and kept in 10 ml volumetric flasks containing methanol in a water bath shaker at 37 ^{0}C for 24 hrs. for measuring the content. The solution was analyzed after dilution for quercetin content using UV- spectrophotometer (UV-2600 Shimadzu, Japan) at λ_{max} 375 nm.

The FTIR spectra of plain optimized bentonite-reinforced starch-gelatin bioplastic and quercetin loaded film were recorded between 4000 and 400 cm^{-1} using a Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific, MA, USA). Samples were scanned 16 times with a resolution of 4 cm^{-1}. (Kotta et al., 2021).

The morphology of optimized quercetin enriched bentonite-reinforced starch-gelatin bioplastic surface was examined with a scanning electron microscope, Tescan Lyra 3 FIB (Center of Nanotechnology, King Abdulaziz University), worked at 10 kV. Images were recorded using secondary electron detector in SEM mode at 12 kV accelerating voltage and with beam intensity set at 80 %. The films were mounted on an aluminum metallic stub using electrically conducting double sided carbon tape and platinum sputter coated for 10 s in order to avoid charging when exposed to high kv electron beam during the imaging process. All images are recorded at specific magnifications for easy comparison.

The biodegradability of quercetin enriched bentonite-reinforced starch-gelatin based bioplastic was determined using soil-burial decomposition test. The bioplastic films were made into 10 x 10 mm^2 pieces and buried in the soil at about 80 mm depth. The test duration varied from 1 to 7 days. The initial weight of the bioplastic film was determined before burial, the weight after decomposition was noted as final weight. The decomposition was calculated for each sample as per the following equation (Di Franco et al., 2004; Shayan et al., 2015; Wahyuningtiyas and Suryanto, 2018).

$$\text{Biodegradability} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} * 100$$

2.6. Antimicrobial activity evaluation

The antimicrobial activity of samples was tested against standard strains of four different bacterial strains and one fungus. The strains were collected from the microbiology laboratory, King Abdulaziz University Hospital, Jeddah, KSA. These strains were *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 6633 (Gram positive bacteria) and also *Escherichia coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 (Gram negative bacteria) and *Candida albicans* ATCC 76615 (fungus).

Agar diffusion technique was used for the preliminary screening of the antibacterial and antifungal activities as described previously (Standards, 2004). In brief, Petri dishes (150 mm) were filled with 50-mL Muller-Hinton agar containing 1 ml bacterial culture (1 × 10^6 CFU/mL). The strains were inoculated separately. For solutions, four holes (4 mm in diameter) were made in the seeded agar plates. The holes were then filled with 50-μL of each sample, standard antibiotic solutions served as a positive control. For films, discs of 4 mm in diameter were made for each film, then placed onto the inoculated dishes, standard antibiotic discs served as a positive control. Petri dishes were then incubated for 24–48 h at 37 °C. Inhibitory activity was defined as the absence of bacterial growth in the area surrounding the holes. The inhibition zone was measured (mm) using a caliper.

3. Results

3.1. Model fit statistics

Upon testing the fitting of the observed responses to different polynomial models, it was found that all the responses best fit to the quadratic model. Fitting to the model was decided based on the highest determination coefficient (R^2) and the lowest predicted residual error sum of squares (PRESS), Table 2. In addition, the good matching between adjusted and predicted R^2 values and the adequate precision values of greater than the permissible limit of 4 supports the model validity.

3.2. Statistical analysis and polynomial equations generation

The significance of the quadratic model for each of the measured responses has been further confirmed by the ANOVA results that showed F-values of 114.00, 572.31, 91.27, and 118.13 for percent moisture uptake, percent elongation, tensile strength, and swelling index, respectively ($P < 0.0001$ for the four responses); such values suggest the significance of the quadratic model with only a chance of 0.01 % that this high value could be credited to noise.

3.3. Influence of the variables on the measured responses

The developed polynomial equations were utilized for assessing the relative influence of the factors by comparing their corresponding coefficients. Furthermore, perturbation and 3D response surface plots were created to demonstrate the trend of the significant variables' effects and interactions, Figs. 1-4.

Table 2
Model fit statistical parameters for the investigated responses of quercetin bioplastics based on the quadratic model*.

Response	Model P-value	R^2	Adjusted R^2	Predicted R^2	PRESS	Adequate precision
Percent moisture uptake (%)	< 0.0001	0.9932	0.9845	0.9237	33.36	34.23
Percent elongation (%)	< 0.0001	0.9986	0.9969	0.9879	350.00	70.24
Tensile strength (N/m^2)	< 0.0001	0.9916	0.9807	0.8651	60285.31	39.21
Swelling index	< 0.0001	0.9935	0.9850	0.9081	18418.08	39.22

* The model fit statistics includes determination coefficient (R^2), adjusted and predicted R^2, predicted residual error sum of squares (PRESS), and adequate precision values.

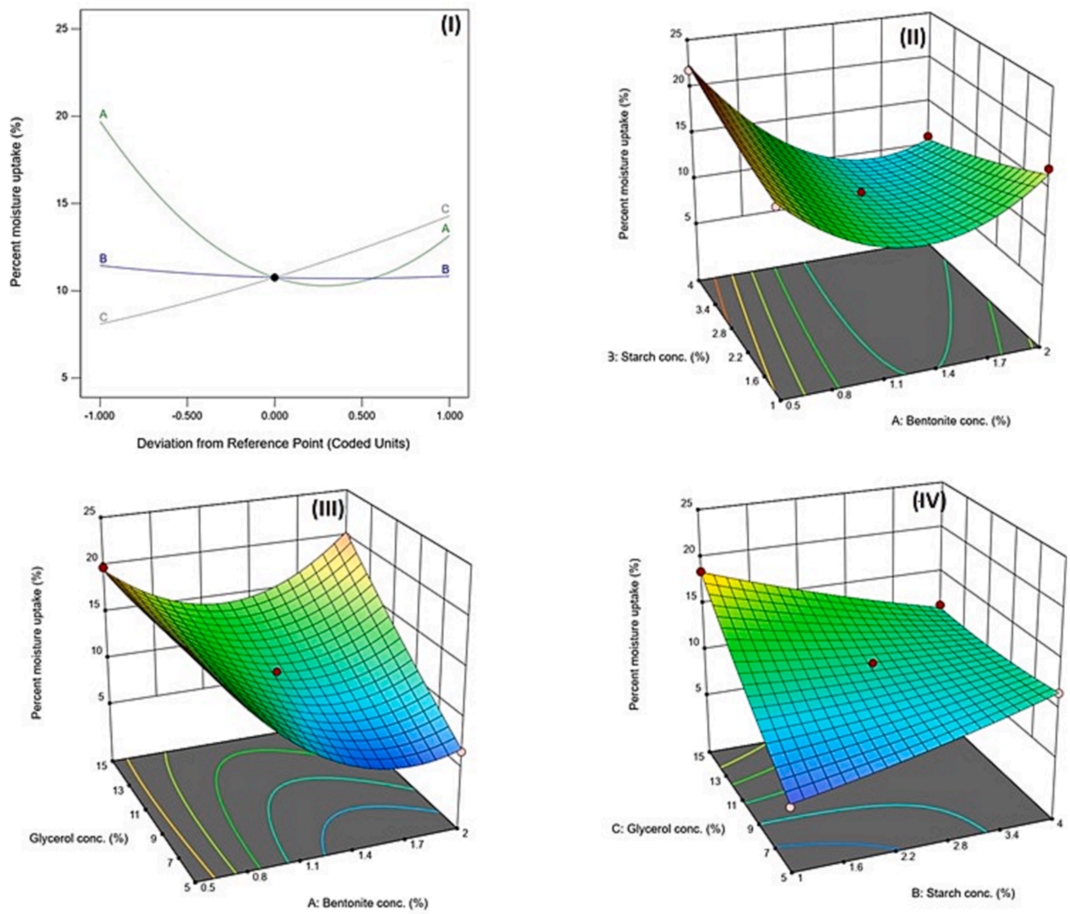

Fig. 1. Perturbation plot for the effect of independent variables (I) and 3D response surface plots (II-IV) on the percent moisture uptake of quercetin bioplastics.

3.4. Optimization of quercetin bioplastics

Based on numerical optimization following desirability technique, the anticipated levels of the optimized formulation that could yield maximized moisture uptake percentage, elongation percentage, tensile strength, and swelling index were 0.50 % bentonite, 3.74 % starch, and 5.00 % glycerol. The combination of such levels could achieve the desirable goals by a desirability of 0.917. The observed responses of 26.02 % 145.14 %, 612.85 N/m^2 and 529.92 for moisture uptake percentage, elongation percentage, tensile strength, and swelling index, respectively coincide well with the predicted values of 25.80 %, 132.96 %, 619.31 N/m^2, and 533.99. The relatively low percentage errors between the measured and predicted responses (less than 5 %) confirm the reliability of the optimization process adopted in the study.

3.5. Characterization of the optimized bioplastic film

Thickness is considered as a physical property that can affect the quality attributes of bioplastics. Thickness must be adjusted as per the required application. The thickness of optimized bioplastic was found to be 0.32 ± 0.6 mm. The weight variation test revealed that the optimized bioplastic formulation was uniform in weight from different batches. A value of 2.59 ± 0.23 % for the result of weight variation indicated the uniformity of the formulation and an acceptable variation between different batches (Mamatha et al., 2010). The water vapor transmission rate of the optimized formulation was found to be 2.14 ± 0.102 $g/cm^2/h$. The optimized quercetin enriched bentonite-reinforced starch-gelatin bioplastic possesses a good folding endurance. A folding endurance value of 122 ± 3.5 was observed for the optimized quercetin enriched bentonite reinforced starch-gelatin bioplastic. This, which is in agreement with the previous reported studies (Joshi and Garud, 2021).

The drug content uniformity was found to be satisfactory. The optimized quercetin enriched bentonite-reinforced starch-gelatin bioplastic showed a drug content uniformity of 97.27 ± 0.638 %.

The FTIR spectra of the optimized quercetin enriched bentonite-reinforced starch-gelatin bioplastic is shown in Fig. 5. SEM analysis revealed the micro composite structure of the bioplastic (Fig. 6). The surface seems to be not homogenous and the surface was visibly uneven with signs of particle aggregation. The biodegradability study confirms the decomposition potential of the bentonite-reinforced starch-gelatin based bioplastic formulation. So here it is clear that the nanoclay in the bioplastic facilitated the decomposition of this nanocomposite.

3.6. Antimicrobial activity of the optimized bioplastic film

For *in vitro* antibacterial and antifungal assay, the samples were evaluated by agar diffusion assay using representative standard strains of Gram-positive, Gram-negative bacteria and fungus, and the results are listed in Table 3 and Fig. 7.

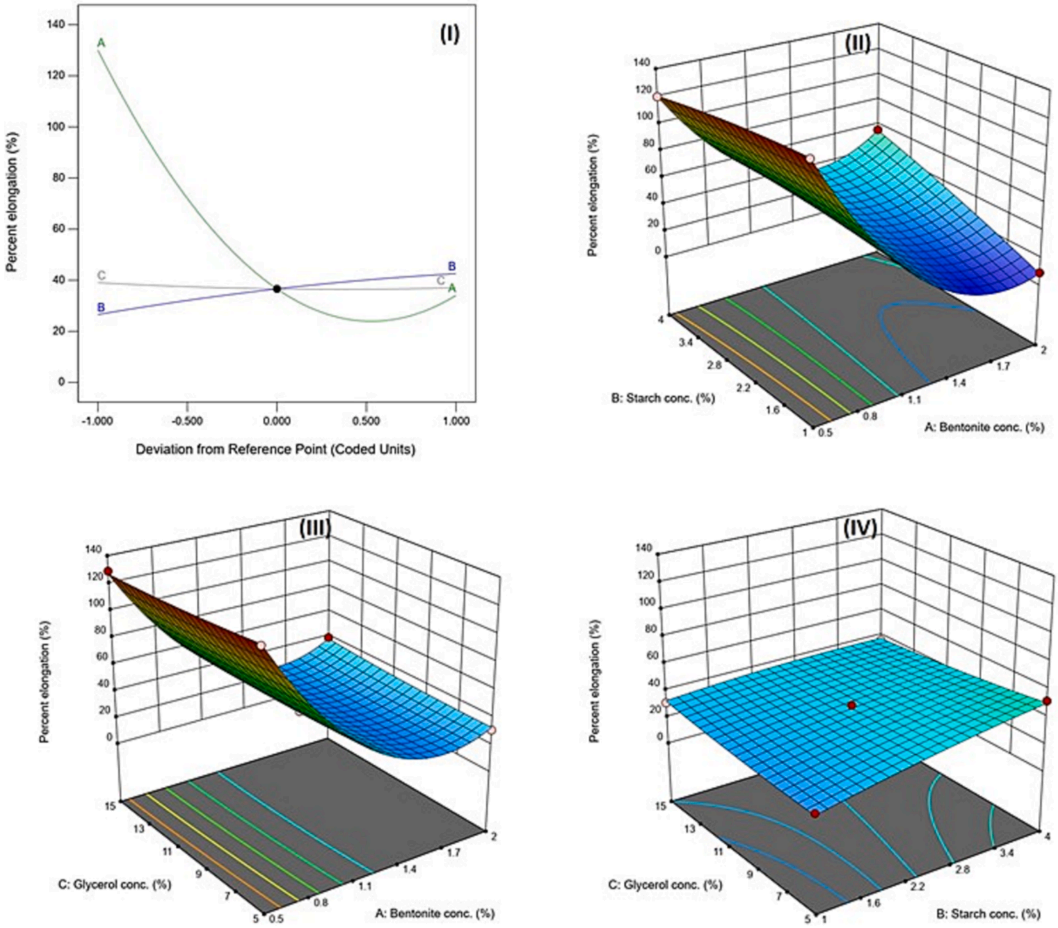

Fig. 2. Perturbation plot for the effect of independent variables (I) and 3D response surface plots (II-IV) on the percent elongation of quercetin bioplastics.

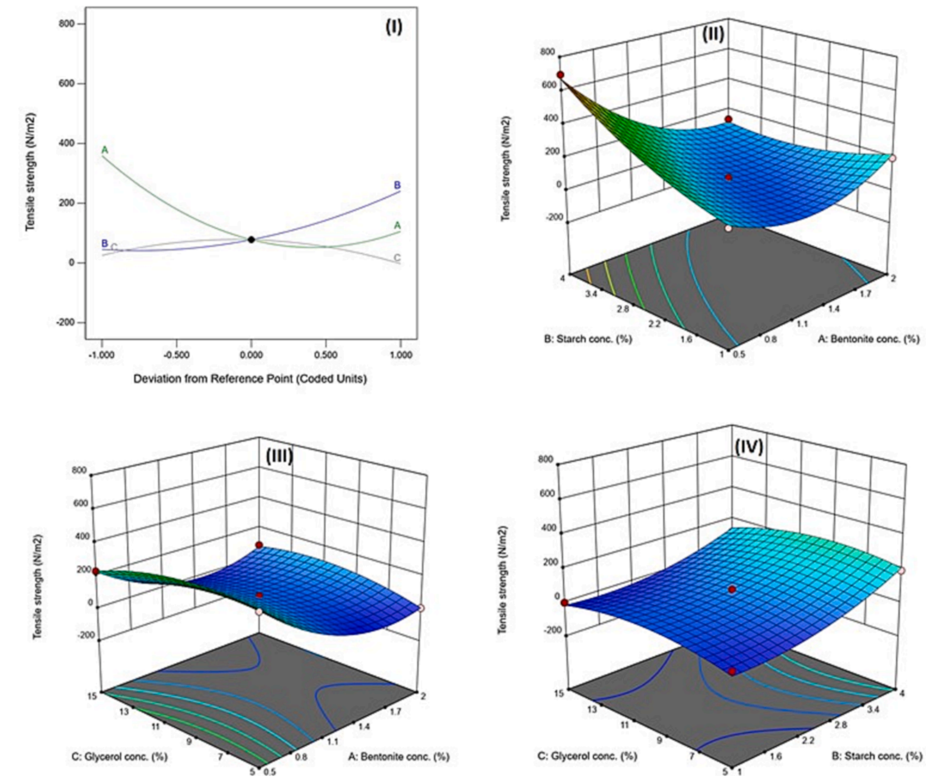

Fig. 3. Perturbation plot for the effect of independent variables (I) and 3D response surface plots (II-IV) on the tensile strength of quercetin bioplastics.

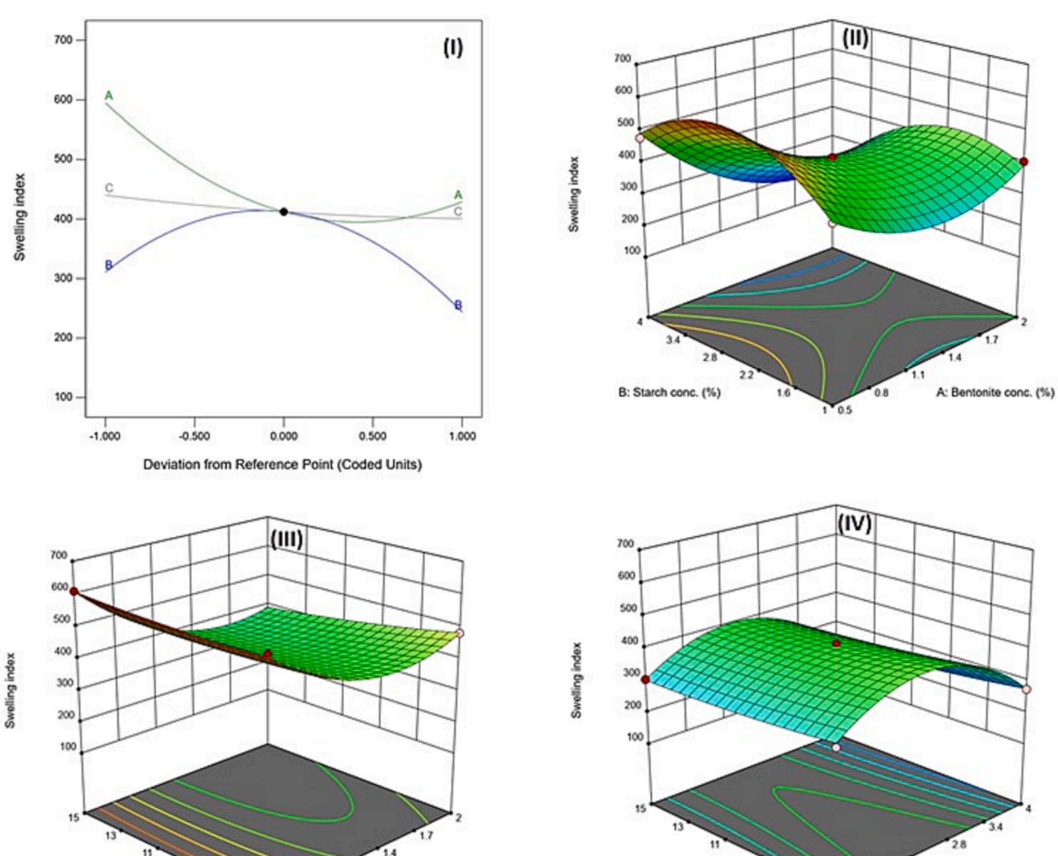

Fig. 4. Perturbation plot for the effect of independent variables (I) and 3D response surface plots (II-IV) on the swelling index of quercetin bioplastics.

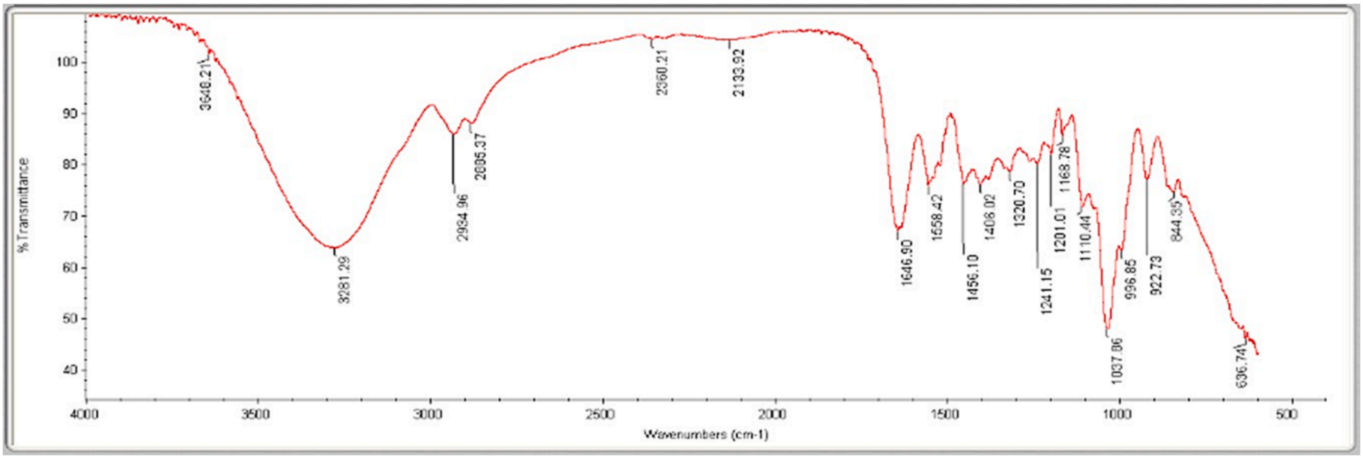

Fig. 5. FTIR Spectra of quercetin enriched bentonite-reinforced starch-gelatin bioplastic.

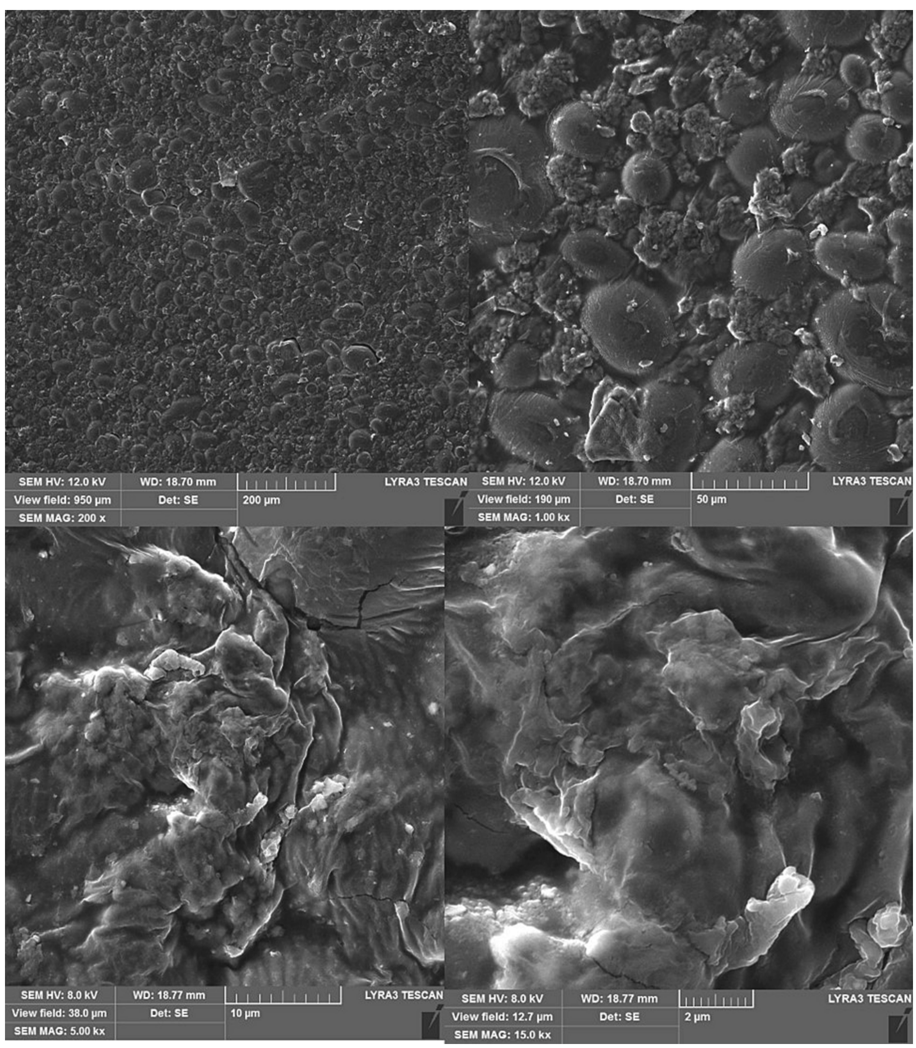

Fig. 6. SEM photograph of the optimized bioplastic film at different magnifications.

Table 3
Antibacterial and antifungal activities of film samples.

No.	Zone diameter (mm)				
	Gram-positive bacteria		**Gram-negative bacteria**		**Fungus**
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
C	12	–	10	10	14
F	14	–	22	18	12
NOR (10 µg)	N/A	N/A	16	16	N/A
KF (30 µg)	11	12	N/A	N/A	N/A
NYS (20 µg)	N/A	N/A	N/A	N/A	15

C: Control bioplastic film, F: Quercetin enriched bioplastic Film, NOR: Norfloxacin, KF: Cephalothin, NYS: Nystatin, N/A: not applicable.

4. Discussion

According to the ANOVA analysis performed at the 95 % level of significance, the linear term A corresponding to the bentonite concentration exhibited a significant effect on all the studied responses. In addition, the linear term B corresponding to the starch concentration exhibited a significant effect on the percent elongation, tensile strength, and swelling index, while the linear term C corresponding to the glycerol concentration exhibited a significant impact on both moisture uptake and swelling index. Furthermore, the results revealed that both percent moisture uptake and percent elongation were significantly affected by all the binary interactions terms (AB, AC, and BC), in addition to the quadratic term A^2. On the other hand, both tensile strength and swelling index were significantly affected by the interactions between bentonite concentration and either starch or glycerol concentrations (AB and AC, respectively), in addition to the quadratic terms A^2 and B^2. Also, the quadratic term C^2 showed a significant effect on tensile strength.

The relationship between each response and the studied variables was presented as a quadratic polynomial equation, which was generated by the software as follows:

$$R_1 \text{ (Percent moisture uptake)} = 10.76 - 3.27\,A - 0.30B + 3.10C - 2.68\,AB + 3.73\,AC - 3.48\,BC + 5.68\,A^2 + 0.3825\,B^2 + 0.4325\,C^2 \quad (1)$$

$$R_2 \text{ (Percent elongation)} = 36.60 - 48.00\,A + 8.00B - 1.00C + 15.50\,AB + 3.50\,AC - 5.50\,BC + 45.45\,A^2 - 2.05\,B^2 + 1.45\,C^2 \quad (2)$$

$$R_3 \text{ (Tensile strength)} = 78.08 - 126.52\,A + 97.46B - 14.01C - 150.93\,AB + 48.33\,AC - 19.25\,BC + 154.31\,A^2 + 65.19\,B^2 - 66.51\,C^2 \quad (3)$$

$$R_4 \text{ (Swelling index)} = 412.12 - 83.19\,A - 33.51B - 19.66C - 63.33\,AB - 25.30\,AC - 3.63\,BC + 100.00\,A^2 - 134.80\,B^2 + 8.18\,C^2 \quad (4)$$

The influence of the variables and the interaction among them are presented as perturbation and response surface plots. The perturbation plot serves as a means to compare the impact of various factors at a specific location within the design space. It illustrates how the response changes while keeping all other factors constant, with the midpoint (coded as 0) set as reference value for all factors. A factor with a steep slope or noticeable curvature in the plot indicates that the response is highly sensitive to variations in that factor. Conversely, a relatively flat line signifies that the response is not significantly affected by changes in that specific factor. Accordingly, the perturbation plot can assist in identifying the factors that exert the greatest influence on the response. Moreover, the direction of the line indicates whether the relation between response and variable is direct or inverse. The response surface plot is a visual representation in three dimensions (3D) that displays the response as it varies in relation to different combinations of numerical factors. It provides a means to illustrate the interdependencies between the response and the investigated variables.

As obvious from the perturbation plot, Fig. 1-I, the percent moisture uptake was negatively influenced by the bentonite concentration, while it was positively influenced by that of glycerol at $P \leq 0.05$. Such observation is supported by the negative coefficient of the term A and the positive coefficient of the term C in equation (1). Further, a significant binary interaction between each two variables was detected as displayed in Fig. 1-II to 1-IV.

Regarding both percent elongation and tensile strength, an inverse relationship was observed with bentonite concentration, while a direct relationship was observed with starch concentration, Figs. 2 and 3, respectively. Such observation is supported by the negative coefficient of the term A and the positive coefficient of the term B in both Eqs. (2)

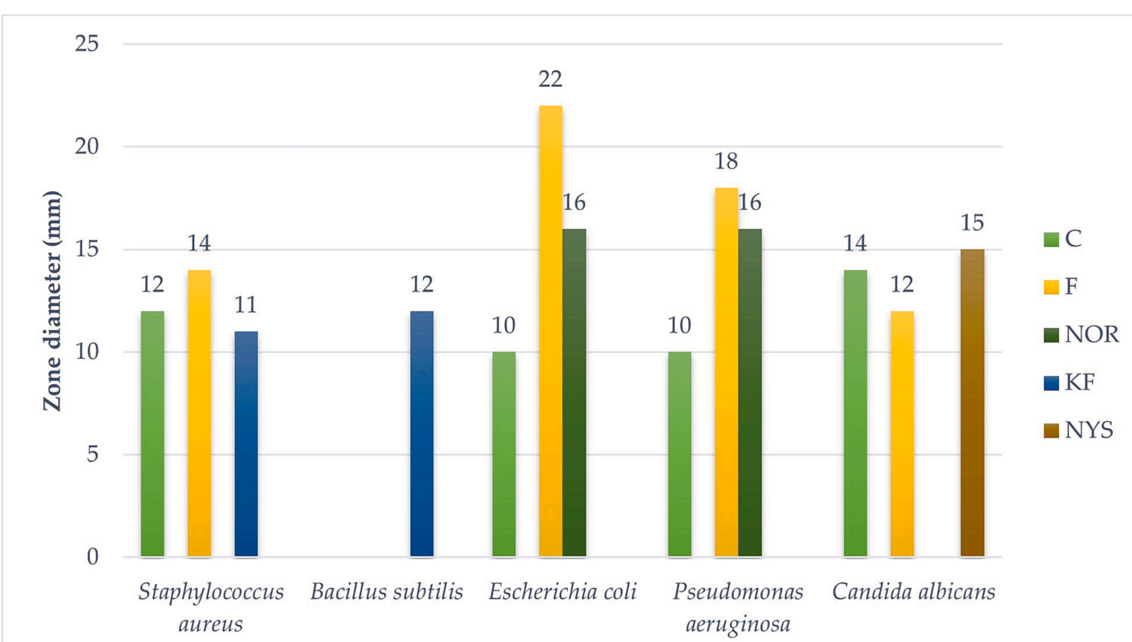

Fig. 7. Zone diameter interpretative histogram for film samples versus five microbial standard strains.

and (3). In addition, the relatively higher magnitude of the term A compared to B reveals a more significant impact of the bentonite concentration on the tensile strength as compared to starch concentration.

As shown in Fig. 4, the swelling index decreases with increasing bentonite and glycerol concentrations, while for starch, a concentration dependent pattern is observed, where the swelling index increases with increasing starch concentration at lower levels and decreases at higher levels. Although the three variables significantly affect the swelling index of the prepared bioplastics, the bentonite concentration was the most influential factor as evidenced by the highest coefficient of the linear term A in Eq. (4).

Regarding the characterization of the optimized film, it was found that as the thickness was increased tensile strength also increased with reduced capacity of elongation. Additionally, as the concentration of ingredients increased, the film thickness also increased. Thickness is also influenced by the area of casting mold as well as the volume of solution pored for casting. Furthermore, a relatively less water transmission rate was observed because of the presence of bentonite. Bentonite act as filler and barrier for water vapor transmission (Joshi and Garud, 2021; Kusum Devi et al., 2003). Another character of the prepared biofilm is its folding endurance, which intends to evaluate the capacity of the bioplastic to survive break-up when as per the usage or application. The higher value of folding endurance suggests that the prepared bioplastic film would maintain its integrity as well as shape during the desired usage.

Regarding the FTIR spectrum of the optimized formulation, the peaks at 2885 cm^{-1}, 1037 cm^{-1}, and 1558 cm^{-1} are found to be characteristic for glycerol as previously reported (Dou et al., 2016; Musa et al., 2013; Omrani-Fard et al., 2020). Many peaks corresponding to different functional groups are also observed due to presence of starch in the bioplastic film. This includes a broadband due to − OH stretching at 3281, aliphatic $-CH_2$ at 2934 cm^{-1}, C = C stretching at 1646 cm^{-1}, −COOH stretching at 1456 cm^{-1} and etheric bond at 1168 cm^{-1} (Ahmad et al., 2012; Omrani-Fard et al., 2020).

As far as the morphological characteristics of the prepared bioplastic film are concerned, SEM image depicts that it contains insoluble remnants or ghosts due to swelling of starch granules. A continuous uniform pattern confirms the sufficient tensile strength. The analysis revealed that bioplastics have grooves and some ridges and appear as an irregular structure. The findings are consistent with previous reports (Harunsyah, 2018) stating that non-homogenous surface was observed for bioplastic from cassava starch with glycerol and clay. Amin et al. also observed a non-homogenous nature with voids, holes, edges and ridges on composite bioplastic made out of titanium dioxide nanoparticles with corn starch (Amin et al., 2019).

Biodegradability is an important parameter as per the new environmental regulations and also the excessive demand of biodegradable and environmentally friendly products these days. Bentonite can potentiate the effect of decomposition where the hydrolysis reaction can be catalyzed by the aluminium Lewis acid site in the nanoclay (Shayan et al., 2015). It is worth noting that the substrate polarity and water absorption capacity is also increased by the addition of bentonite, which in turn leads to increased rate of heterogenic hydrolysis decomposition (Nazir et al., 2016). Moreover, the bentonite-reinforced starch-gelatin based bioplastic accelerated the attack and damage by microorganism into the bulk of bioplastic due to increased hydrophilicity by the elimination of regular crystalline structures. The heterogenous hydrolysis is initiated by the –OH groups in the silicates layers by absorbing water and lead to decomposition into tiny fragments and ultimately disappeared in the soil (Lee et al., 2005). Biodegradation in soil is dependent on the diffusion of living microbes in watery soils (Shayan et al., 2015; Wahyuningtiyas and Suryanto, 2018).

Finally, the results of antimicrobial activity indicate that bentonite-reinforced starch-gelatin based bioplastic films are able to reduce the presence of microorganisms. The antimicrobial activity showed the highest activity against Escherichia coli followed by Pseudomonas aeruginosa and Staphylococcus aureus. The incorporation of quercetin resulted in a larger zone of inhibition as compared to placebo films. This could be attributed to the fact that the incorporation of quercetin in the bioplastic films might interfere with the cell division, replication and also lowering the cell vitality of bacteria. additionally, there might be an interaction between negatively charged bacterial surface and the cationic polymers present in the film leading to an adsorption effect. This in turn can cause diffusion of these polymers across the cell membrane and leads to cell death by the leakage of cytoplasmic constituents (Mandapalli et al., 2017; Souza et al., 2015).

5. Conclusion

Quercetin incorporated bentonite-reinforced starch-gelatin based bioplastic films were formulated and optimized using Box Behnken experimental design. Based on numerical optimization following desirability technique, the anticipated levels of the optimized formulation that could yield maximized moisture uptake percentage, elongation percentage, tensile strength, and swelling index were found out. The optimum levels of variables were found to be 0.50 % bentonite, 3.74 % starch, and 5.00 % glycerol to achieve the desirable goals by a desirability of 0.917. It was found that for the optimized films the observed responses for moisture uptake percentage, elongation percentage, tensile strength, and swelling index were 26.02 %, 145.14 %, 612.85 N/m^2 and 529.92 respectively Scanning electron microscopy revealed the non-homogenous nature of the film. A folding endurance value of 122 ± 3.5 shows the sufficient endurance of the film and 97.27 % drug content uniformity ensures the uniform and efficient entrapment of quercetin in the optimized film. Moreover, the test for biodegradability was satisfactory. The antimicrobial activity showed the highest activity against Escherichia coli followed by Pseudomonas aeruginosa and Staphylococcus aureus. The overall results of the study revealed that quercetin loaded bentonite-reinforced starch-gelatin based biofilms could be used as in ecological friendly active food packaging applications as well as pharmaceutical application with significant antimicrobial properties.

Funding

The project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant no. (G: 380–249-1442). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

CRediT authorship contribution statement

Hibah Mubarak Aldawsari: Conceptualization, Project administration, Funding acquisition. **Sabna Kotta:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Hani Z. Asfour:** Validation, Formal analysis, Supervision. **Sajith Vattamkandathil:** Methodology, Writing – review & editing. **Mahmoud Abdelkhalek Elfaky:** Methodology, Writing – original draft. **Lubna Y. Ashri:** Formal analysis, Writing – review & editing. **Shaimaa M. Badr-Eldin:** Validation, Formal analysis, Writing – original draft, Project administration.

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.

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