



Preparation of nanobiocomposite 45S5 bioactive glass with nano-hydroxyapatite-chitosan powders and characterization, mechanical and biological properties *in-vitro*

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In bone tissue engineering, combining bioceramics and biopolymers is highly promising. In this study, bioactive glass (BG), nano-hydroxyapatite (nHA), and chitosan nanoparticles (CS) are used to develop (BG-HA-CS) nanobiocomposites with enhanced bio-functional properties for promoting bone tissue formation and regeneration due to their excellent biocompatibility and ability to support cell proliferation. BG is synthesized at nano-scale using the sol-gel method, and different ratios of BG/HA/CS are prepared using hydraulic pressing. The nanobiocomposites are sintered at 1000 °C under controlled heat treatment. Characterization techniques including X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), and Fourier transform infrared spectroscopy (FT-IR) are used to evaluate apatite formation in simulated body fluid (SBF). In vitro bioactivity and biodegradation are assessed by soaking samples in SBF for 1, 7, 14, 21, and 28 days. Physical properties such as apparent porosity, water absorption, and apparent density, along with mechanical properties (Vickers micro hardness), are evaluated. Antibiofilm activity is tested against Gram-positive and Gram-negative bacteria. Results showed porosity of 57.5%, water absorption of 41.3%, density of 1.29 g/cm³, and micro hardness of 72.52 MPa at 20% CS content. Increasing CS enhanced bioactivity and biodegradation. The nanobiocomposites exhibited high antibiofilm activity (93.5% against *Staphylococcus aureus* and 87.8% against *Klebsiella pneumoniae*), indicating their potential for bone repair and regeneration.

Keywords: Biological activities; Nanobiocomposites; Bioactive glass; Nano-Hydroxyapatite.

1. INTRODUCTION

Bone is essential and serves several functions inside the body; it supports the whole human structure and offers rigidity, strength, and a degree of flexibility to the living body. Additionally, its structural function, bone serves as a metabolic reservoir, storing calcium, phosphate, magnesium, and other elements [1]. Bone defects caused a great challenge to orthopedic surgeons despite the self-healing capacity of bone tissue, repair of a significant bone defect requires the use of a bone filler. The extent and severity of the defect are the primary factors that dictate the clinical techniques that are utilized for dealing with of bone healing problems. These techniques may include the implantation of graft material at the site of the defect to accelerate a repair procedure, which can be synthetically fabricated [2]. Biomaterials are synthetic materials produced by humans that are used to repair or restore bodily functions that have been injured or damaged. To serve as a bone tissue substitute, a biomaterial must be non-toxic and capable of generating a hydroxyapatite (HA) coating on its surface, which decreases the possibility of rejection [3,4]. Ceramic materials are non-metallic solids, inorganic substances composed of natural or synthesized raw substances, processed through heat and often pressure, are utilized across various industries according to their versatile characteristics including electrical insulation, thermal insulation, and corrosion resistance [5-7]. Its essential biomaterials sources for biomedical engineering applications. Ceramics designed to come into contact with living tissues are known as bioceramics [8,9]. These materials are offering characteristics including biological compatibility, bioactivity, and strong mechanical properties. Bioceramics are used in medical and dental applications where they replace or augment hard tissues like bones and teeth, Drug Delivery and Tissue Engineering [10, 11]. Bioactive ceramic improves the possibility of production of hydroxyapatite (an inorganic component that comes from natural bone), on biomaterial surfaces [12]. Bioactive glasses (BGs) are the most extensively bioceramics, since they interact with the biological environment, promoting tissue adhesion and gradually dissolving as new tissue regenerates and heals [13, 14], The most often utilized form of BG is 45S5, which is made up of (6 % P₂O₅, 24.5 % CaO, 24.5 % Na₂O, and 45 % SiO₂). This form of bone graft is osteoconductive, osteogenic, and biodegradable [15]. Hydroxyapatite (HA) is a bioceramic that is a primary in-organic constituent found in natural bones, contributing to 65% of total bone mass. It has been widely utilized in clinical uses according to its superior biological compatibility, bioactivity, osteoconductivity, and also osteoinductive properties, as well as its chemical similarity to bone [16]. The natural polymer chitosan (CS) is derived from a shell of crustaceans, acts as a hydrophilic biopolymer that is highly biocompatible and enhance cell adhesion, proliferation, promoting bone formation offers several beneficial properties for bone repair and healing [17,18]. Chitosan enhances cell adhesion and proliferation because of to its chemical similarity to glycosaminoglycans, which are primary constituents of bone [19]. The combination of nanobiocomposites consist of ceramics and polymers matrix (bioactive glass, hydroxyapatite and chitosan) can showed enhanced biological, chemical, and physical characteristics for excellent bone tissues regeneration, in addition increase osteoconductivity and biodegradability with significant achieve better mechanical response and a faster bioactive action [20]. The term Biofilms are complicated microbial communities enclosed in an external macro-Molecular matrix synthesized by their inhabitants. Biofilms are significantly important for the overall health due to their major role in specific infectious diseases and their importance in various infections associated with medical devices [21]. This research aimed to create a nanobiocomposite material consisting of bioactive glasses/ hydroxyapatite/ chitosan for repairing bone and to obtain excellent biocompatibility and good porosity. Also, to provide promising solutions to establish the optimum equilibrium between flexibility and mechanical strength, thereby enhancing osseointegration and improving the performance of bone repair in clinical applications. In addition, evaluate the anti-biofilm activity of BHC nanobiocomposite.

2. MATERIALS AND PROCEDURES

2.1. Manufacture of Nanobiocomposites

45S5 bioactive glass is synthesized using sol-gel technique, precursors including Tetraethyl orthosilicate (23.5 ml) and Triethyl phosphate (3.35 ml) are added dropwise to an acidic solution (10.4 ml) under magnetic stirring, followed by Sodium nitrate (16.5 g) and Calcium nitrate tetrahydrate (15.5 g). The mixture is stirred for 1 h until a clear sol is obtained and then transformed into gel by Polycondensation. The gel is dried at 60 °C for 12 hrs. and heat-treated at 200 °C for 5 hrs. and 700 °C for 2 hrs. to obtain BG 45S5 powder. Then BG mixed with different ratios of each nHA powder 10, 20, 30, 40 (wt. %) (Hualanchem, purity 96%, made in China), had nanoparticle size ± 20 nm and chitosan powder 5,10,15,20 (wt. %) (Nanochemazone, purity 90.33%, made in Canada), had nanoparticle size 30-50 nm. After that a mixture are stirred for 12 hrs. to ensure homogeneity after that nanobiocomposite are prepared by uniaxial pressing under a compaction pressure of 312 MPa using a hydraulic press in a cylindrical mold. The nanobiocomposites specimens had disc shape with diameter 20 mm and 4 mm thickness sintered using furnace (BSM-F40, china) at 1000°C to promote densification this is consistent with previous study [22]. The mixture ratios of BG, nano-HA and CS used to prepare the nanobiocomposites samples as shown in (Table 1 and Figure 1) illustrated Flow chart of preparation nanobiocomposites specimens.

Table 1 Mixture ratios of (Bioactive glass, nano-Hydroxyapatite and Chitosan) used to prepare nanobiocomposites samples.

Nanobiocomposite specimens Code	Composition of each element for nanobiocomposite (wt. %)		
	Bioactive glass (45S5) BG	Hydroxyapatite (nHA)	Chitosan (CS)
BHC1	85	10	5
BHC2	70	20	10
BHC3	55	30	15
BHC4	40	40	20

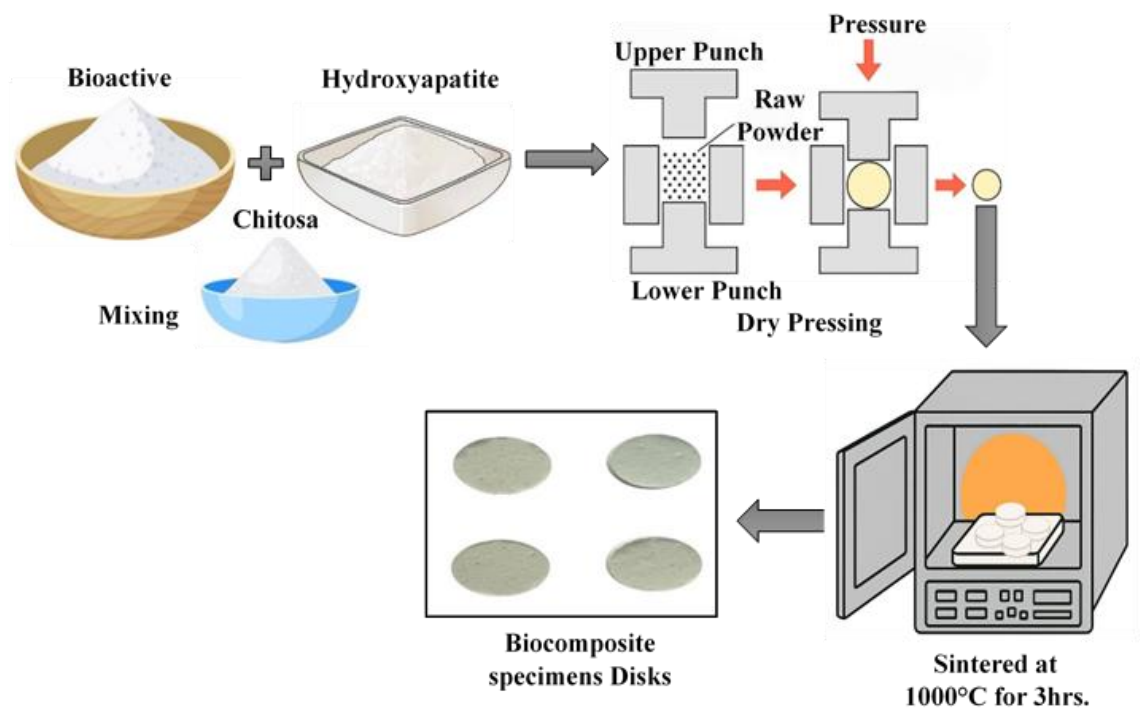


Figure 1 Flow Chart of Preparation Nanobiocomposites Specimens.

2.2. PREPARATION OF IMMERSION SIMULATED BODY FLUID (SBF):

A simulated bodily fluid utilized in this investigation is procured commercially and prepared according to the Kokubo and Takadama method [23]. The solution's ion concentrations are presented in (Table 2) [24].

Table 2 Artificial (SBF) Ion Concentrations Compared to Human Blood Plasma.

Ions	SBF (mmol/l)	Human Blood Plasma (mmol/l)
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ⁺	1.5	1.5
Ca ⁺	2.5	2.5
Cl ⁻	148.8	103.0
HCO ₃ ⁻	4.2	27.0
HPO ₄ ²⁻	1.0	1.0
SO ₄ ²⁻	0.5	0.5

2.3. Immersion

Nanobiocomposites specimens from bioactive glass, Hydroxyapatite and chitosan (prepared in paragraph 2.1). It is immersed in artificial SBF for periods of 1, 7, 14, 21, and 28 days, after which it is removed from the artificial simulated body fluid and then ished with distilled water well.

3. CHARACTERIZATION OF NANOBIOCOMPOSITES BHC

The samples of Nanobiocomposite are submitted to (XRD) by using a diffractometer (PW1730, Philips, Netherlands) that utilizes Cu-K α radiation ($\lambda = 1.5405\text{\AA}$). The patterns of diffraction have been determined in the 2 theta range, which extended from 20°- 80°, with a step size of 0.05. While For the purpose of performing structural characterizations of the Nanobiocomposites samples, (FT-IR) spectroscopy (PerkinElmer Spectrum IR-10.6.2-USA) is utilized in conjunction with a Fourier transform imaging system. The wavenumber range utilized for the recording of the spectrum is between 500 and 4000 cm^{-1} . Also, a morphology of the surface and topography of Nanobiocomposites are observed using a FESEM (MIRA 3 TESCAN, Czech) both before and after the process of immersion in SBF solution.

4. PHYSICAL AND MECHANICAL PROPERTIES FOR NANOBIOCOMPOSITES

is include apparent porosity (A.P), water absorption ratio (W.A), apparent density (A.D), and Vickers Micro Hardness, Powders are formed and burned, resulting in the formation of pores in each ceramic body. The porosity corresponds with water absorption and inversely with an apparent density [25]. And to determine the material's hardness, a constant force of (9.8 N) is applied for a period of ten seconds during the test. According to the American specifications AS-TM (C 1327-99), the investigation is conducted at the University of Technology's Department of Production and Minerals Engineering.

5. BIOLOGICAL EVALUATIONS IN VITRO

pH change and weight loss to investigate the bioactivity evaluation and biodegradation behavior *in vitro*, nanobiocomposites samples are immersed in a simulated body fluid (SBF) and maintained in an incubator shaker at 37°C for durations of 1, 7, 14, 21, and 28 days. The temperature is sustained at 37°C during the experimental period. At the conclusion of the predetermined time periods, by using a pH -meter (Digital pH -meter/ PH-600, Metravi, India), the SBF solutions' pH is measured. Additionally, the weight of the nanobiocomposite specimens is recorded both before and after they are placed in artificial simulated body fluid (SBF). Then, Anti-biofilm activity determined according microtiter plate (MTP) technique to evaluate the inhibitory concentrations of nanobiocomposites BHC against biofilm forms with a 1:1:1 ratio from stock solution [26]. Sterile flat-bottom polystyrene microtiter plates (100 wells) are used to evaluate biofilm formation, which begins with bacterial adhesion to the well surface and is influenced by tested chemicals. Bacterial isolates are activated overnight on nutrient agar at 37 °C, then suspended in sterile normal saline to achieve a 0.5 McFarland standard (1.5×10^8 CFU/ml). Each well contained 90 μl of BHI supplemented with 2% glucose and 10 μl of bacterial suspension. After incubation, wells are emptied, ished three times with sterile PBS to remove non-adherent cells, and dried. Then, 150 μl of 1% crystal violet is added and incubated at room temperature for 20 min. Excess stain is removed by ishing with PBS, and adherent cells are re-solubilized with 150 μl of 95% ethanol. Optical density (OD) is measured at 580 nm using a microplate reader. All experiments are performed in triplicate and repeated three times.

6. RESULTS AND DISCUSSION

6.1. Characterization of nanobiocomposites bhc

Figure (2), showed an XRD pattern for the prepared nanobiocomposites specimens. The X-ray diffraction analysis indicates that the characterization of HA diffraction peaks can be found at $2\theta = 26.7^\circ$, 31.4° , and 34.2° . The characteristic peak approximately at 23.7° may be attributed to amorphous phases or residual products resulting from the thermal decomposition of chitosan. This

indicates a reduction in CS due to thermal decomposition, whereas BG/HA phases remained unchanged. The overall structural characteristics of (BG/HA/CS) nanocomposites material is preserved, and no new crystalline phases is formed. This is consistent with research [27].

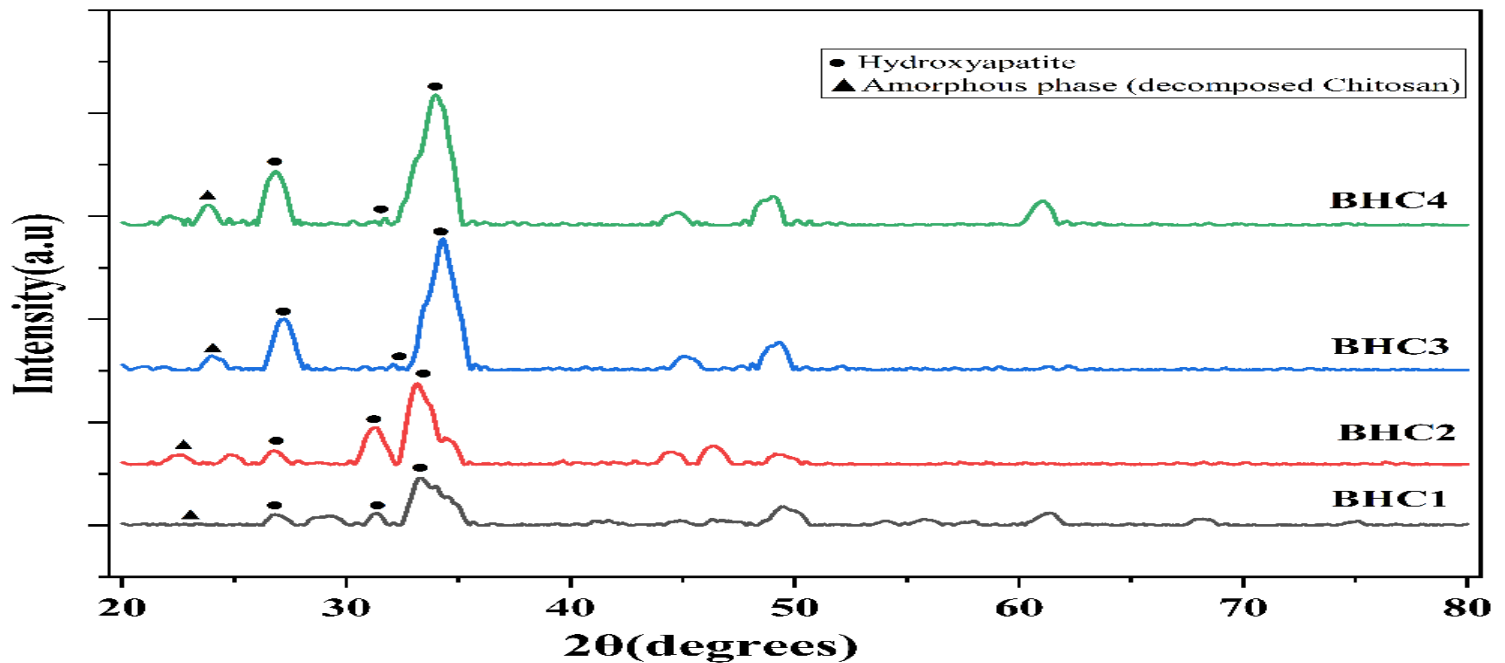


Figure 2 X-Ray Diffraction of the Prepared Nanobiocomposites Specimens (BHC1, BHC2, BHC3, and BHC4).

Figure 3, showed the FTIR spectrum of the prepared nanobiocomposite specimens prior to soaking in SBF. The results indicated the peaks of absorption of CO_3^{2-} are located at approximately 1412, 1560, additionally 1637 cm^{-1} . However, a peak of BG, observed at 701 cm^{-1} , dispersed across the nanobiocomposite and is attributed to (Si–O–Si) stretching vibrations. A phosphate group (PO_4^{3-}) hydroxyapatite (HA) is responsible for an absorption peak at 567, 601, 921, and 1080 cm^{-1} . Additionally, the group of amines (N–H) in CS is discovered at a frequency of approximately (3300–3400 cm^{-1}). FTIR spectrum confirms the presence of HA and suggests the existence of residual functional groups associated with chitosan decomposition products. HA chemical structure and residual products formed during thermal decomposition from chitosan is not changed by bioactive glass. This is consistent with research [28]. And, (Figure 4) showed the (FT-IR) spectral of the prepared nanobiocomposites specimens after soaking in an SBF solution, The results demonstrate that the development of the hydroxyapatite (HA) layer has occurred due to the dissolving of a BG particles, leading to release and diffusion of calcium and phosphorus ions. Additionally, the exchange of ions contributes to the production of these apatite layers. The dissolution of BG is apparent in the FT-IR spectral, as indicated by the decrease in silica peaks intensity. This phenomenon is attributed to the initiation of the HA layer after soaking in SBF solution. The FT-IR spectrum shows that BG-related (Si–O–Si) elongate peak patterns reduced after 28 days of soaking in the SBF solution, as the amplitude of the silicate peaks decreased with continuous soaking, while the phosphate bands' intensity increased. Additionally, the carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) peaks associated with the production of hydroxyapatite (HA) in SBF solution are observed. The carbonate peaks that are discovered at 680 and 1415 cm^{-1} are found to be intensified. Furthermore, the phosphate group exhibited broad and more intense peaks at 550 and 980 cm^{-1} respectively. This is consistent with research [29, 30]. Functional groups infrared frequencies on a nanobiocomposites BHC surface prior to and after SBF reaction are listed in (Table 3).

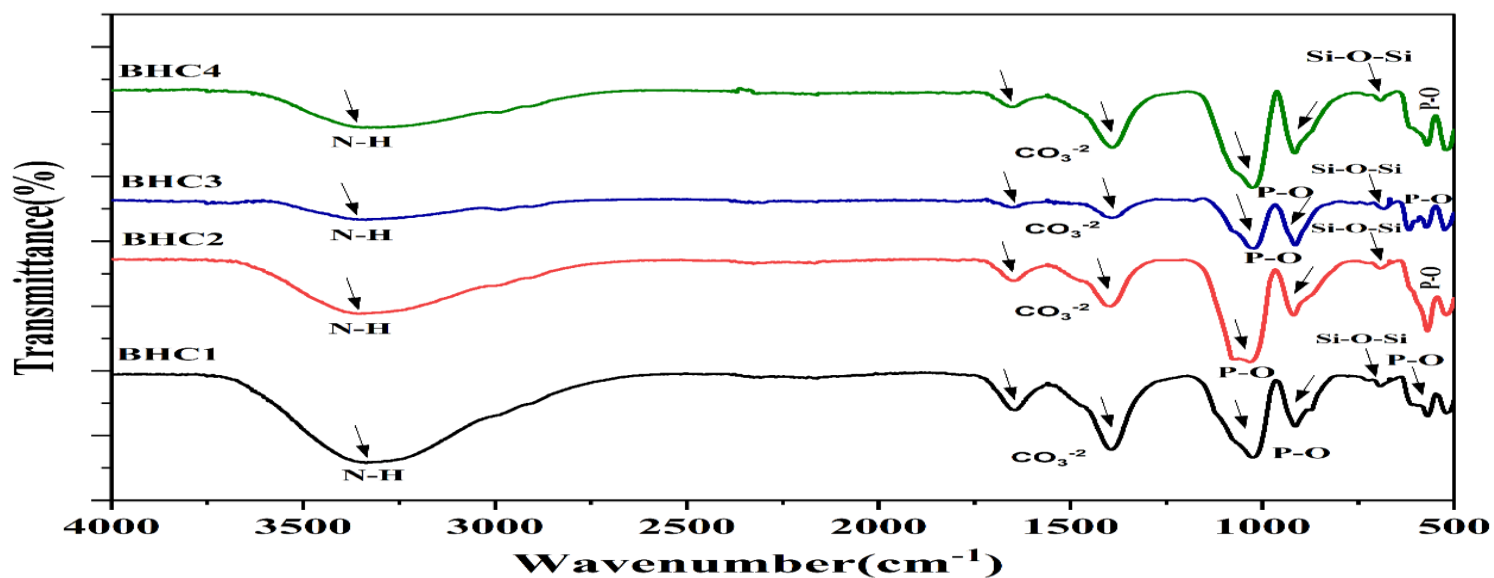


Figure 3 FT-IR Spectrum of the Prepared Nanobiocomposites Specimens (BHC1, BHC2, BHC3, and BHC4) Prior to soaking in SBF.

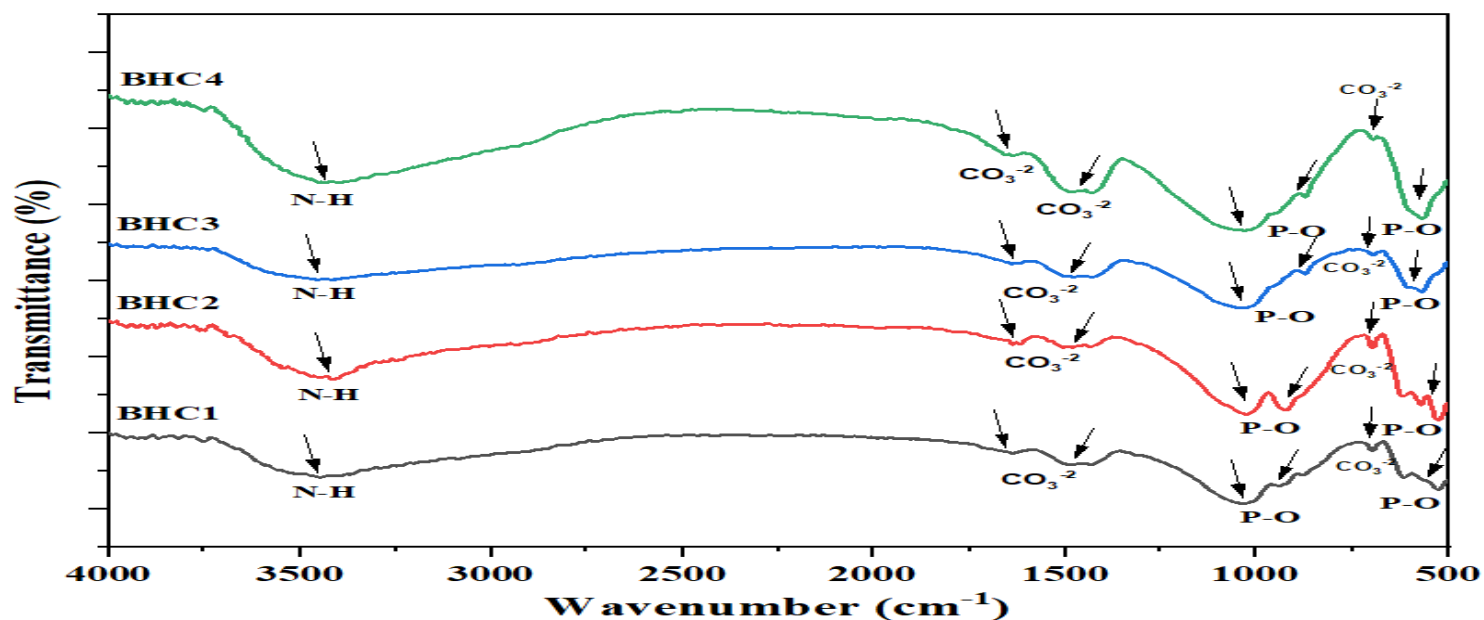


Figure 4 FT-IR Spectrum of the Prepared Nanobiocomposites Specimens (BHC1, BHC2, BHC3, and BHC4) after Immersion in SBF.

Table 3 Functional Groups Infrared Frequencies on a Nanobiocomposites BHC Surface Prior to and After SBF Reaction.

Bands	Wavenumber cm^{-1}
(Si-O-Si) symmetric stretching	701
(P-O)	567, 601, 921, and 1080
carbonate groups (CO_3^{2-})	1412, 1560, and 1637
(N-H) groups	3300-3400

Figures 5 and 6, showed the morphology of the surface for the fabricated nanobiocomposites specimens BHC prior to and after immersion in simulated body fluid, the microporous membrane structure of the nanobiocomposites is illustrated by the presence of interconnecting open porous. FT-IR measurement validated the existence of both BG and HA particles within polymeric matrix of nanobiocomposites, which displayed a uniform distribution of both types of particles. A combination of BG and HA particles can be seen during the FESEM images in (Figure 5). HA particles are induced and imbedded within the polymeric matrix to exhibit semi-spherical morphologies. The mechanical stability of the nanobiocomposites is eventually improved as a result of a Particles of HA and their aggregates filling pores that are present inside the polymeric matrix, this is consistent with the results of [30, 31].

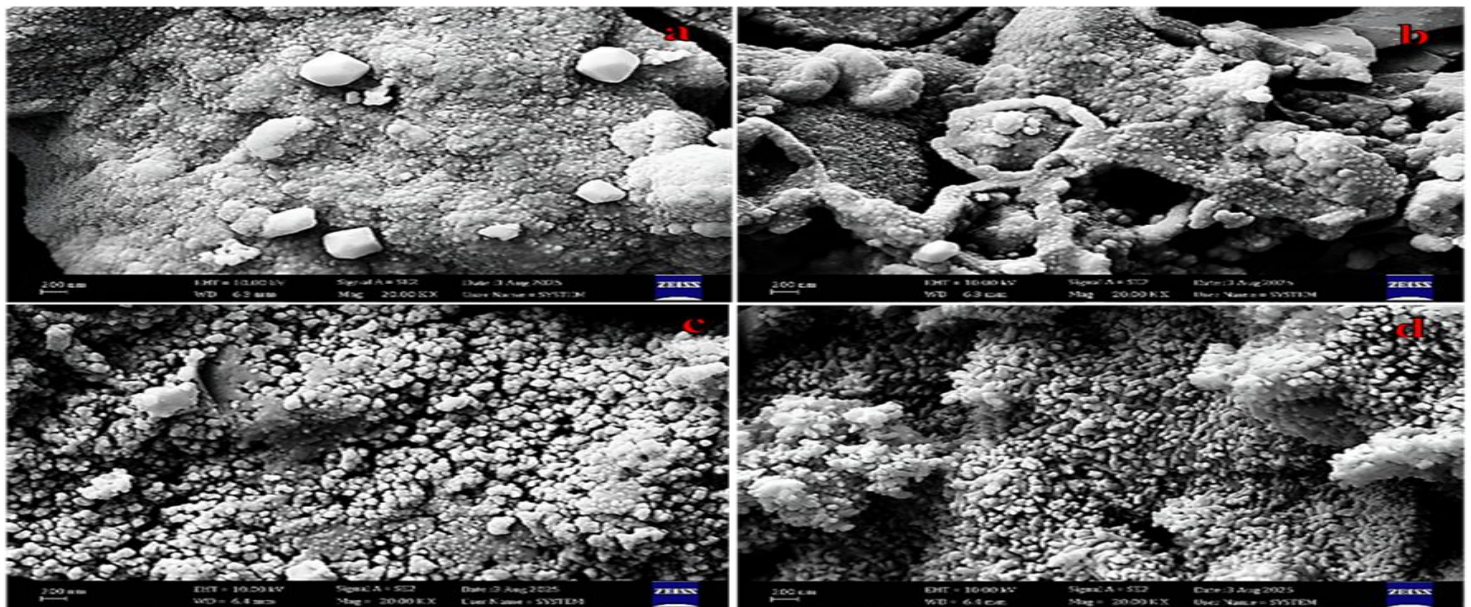


Figure 5 FE-SEM Images of the Prepared Nanobiocomposite Specimens before Immersion in SBF (a) BHC1 (b) BHC2 (c) BHC3 (d) BHC4.

After soaking in SBF, the results, which are illustrated in (Figure 6) observed presence of the clear spherical apatite crystals are seen on the surface, attributed to a release and dispersion of calcium and phosphorus ions resulting from the BG particles' decomposition. Subsequently, an ionic exchange process promotes the development of an appetite- like structure on the surface of the nanobiocomposites. After a period of 28 days of immersion in simulated body fluid, the surface of the nanobiocomposites are covered with a coating of flakes-like hydroxyapatite (HA) crystals, as demonstrated by the images. In summary, the nanobiocomposites surface is partially covered by HA [32]. Form (c and d) images nanobiocomposites, shows the existence of rich spherical shapes stacked over one another to produce an appetite layer that is similar to bone for both nanobiocomposites. Nanobiocomposites BHC's pores are linked together, resulting in a 3-D structure that is favorable to the growth of cells and tissues. This is consistent with research [27,33].

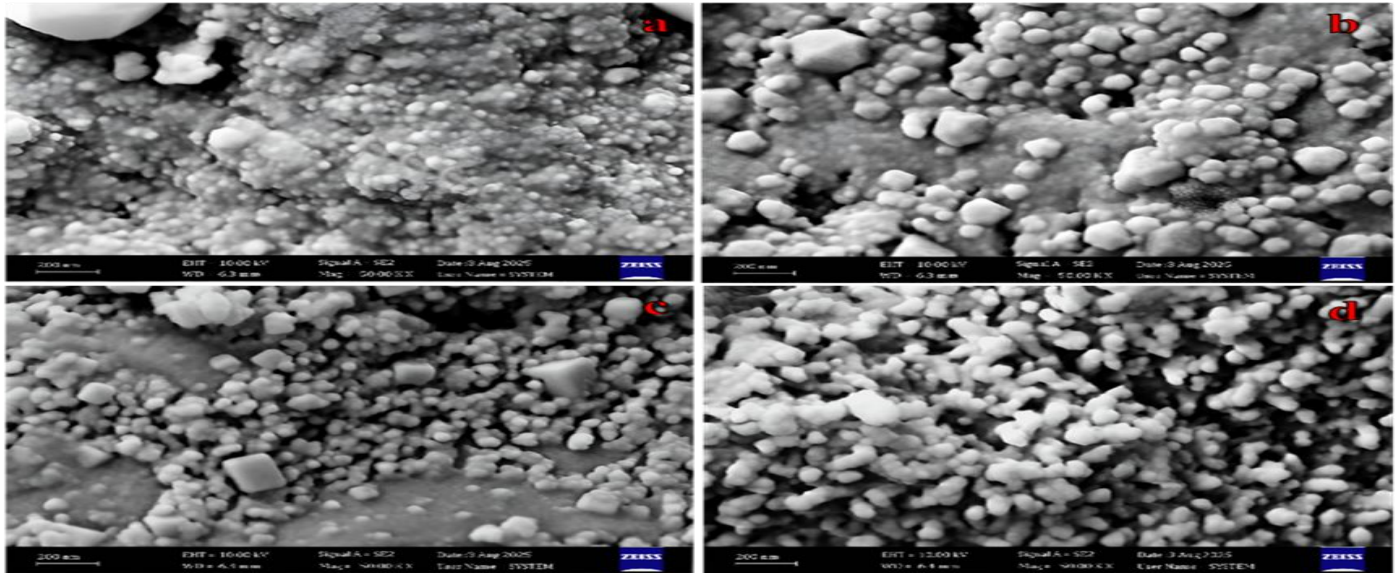


Figure 6 FE-SEM Images of the Prepared Nanobiocomposite Specimens after to soaking in SBF for 28 Days (a) BHC1 (b) BHC2 (c) BHC3 (d) BHC4.

6.2. Nanobiocomposites' physical and mechanical characteristics

An apparent porosity and apparent density are shown in (Figure 7a), figure demonstrates the effects of chitosan (CS) ratio on the apparent porosity of nanobiocomposite specimens (BG-HA). The result shows the increasing addition of chitosan (CS) ratio in (BG-HA) nanobiocomposites increases in the apparent porosity of nanobiocomposites specimens more than nanobiocomposites consist of (BG+HA) only, due to pore formation resulting from the thermal decomposition of chitosan during sintering, which creates additional voids within the nanobiocomposite structure this leads to increase in the apparent porosity. As a result, apparent density is decreased because of chitosan acts as a spacer between the bioactive glass and hydroxyapatite particles, reducing of the particle packing and creating more voids between particles. Apparent density has an inverse correlation with apparent porosity and water absorption; therefore, density reduces as porosity increases. This is in consistent with research [34]. From (Figure 7b), the results show the increasing addition of chitosan (CS) ratio in (BG-HA) nanobiocomposites increases in the water absorption ratio of nanobiocomposites specimens more than nanobiocomposites consist of (BG+HA) only, due to the hydrophilic nature of chitosan and including functional groups such as amino ($-NH_2$) and hydroxyl groups ($-OH$) have the ability to create a strong hydrogen bond with molecules of water. In addition, chitosan is biodegradable and its polymeric chains undergo degradation when immersion it's in aqueous environments facilitates more water penetration this consistent with other researches [35].

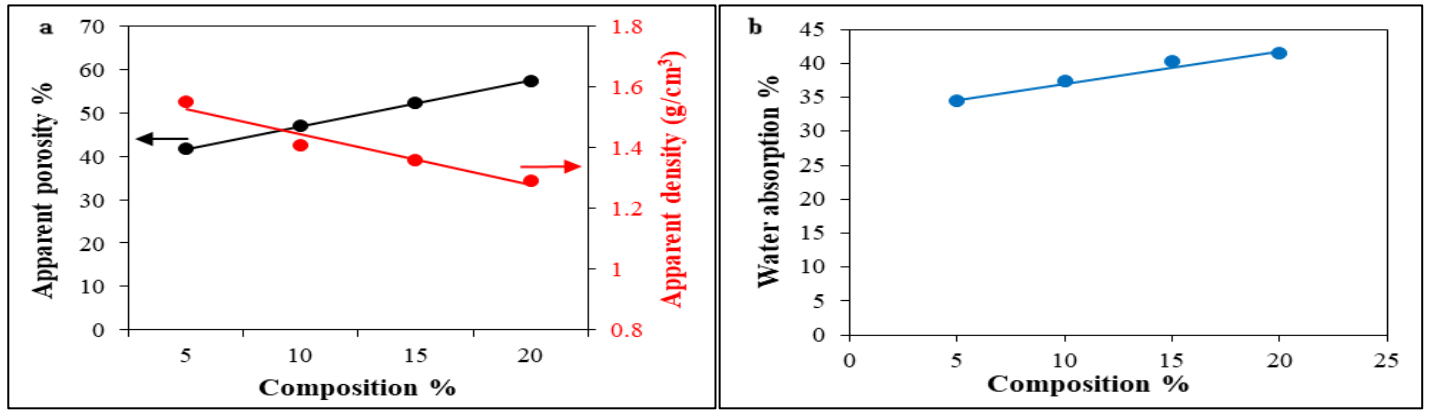


Figure 7 Effect of (CS) Addition Ratio on a: Apparent porosity and Apparent density, b: Water Absorption Ratio of Nanobiocomposites Specimens (BG-HA).

Figure 8, shows effect of chitosan (CS) ratio on the Vickers hardness of nanobiocomposites specimens (BG-HA) before soaking in artificial SBF. Results shows that an increased addition of chitosan (CS) ratio in (BG-HA) nanobiocomposites decrease in the Vickers micro hardness of nanobiocomposites specimens before immersion in artificial simulated body fluid due to the polymer's softer and flexible nature than both bioactive glass and hydroxyapatite which leads to increased porosity and reducing interparticle bonding. Additionally, a reduced apatite/biopolymer mass proportion in a composite relates with smaller apatite nanoparticle size, which indicates a reduced degree of hydroxyapatite crystallinity. This is consistent with research [36]. Chitosan forms a polymeric coating surrounding the bioactive glass particles, which hinders particle-to-particle cohesion and reduces load transfer efficiency these leads to decreased hardness compared to pure bioactive glass [37].

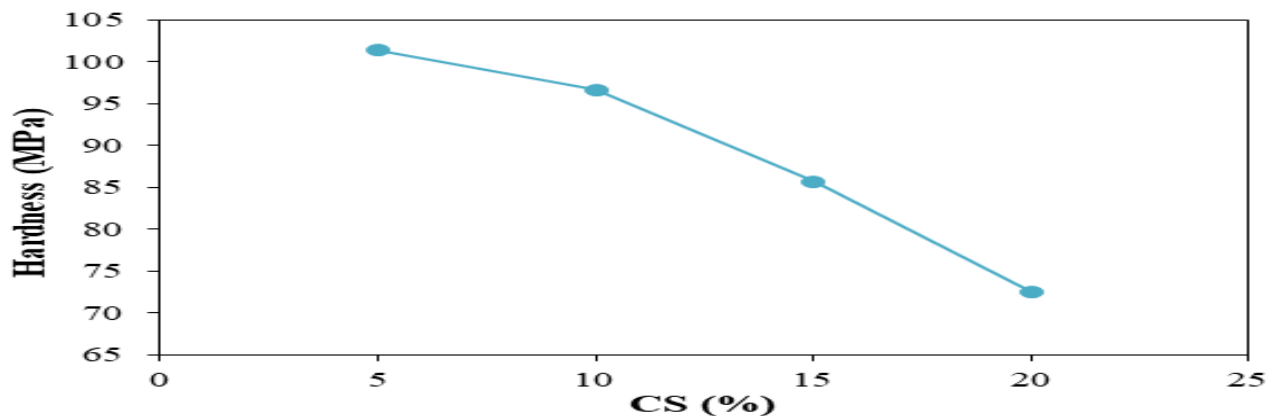


Figure 8 Effect of (CS) Addition Ratio on the Hardness Vickers of Nanobiocomposites Specimens (BG-HA) before Immersion in SBF.

6.3. Biological evaluations in vitro:

(Figures 9a, b) illustrates the alterations in a pH value of SBF and weight loss respectively after immersion of nanobiocomposites for 1, 7, 14, 21, 28 days. Results shows that the highest pH values occurring during the first day of immersion. As increases the chitosan content around the bioactive glass particles pH values decrease, because it acts as a diffusion barrier, limits Ca^{2+} , Na^{2+} ions release

from bioactive glass and contributing to a more stable pH environment with reduced fluctuations, therefore decreased the pH values compared with pure bioactive glass and BG/HA-based nanobiocomposites without chitosan in SBF. The composite containing chitosan is more favorable for bone regeneration, as it helps maintain the pH within a physiologically safe range (7.0-7.8), which is suitable for the bone tissue growth environment in consistence with another research [38].

From (Figure 9b), the results demonstrate the weight loss of nanobiocomposites specimens increases with increase immersion time, but at slower rate than of the first 7 days. During the first 7 days nanobiocomposites specimens have undergone rapid weight loss due to the degradation and dissolution of bioactive glass and release of ions (Ca, P) leads to formed apatite layer acts as a semi-protective coating. When increases the addition of (CS) ratio enhances apatite formation and slows the degradation of the bioactive glass in consistence with another researcher [30, 39]. Additionally, the nanobiocomposites containing chitosan exhibits a lower degradation rate (weight loss) and more advantageous for bone repair compared to nanobiocomposites (BG+HA) only because the chitosan absorbs water and improves stability of the nanobiocomposites, provides a better balance between bioactivity [40].

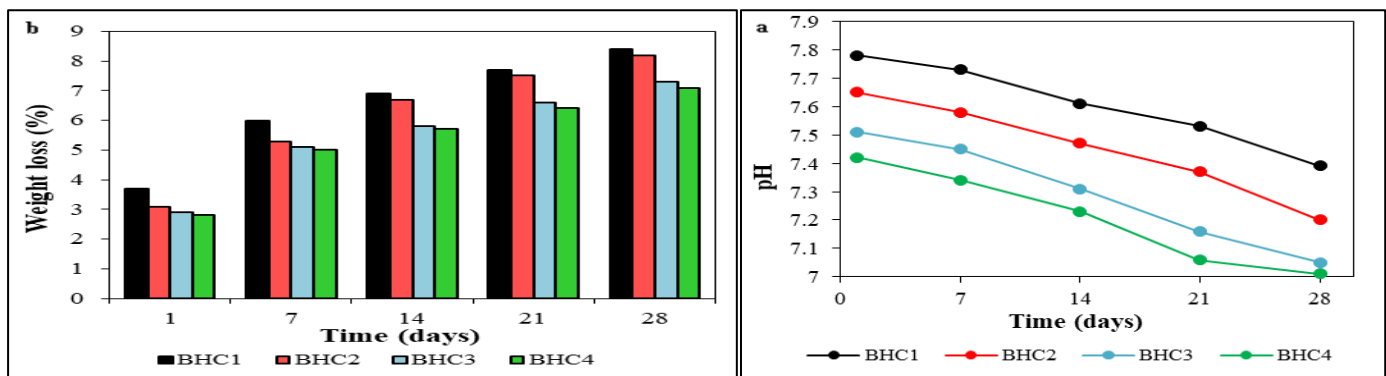


Figure 9 a: pH Behavior of the SBF, b: Weight loss of the nanobiocomposite specimens (BHC1, BHC2, BHC3, and BHC4) after immersion in SBF.

7. CONCLUSIONS

The results of anti-biofilm activity for BG, HA, CS and BHC samples was significantly improved against all four bacterial strains. The effects of BG, HA and CS in stock conc. on bacterial isolates used in this study, show that the all BG, HA and CS effectively inhibited biofilm development for all types of pathogenic bacterial isolates (Figure 10 a - f). BHC sample showed a maximum anti-biofilm activity was 93.5% and 87.8% against *S.aureus* and *K. pneumoniae* respectively, as illustrated in (Figure 10g). Also, BG-HA significantly inhibiting the capacity of bacteria to create a biofilm (Figure 10g, h). The anti-biofilm formation activity accomplished as a result of the bioactive glass 45S5 undergoes partial dissolution when contact with an aqueous environment and releasing Na^+ , Ca^{2+} , Si^{4+} , and PO_4^{3-} ions. This ionic exchange increases the pH, creating an alkaline environment, and elevates the osmotic pressure of the medium creating an inhospitable environment for the proliferation of bacteria [41, 42]. Additionally, according to presence of HA can inhibit bacterial cell wall formation and growth due to factors including the production of oxygen species that are reactive, electrostatic reactions among HA nano-particles and a cell wall, entry of HA nanoparticles into a cell, and subsequent reformation of HA within a cell [43,44]. Also, when combined chitosan with BG and nHA, CS can form a protective coating on surfaces, which inhibits bacterial adhesion and prevents biofilm formation [45,46].

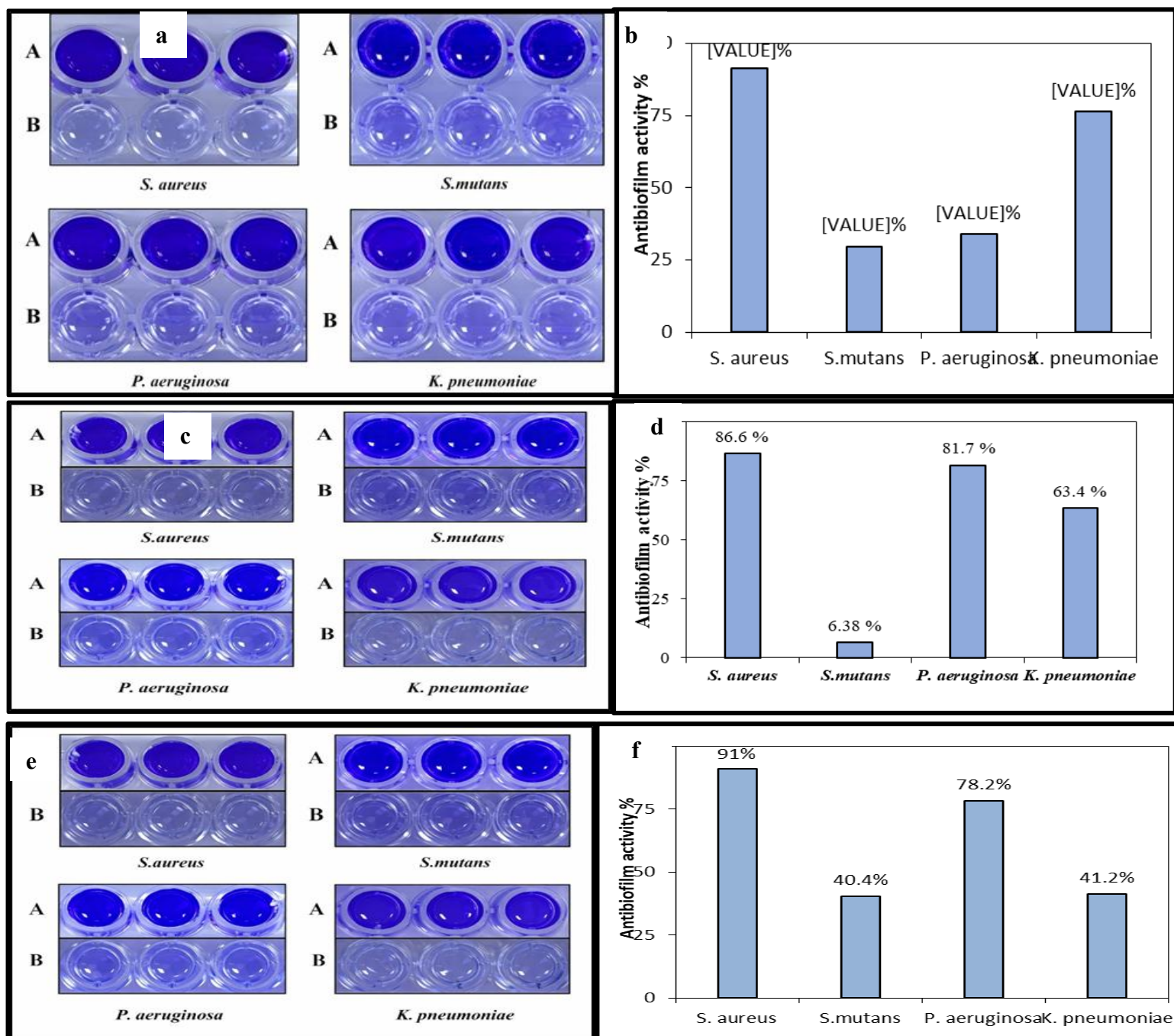


Figure 10 Antibiofilm Activity.

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