RESEARCH PAPER

Development of Sustainable Chitosan–Collagen Composite Scaffolds from Bio-Waste for Tissue Engineering Applications

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ABSTRACT

In Tissue Engineering, a Bio-scaffold restores the function or regenerates the tissue. Bio-based scaffolds offers an ideal matrix for proliferation of cells and deposition of extracellular matrix They ultimately contribute to the repair and regeneration of tissues in the specific regions of the biological system. This current work aims to make a chitosan scaffold coated with collagen extracted from fish waste, by the method of freeze drying and carry out eukaryotic (yeast) cell growth and mechanic studies and degradation assays on the scaffold. Characterization of scaffolds is done using bio analytical techniques FTIR, SEM and XRD. Estimation of protein content was done using Lowry's method and Bradford method. In addition, yeast cell growth on the scaffold is also studied. The total collagen extracted was 2.84 mg/10 gm of the bone fraction. Chitosan-collagen blend of 5% gave the highest swelling % when compared to other blends. 20% of the yeast cells were dies when grown on 4% blend of chitosan and collagen when compared to 4% viability loss with 5% scaffold. The physico-chemical and biological studies proved the chitosan-collagen scaffold to of significant importance in bio-medical applications.

Keywords: *Chitosan, Collagen, Tissue Engineering, yeast culture, upcycling of fish waste, bio-scaffold.*

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INTRODUCTION

Every day thousands of medical procedures are being performed in hospitals in order to repair or rather replace tissue through either disease or trauma. Hence, this highly advancing field of tissue engineering aims to regenerate these tissues by combining cells from the human body with highly porous scaffold biomaterials. Disease, trauma, or injury of any kind can damage these tissues deeply, which further requires help with respect to 3 regenerations. Mostly the treatment for these problems involves transplanting tissues from one site in the patient's body to another site called- Auto graft or from one individual to another- Allografts. Although these treatments have been majorly successful in solving most of these issues, there are major problems associated with both the techniques and methods.

A great number of fishes, which is a rich source of marine collagen, are underutilized and dumped away as waste, which is more expensive to dispose of because of its high organic content. The parts of the crustaceans such as bone, fins and skin are the potential source of collagen and the extraction can result in yield of 36-54%. The crustacean shell waste gives chitosan remarkable biological properties such as bioresorbable degradation products, hydrophobicity, biocompatibility, cellular binding capability, and wound healing acceleration, which explores their wide range of application. Currently in pharmaceutical, food and cosmetics, the collagen is extensively used in making scaffolds and finding key applications in tissue repair and regeneration. The waste generated by the marine industries can be up cycled and used in the medical sciences; one of the applications can be in tissue engineering.

The scaffolding process involve many strategies which include extracellular matrix of allograft or xenograft imbedded with cell-seeding, the pre-made porous scaffolds implanted with cell-seeding, the extracellular matrix secretions added to laminated cell sheets and administering self-assembled hydrogels injected with cells. The raw materials for the preparation of scaffolds include natural and synthetic polymers wherein the bio-based polymers are comparatively of much use. Polymeric substances such as proteins and carbohydrates derived from plants and animals are of main use which can influence cell behavior, particularly in terms of chemical signaling and biocompatibility. However, poor mechanical qualities and quick biodegradability limit their effectiveness. However, these drawbacks can be overcome by using appropriate cross linkers after spinning, or by using a blend of synthetic polymers with naturally-derived ones thus potentially creating an ideal scaffold for bio-medical applications. Synthetic biopolymers such as

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PLA (Polylactic acid), PLGA (poly (lactic-co-glycolic acid), and PLCL (poly (l-lactide-co-ε-caprolactone)) fabricate into a scaffold. Here we have used Natural biopolymers such as Chitosan and protein such as Collagen because of their properties such as Biocompatibility, Cytocompatibility, and adequate mechanical strength.

Scaffolds are prepared using Chitosan one of important bio-polymer produced from chitin as derivative. Chitin is regarded to be second largest biopolymer which is found in the different parts of crustaceans, arthropods, mollusks and even in fungi.

Apart from animal and microbial derived polymers, plant also serve as excellent source of polymers which are produced in the form of carbohydrates such as cellulose, starch and pectin, the proteins such as soy protein, gluten, camelina and zein, a complex polymer such as lignin. Most of these find potential use in the current sundry biomedical applications. Because of its great biological compatibility, biological degradability, and ability to rapidly remodel in vivo, the extracellular matrix (ECM) has always piqued researchers' interest in scaffold creation. Proteins, such as collagen, fibrinogen, gelatin, elastin, and polysaccharides, such as alginates, hyaluronic acid, cellulose, and chitosan, make up the majority of the ECM. This complex mixture provides mechanical and metabolic support to neighboring cells while also regulating their regeneration performance. Scaffolds can be used as cellular systems or as delivery vehicles for cells and pharmaceuticals in cell and tissue regeneration; as a result, the cellular material must be able to colonize the host cell effectively to meet the needs of regeneration and repair.

Narendra Reddy, et al. (2021), confirmed that treating the freeze-dried 3D chitosan scaffolds with alkali will enhance its stability and strength. It was also observed that the strength, water resistance and other characteristics were further enhanced with higher concentrations. The scaffolds exhibited an optimal inhibition of 97% against both gram negative and gram-positive bacteria. This work reveals a new method for developing chitosan scaffolds with their features ideal for food, medicine and related applications. Gokula Krishna Sivasundari Arumugam, et al. (2016) extracted the collagen from Sole fish skin which is in the form of fibrils and was very similar to commercially available calfskin type I collagen. Peck Loo Kiew, et al. (2013) extracted a pepsin soluble collagen and an acid soluble collagen and from the *Clarias* species, a freshwater catfish which is extensively consumed in Malaysia. This work demonstrated that collagen extracted from any source can possibly be governed and regulated through collagen yield measurement using a modified Lowry's method. Takeshi Nagai, et al. (2000) extracted the collagen type I from from fish fin, bone, and skin and found a very decent yield. This work concluded that processed fish waste can be a probable alternate source of collagen. H J Khamna, et al. (2000) designed a chitosan-collagen scaffold in a cylindrical shape with radiated pores which can cater transplantation of hepatocyte for bioartificial liver implantations. The scaffold was developed through precise freezing and lyophilization, which could provide a microstructure for effective cell seeding. Mahboob, et al. (2015) isolated and characterized the collagen from the scales, fins and skin of *Cirrhinus mrigala* and *Catla catla. This study revealed that distinction in* imino acid leads to a substantial variation in collagen and through proximate analysis, higher protein was found in fins. Divya Natraj, et al. (2018) formed a sustainable and eco-friendly chitosan collagen and made them biocompatible with the citric acid cross linkage to enhance its stability and mechanical features. This study confirmed a sustainable alternative source of biofilms.

However, the prior research confirmed that collagen can be effectively extracted from fish waste and production of scaffolds from chitosan, steam remains in effective development of scaffold, uniform porosity, scalability, reproducibility and long-term cell viability which limits the practical application. The present research work emphasizes on development and analysing an effective and sustainable biomaterial from collagen from fish waste integrated with chitosan scaffold. Saccharomyces cerevisiae serves as a model organism to estimate the compatibility of scaffold towards cell proliferation and interactions on surface there by influencing towards the development of economically feasible, sustainable tissue engineering framework.

Materials and methods

Extraction of Collagen and Characterization

The fish waste was collected from the local market and the restaurants as well. The waste was thoroughly cleaned from blood and other impurities with clean water. Then cleaned fish waste was dried.

Pre-treatment to Collagen Substrate

This procedure was done to remove the non-collagenous proteins and fats. Cleaned fish skin was treated with 0.3M NaOH w/v in the ratio of 1/10. The prepared mixture was stirred using a magnetic stirrer for 4 h and NaOH solution was changed for every hour. The treated skin sample was washed with distilled water until neutral pH was attained. The treated sample was kept in 20% butanol solvent for 30 h at 1/10 (w/v) and every 10 hours skins were shifted into fresh butanol solvent. The treated skin samples were washed with distilled water until neutral pH was attained.

Extraction of Collagen

The pre-treated sample from the previous was treated with 1.5M (w/v) acetic acid solution and filtered. The filtrate was treated with 0.05 M tris-HCl and NaCl and adjusted to basic pH of 8.0 using 5M NaOH. The filtrate was centrifuged at 4000rpm for 30 min. The pellet was collected and dissolved in 0.1M acetic acid and dialyzed against 1L of 0.1M acetic acid for 24 h. The samples were further dialyzed in distilled water for another 24 h.

Estimation of Extracted Protein Using Modified Lowry's Method.

The reagent A and B were prepared accordingly as reported elsewhere. The Folin - Ciocalteu reagent was diluted with distilled water at the ratio of 1:15. The extracted collagen suspension mixed with reagent A and B in appropriated concentrations. Blank solution was mixed in a similar way without collagen sample. The solution prepared was incubated at 50°C for 20 min. The sample solution was cooled to RT and 2.4 mL of FC reagent was added, shaken vigorously and incubated again at 50°C for 10 min. After cooling to room temperature, the absorbance was recorded at 650 nm against the reference.

Estimation of Extracted Protein using Bradford assay

Preparation of Bradford's Reagent:

BSA solution of 0.1% (w/v) which is a reference was prepared in distilled water. Varying conc. of BSA solution was pipetted into which 1mL of Bradford reagent was added. The color developed upon incubation at RT was recorded at 595 nm using UV- Visible Spectrophotometer.

Preparation of Chitosan Scaffold:

Preparation of 500mL 2% Acetic acid solution:

Varying concentrations of chitosan from 1 to 5% (w/v) was prepared in 2% (v/v) acetic acid solution. The suspension was further incubated in shaking incubator at 90 °C for 30min. Into 24 well plate, the prepared solution was transferred and the solution was incubated at -65 °C for two days. The set scaffold structure was lyophilized at -108 °C for 48 h. Further scaffolds were kept in hot air oven at 50 °C for 5h the scaffold was nutralised with 5% NaOH for 24h.

Characterization of Chitosan Scaffold

Swelling Studies using Phosphate Buffer Saline

The different scaffolds prepared weighed dry and each of them immersed in of PBS and incubated at 37 ° C for 24 hours. After wiping the scaffolds to remove water droplets, the wet weight was measured. The % of swelling was calculated using the formula

$$Sw(\%) = \frac{(Ww - Wd}{Wd} x100$$

Where,

Sw (%) is swelling percentage.

Ww= Wet weight and Wd= Dry weight.

Structural characterization of Chitosan scaffolds

The prepared scaffolds were analysed for their structural features by FTIR, SEM and XRD. The interaction of chemical functional groups, the networking of scaffold with porosity and crystalline behaviour were studied respectively.

Determination of effect of chitosan on yeast cell viability

The yeast cells (Saccharomyces cerevisiae) were grown in YPD media and the culture was preserved for future use. For the viability assay the mother culture of yeast was prepared by inoculating YPD broth at 1% concentration and incubating for 48 h at 30 oC in shaking incubator. The inoculum developed was treated with varying conc. of chitosan for 48 h and later subjected for determination of cell viability. 200µL of yeast broth was taken in microcentrifuge tube and mixed with distilled water in 1:1 ratio. Then 1 drop of crystal violet stain was added for visualisation under light microscope. The number of cells in each box of 4*4 was visualised and counted using Haemocytometer.

The viability assay was also performed with tryphan blue dye (0.4% w/v in distilled water). The scaffolds were sterilized in UV light. The scaffold was immersed in 10 mL of YPD broth and kept in a shaking incubator at 25℃ for 24 h. Broth and trypan blue are mixed in 1:1, and incubated at room temperature for 3 minutes. Cell counting is carried out using Hemocytometer.

RESULTS AND DISCUSSION

Amount of collagen extracted from the cartilaginous waste

30g of fish skin (raw material) was processed after defatting and removing the proteins other than collagen in acetic acid solution. Both the Lowry's assay and Bradford assay were performed to estimate the amount. The total collagen determined was found to be 0.4 mg/mL which amounts to 2.84 mg/10 gm of the extracted collagen fraction.

Characterization of Chitosan Scaffolds

The blend of chitosan-collagen suspension was processed to prepare them scaffold. The scaffolds of different concentrations were obtained by lyophilization after 6 hours for 1mL, 10 hours for 3mL, and 24 hours of lyophilization for 5mL well of the scaffold, as shown fig 1. A blend concentration of 1% - 3% did not give scaffolds, even in 3mL volume, may be because of the high amount of water that was sublimed in the lyophilization process. Further studies were only carried out with the blend of 4 and 5% concentrated scaffolds.

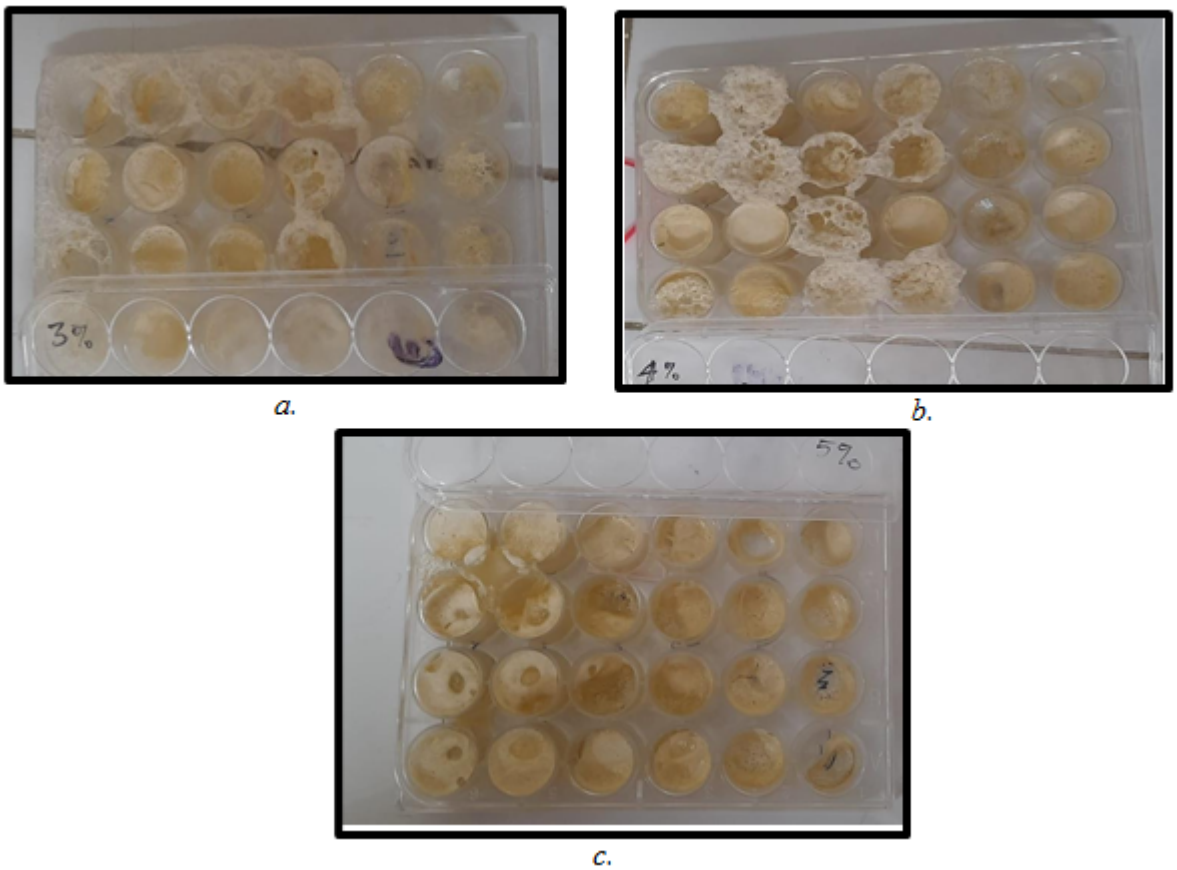


Figure 1: Preparation of scaffolds with blending proportions a. 3% b. 4% and c. 5 % chitosan-collagen components

Chitosan Scaffold Studies

Swelling Studies

Scaffolds have a tendency to swell in contact with the physiological fluids of the human body affecting their shape and mechanical stability. Chitosan is a hydrogel having a higher percentage of swelling, this being a larger drawback in the case of load-bearing scaffolds. As calculated the scaffold conc. of 5% showed the swelling percentage to be 85.5%. The scaffold formed with conc. of 3 and 4% did not withstand the swelling process rather dissolved completely.

Ph Stability of Scaffold

The pH stability of the scaffold was performed to know the intactness of the scaffold at varying pH. This helps one to explore the options to use the scaffold for various biomedical applications which functions at different pH . As revealed from the table 1, the scaffold at different conc. showed no significant changes at extended time durations. The stability of the scaffold also demonstrates that the chitosan blend does not collapse or no chemical interventions that can vary the pH.

Table 1: The pH stability studies of the scaffold in different time intervals demonstrating the intactness and suitability for bio-medical applications.

Concentration	0 hrs	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs	24 hrs	48 hrs	72 hrs
3%	7.2	6.8	6.77	6.88	6.54	6.4	6.5	6.62	7.2
4%	7.2	7.23	7.18	7.02	6.97	6.8	6.5	6.59	7.05
5%	7.2	6.2	6.57	6.63	6.51	6.4	6.54	6.6	6.74

Structural properties of chitosan-collagen scaffold

The prepared scaffold blend formed from chitosan and collagen was determined for structural properties. The FTIR, SEM and XRD analysis revealed significant features of the blend material. All the three conc.of blends were subjected to FTIR analysis as shown in fig.3. The wave numbers revealed the respective functional groups characteristic of chitosan and collagen as well. The key findings include the presence of significant peaks corresponding to amide (~1650–1630), hydroxyl amino (~3400–3500) and glycosidic linkages (~890–800) (Table 2). No significant differences were seen in three blends of varying concentrations. A strong broad peak corresponding to hydroxyl and amino group revealing broad stretching was seen in all the three scaffolds.

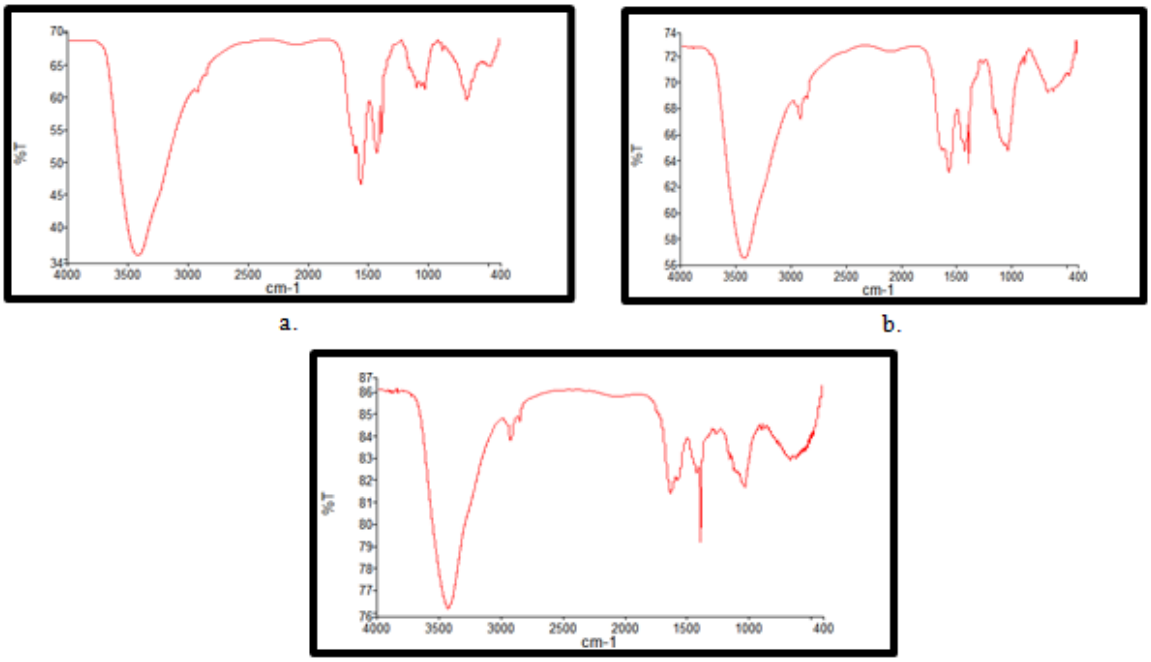
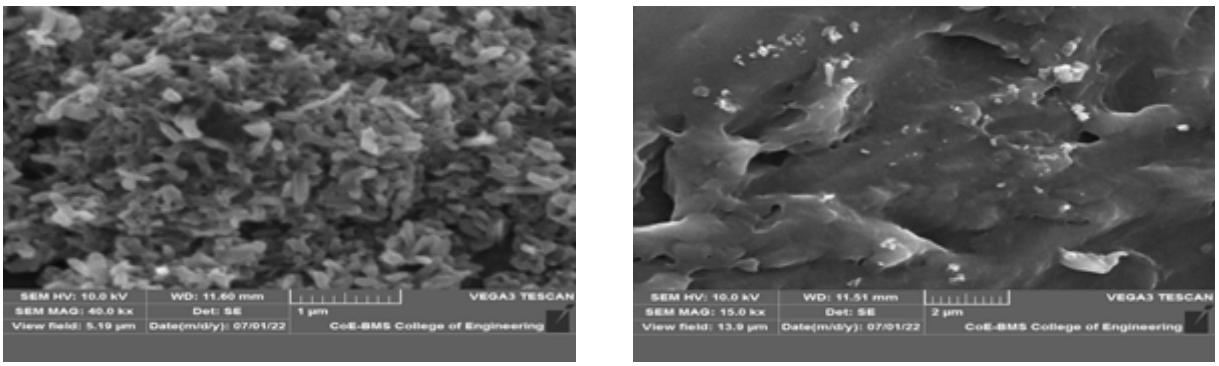


Figure 2: FTIR spectra of varying conc. of chitosan -collagen scaffold a. 3%, b. 4% and c. 5 %.

Table 2: IR spectra of different conc.of Chitosan-Collagen blend

Wavenumber (cm^{-1})	Functional Group	Assignment / Vibration
~3400–3500	–OH, $–NH_2$	Broad stretching (hydrogen bonding)
~2920–2850	–CH	Aliphatic C–H stretching
~1650–1630	Amide I	C=O stretching
~1590–1560	$–NH_2$	N–H bending (amide II)
~1420–1380	$–CH_3$ / $–CH_2$	Bending vibrations
~1320–1260	Amide III	C–N stretching
~1150–1020	C–O–C / C–O	Glycosidic bond stretching
~890–800	β-glycosidic linkage	Ring vibration

Morphological Features of Chitosan-Collagen Blends



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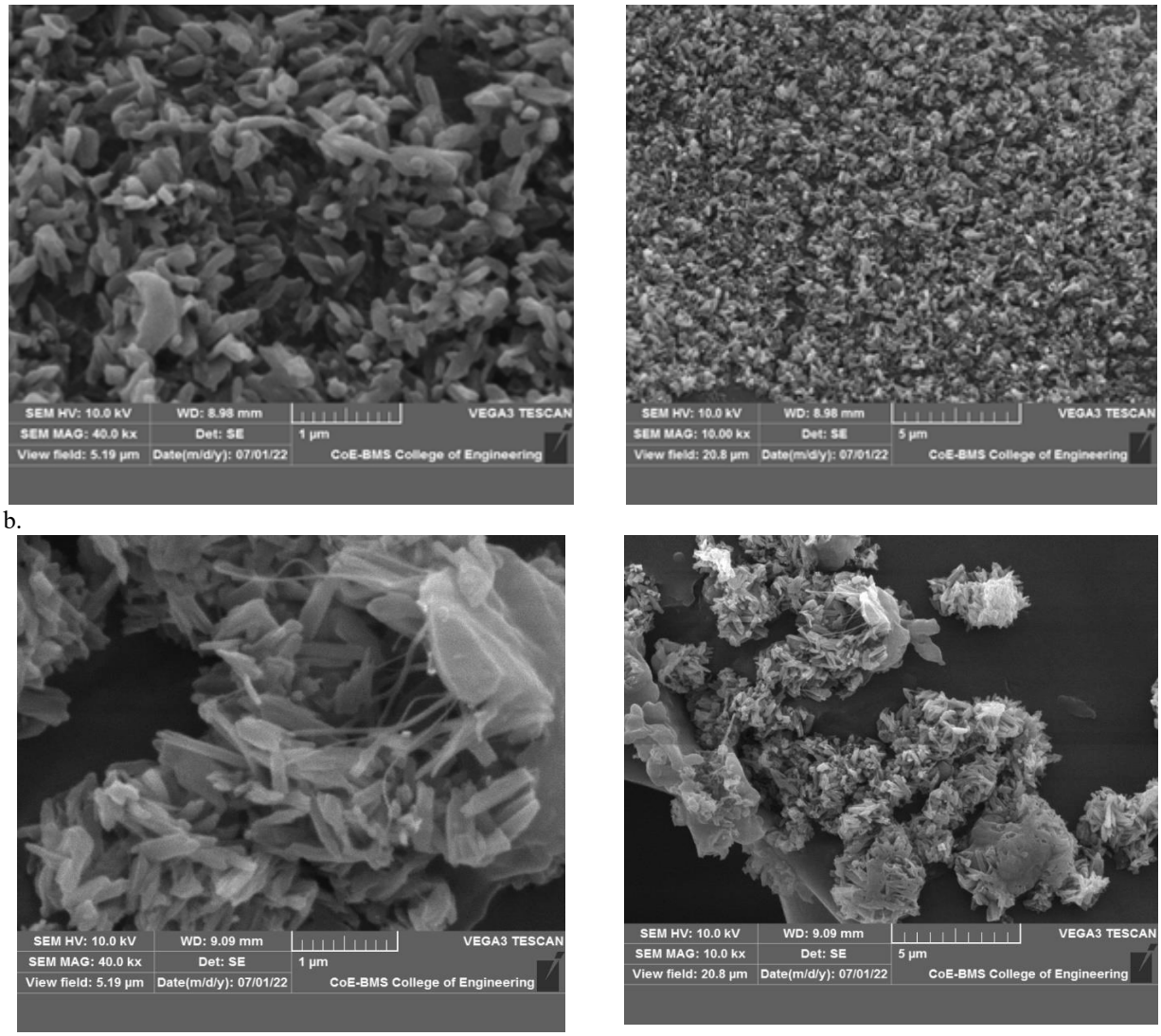

Figure 3.SEM images of chitosan-collagen blend of varying proportions. a. 3% b. 4% and c. 5%. The left and the right panel images in each is corresponding to scanning magnification of 40kx and 10kx respectively

All the three scaffolds were studied for morphological features using scanning electron microscope. The surface morphology of the chitosan–collagen blend was analyzed using scanning electron microscopy (SEM). The SEM images revealed a rough, porous, and network structure (Fig 3). At higher resolution, the scaffold showed flake-like and rod-shaped microstructures, indicating strength of interactions between chitosan and collagen. At lower resolution, the scaffold showed homogeneous distribution indicating no phase separation and hence maximum interaction between scaffold monomeric units. The scaffold had a porous dimension of 1-6 µm suggesting its applications in tissue engineering and drug delivery.

Crystalline Properties of Chitosan-Collagen Scaffold

XRD spectrum of 3% chitosan scaffold showed diffraction peak at 5.5358, 7.5489, 11.7486, 16.7410, 22.8888, 53.0128 and the compound name was 2,4,6, -tris(difluorosulfoximido)- 1,3,5-triazene, whose chemical formula is $C_3 F_6 N_6 O_3 S_3$ attributed to presence of chitosan polymer. XRD spectrum of 4% chitosan scaffold shows diffraction peak at 5.1871, 6.9257, 11.4368, 20.3929, 27.0662, 51.5344, 52.3950, 73.4790, 76.6260 and the compound name was Tetraethylammonium Thiourea Hydrogensulfate Hydrate, whose chemical formula is $C_9 H_{27} N_3 O_5 S_2$. XRD spectrum of 5% chitosan scaffold showed diffraction peak at 5.2311, 6.9488, 11.4934, 23.3191, 25.6596, 29.7838, 35.5241, 37.3540, 44.6813, 47.0676, 59.9850 and the compound name was 2,4,6, -tris(difluorosulfoximido)-1,3,5-triazene, whose chemical formula is $C_3 F_6 N_6 O_3 S_3$.

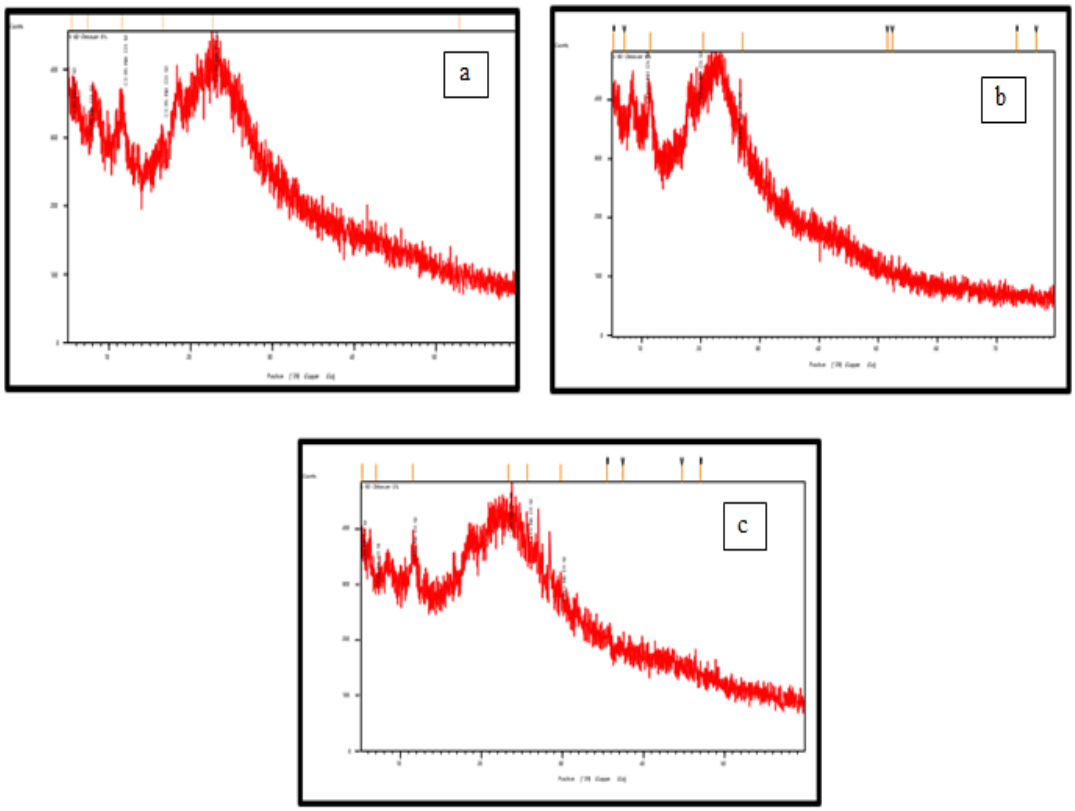

Figure 4.XRD spectrum of chitosan-collagen blend of varying proportions. a. 3% b. 4% and c. 5%.

Cell viability of Yeasts on Chitosan-Collagen Scaffold

The yeast cell viability on the prepared scaffold was performed with Triphon blue assay. The method relies on the principle of dye exclusion wherein only the dead cells will take up the stain. Triphon blue is an anionic hydrophilic azodye and the partially damaged or completely damaged cells are easily percolated by this stain. Table 3 provide the details of the number of yeast cells surviving on the scaffold. It is inferred from the table that the number of dead cells is significantly higher with 4% of chitosan-collagen scaffold when compared to the control.

Table 3: Number of viable and dead cells of yeast cultured with varying conc. of chitosan-collagen scaffold.

Concentration of scaffold	No of live cells	No of dead cells
Control	$3.36x10^6$	$0.28x10^6$
4% Collagen	$5x10^6$	$1x10^6$
5% Collagen	$2.5x10^6$	$1x10^5$

CONCLUSION

Tissue engineering need to provide functional criteria that will aid people who are designing and manufacturing these repairs and replacements. The qualities of scale-up, packaging, storage and handling are equally crucial. Biomedical engineering and nanotechnology will assist eukaryotic cell treatments to overcome their flaws by them to proliferate to an adequate concentration, homogeneity, and proliferation at the desired area. When administered to the human body, however, biomaterials can be hazardous. As a result, numerous strategies for improving biomaterial biocompatibility have been devised. To date, a variety of polymeric scaffolds have been used in tissue engineering. Scaffolds, whether made from natural materials or synthetically generated, must meet particular design requirements in order to be beneficial in this domain. In the present study, chitosan scaffolds were developed for hepatocyte attachment. However, due to the failure to establish a cell line we will be sticking to growing yeast as a model organism on the obtained scaffold.

Various traditional techniques for constructing porous 3D scaffolds were used in the following decade, including fibre bonding, phase separation, solvent casting, particle leaching, moulding, and foaming. All of the traditional approaches have one main flaw: they don't allow enough control over the scaffold design, pore size, and pore network, resulting in an incontinent and less-than-ideal 3D scaffold.

In future we would like to do HepG2 cell line studies, where all the related assays, proliferation, adheration studies will be done to study how it functions on a artificial scaffold made of a natural biopolymer.

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