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Screening of some actinomycetes isolates for their ability to biosynthesize collagen nanoparticles, optimization of fermentation conditions for improved biosynthesis and anti-hemolytic activity

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Abstract: Collagen nanoparticles (collagen-NPs) have several advantages over other natural and synthetic polymeric NPs, including suitable biocompatibility and biodegradability, low antigenicity, a high contact surface, and reduced toxicity. Collagen-NPs appropriate for use in the field of biomedicine in skin wound healing, tissue engineering, drug delivery, in bone grafting, and healing as they promote optimal bone regrowth and for treatment of the damaged nerve tissues and nerve regeneration. Eight strains were assessed for their abilities for the biosynthesis of collagen-NPs using their cell-free supernatant. After incubation, white turbidity appeared as the result of collagen-NPs formation. The most potent strains for maximum biosynthesis of collagen-NPs were identified as *Streptomyces xinghaiensis* strain NEAA-1 and *Streptomyces plicatus* strain NEAA-3. These *Streptomyces* strains were tested for collagen-NPs biosynthesis under different conditions, such as concentrations of collagen and strains cell-free supernatant, temperature, initial pH, and incubation time. The maximum biosynthesis of the collagen-NPs was 4.13 mg/mL and 5.16 mg/mL, respectively, at 35 ºC and 40 ºC for *Streptomyces xinghaiensis* and *Streptomyces plicatus*. The maximum biosynthesis of the collagen-NPs was 5.24 mg/mL and 6.33 mg/mL, respectively, at 7 pH for *Streptomyces xinghaiensis* and *Streptomyces plicatus*. The maximum biosynthesis of the collagen-NPs was 6.51 mg/mL and 7.42 mg/mL after 48 h for *Streptomyces xinghaiensis* and *Streptomyces plicatus*, respectively. The biosynthesized collagen-NPs showed 96% anti-hemolytic activity.

keywords: Collagen-NPs, biosynthesis, *Streptomyces*, optimization, anti-hemolytic.

1.Introduction

Biopolymer-based nanoparticles involving protein nanoparticles have acquired a great of interest in the last few decades due to their many desired properties, such as biodegradability, bioavailability, low toxicity, and relatively low cost. Proteins are a class of natural molecules that are involved in various fields in nanobiotechnology, for example, nano medicine, immunology, pharmacy, toxicology, and other medical applications **[1]**. As a result of the wide range of protein nanoparticles applications, there a wide range of proteins that are used to form protein nanoparticles, such as silk fibroin, collagen, keratin, gelatin, corn zein, elastin, gluten, bovine serum albumin, casein, soy protein, etc., which have received significant attention due to their scalability in biomaterials research.

Collagen is the most abundant protein found in the human and animal body, existing in bones, muscles, and skin. Collagen is useful in a wide range of applications, including wound healing, drug delivery, aging skin, antiviral, anti-inflammatory, and tissue regeneration **[2]** & **[3]**. According to reports, marine collagen is more hydrolyzable than mammalian collagen,

which makes it appropriate for additional processing to produce peptide derivatives **[4]**. Collagen has been demonstrated to possess both structural and functional qualities that make it an ideal substrate for cell adhesion, proliferation, and differentiation **[5]**. Collagen-NPs are more useful than other natural and synthetic polymeric NPs as they have favorable biodegradability and biocompatibility, a high contact surface, low antigenicity, reduced toxicity, and a high cationic charge density potential due to the high number of amino groups in the absence of surface modification with target compounds **[6]**. There are a massive number of chemical, physical, biological, and hybrid techniques available to synthesize nanoparticles of different types **[7].**

Biological techniques involve the use of natural sources like plants or microorganisms to synthesize nanoparticles while avoiding toxic chemicals and hazardous byproducts and lowering energy consumption **[8]**. Through the last few years, microorganisms, including bacteria (such as actinomycetes), fungi, and yeasts, have been utilized for the synthesis of nanoparticles, both extracellular and intracellular. Actinomycetes share important characteristics with fungi and bacteria and also have the ability to produce secondary metabolites through various biological activities **[9]**. Actinomycetes are thought to be effective candidates for producing metal nanoparticles through extracellular or intracellular synthesis **[10]**. Among actinomycetes genera, *Streptomyces* is the largest genus that can produce a wide variety of bioactive secondary metabolites, and extracellular enzymes that frequently used in the manufacture of nanoparticles **[11]**, **[12]**, **[13]**, **[14]** & **[15]**. The cell-free supernatant of *Streptomyces flavolimosus* was used as a reducing and stabilizing agent for gold nanoparticle biosynthesis **[16]**. The cell-free supernatant of *Streptomyces microflavus* strain NEAE-83 was used for the production of chitosan nanoparticles **[17]**.

The aim of the present work is to determine the strains that were more efficient in biosynthesizing collagen-NPs out of eight newly isolated strains, optimize the variables affecting collagen-NPs biosynthesis, and to detect their potency as anti-hemolysis agent.

2. Materials and methods

2.1. Streptomyces strains culturing

The eight strains of *Streptomyces* (coded as NEAA-1, NEAA-J, NEAA-1F, NEAA-26, NEAA-29, NEAA-6B, NEAA-82, NEAA-U3) were generously donated by the second author, Prof. Noura El-Ahmady El-Naggar. *Streptomyces* strains have been cultured on starch-nitrate agar medium **[18]** slopes composed of (g/L) agar, 20 of soluble starch, 0.5 of $MgSO_4.7H_2O$, 3 of $CaCO_3$, 2 of KNO_3, 0.5 of NaCl, 1 of K_2HPO_4, and 0.01 of $FeSO_4.7H_2O$. With the same previous medium, the eight strains were cultivated for seven days at 30ºC then kept as spore suspensions in 20% (v/v) glycerol solution at - 20ºC.

2.2. Cell-free supernatant preparation

250-mL Erlenmeyer flasks holding 50 mL of the medium composed of (g/L) of 20 soluble starch, 0.5 $MgSO_4$, 0.5 KNO_3, 1 NaCl, 0.5 K_2HPO_4, 0.5 $FeSO_4.7H_2O$, 0.1, and 0.3 yeast extract were prepared and sterilized. Three 9-mm-diameter disks taken from *Streptomyces* strains growth cultivated on petri dishes containing starch nitrate agar medium were used to inoculate the previously prepared flasks. The flasks had been incubated at 30ºC and 150 rpm for five to seven days in a rotatory incubator shaker. The cell-free supernatant was separated from the mycelia cells by centrifugation for 15 minutes at 4ºC and 8,000 rpm.

2.3. Collagen-NPs biosynthesis

The process of synthesizing collagen-NPs involved mixing 1 mL of 10 mg/mL marine collagen solution (MM Ingredients Ltd., UK) with 1 mL of cell-free supernatants of each of the eight actinomycetes strains, and then incubating the mixture for 48 hours at 37ºC. The procedure has been carried out in triple or quadruple and then gathered collectively. Following centrifugation at 13,000 rpm, the supernatant was discarded, and the pellets (collagen-NPs) were washed by distilled H_2O. The washing step was duplicated to eliminate all the cell-free supernatant of *Streptomyces* stains. Finally, the pellets were dispersed in dist. H_2O then go for freezing dried. The generated dried collagen-NPs were weighed.

2.4. Optimization of the variables affecting the biosynthesis of collagen-NPs

To optimize the variables influencing the biosynthesis of collagen-NPs by the cell-free supernatants of *Streptomyces* sp. strain NEAA-1 and *Streptomyces* sp. strain NEAA-6B, the process involved mixing different volumes of both marine collagen solution (0.5, 1, 2, 3, 4, 5, 7, 8, 9, 9.5 ml of 10 mg/mL) with cell-free supernatant (9.5, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 mL) and then incubating the mixtures for 48 hours at 37ºC. To evaluate the biosynthesis of collagen-NPs at different temperatures, 1 mL of cell-free supernatant was added to 9 mL of collagen solution; the mixtures were incubated for 48 hours at different temperatures (20, 25, 30, 35, 40, 45 and 50ºC). Then biosynthesis at different pH levels (5, 6, 7, 8, 9), and incubation times (1, 24, 48, 72, and 96 h) were evaluated.

2.5. Anti-hemolytic properties of collagen nanoparticles

Rat hearts were punctured to get a blood sample, which was then placed in a heparin tube. The erythrocytes have been separated from the plasma. Erythrocytes have been centrifuged at 2500 rpm/min for 10 minutes during the final wash to obtain packed erythrocyte cells. Peroxyl radicals were used to promote hemolysis in this test procedure. A 10% suspension of phosphate buffered saline pH 7.4 (PBS) containing erythrocytes was combined with 200 mM 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) in PBS at various concentrations. The mixture was gently shaken for two hours at 37ºC, diluted with PBS (eight volumes), and then centrifuged for ten minutes at 1500 rpm. The supernatant's absorbance was measured at 540 nm. Eight volumes of dist. H_2O have been combined with the mixture and treated the same way. After centrifugation, the supernatants' absorbance was measured at 540 nm along with H_2O to evaluate if complete hemolysis occurred. The positive control that was used was L-ascorbic acid. The proportion of hemolysis was determined using the formula (1-A/B) × 100%. The average and standard deviation of the results were displayed. Vitamin C has been utilized as a positive control **[19]**.

3. Results and Discussions

3.1. Capability of actinomycetes isolates to produce collagen-NPs

The capabilities of eight isolates to produce collagen-NPs using their cell-free supernatants have been detected after 24 h of incubation at room temperature. Collagen-NPs formed as white turbidity, as depicted in **Figure 1**. The most effective strains for collagen-NPs biosynthesis were the cell-free supernatants of both *Streptomyces* sp. strain NEAA-1 and *Streptomyces* sp. strain NEAA-6B **(Figure 2)**, which yielded 2.5 and 2.43 mg/mL of collagen-NPs, respectively, before optimizing. *Streptomyces* sp. strain NEAA-1 was identified as *Streptomyces xinghaiensis* strain NEAA-1 **[20]** and *Streptomyces* sp. strain NEAA-6B was identified as *Streptomyces plicatus* strain NEAA-3

Figure 1: (A) collagen solution; (B) collagen-NPs solution (white turbidity)

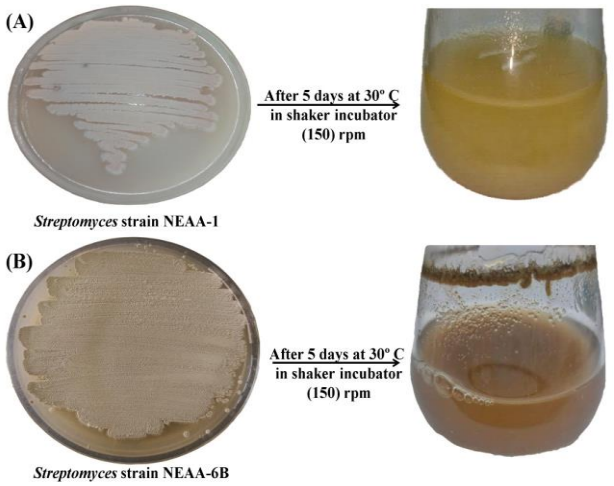

Figure 2: (A) *Streptomyces xinghaiensis* strain NEAA-1 and (B) *Streptomyces plicatus* strain NEAA-3

3.2. Effect of different volumes of both collagen solution and cell-free supernatant on collagen-NPs biosynthesis

No collagen-NPs produced when 0.5 mL of cell-free supernatant was added to 9.5 of the marine collagen solution (**Figure 3**); this is because of the inability of the cell-free supernatant of the *Streptomyces* strain to reduce the collagen (bulk) to form nanoparticles. The maximum biosynthesis of collagen-NPs **(Figure 3)** appeared after adding 1 mL of cell-free supernatant to 9 mL of collagen solution. The behavior was the same of the both *Streptomyces* strains.

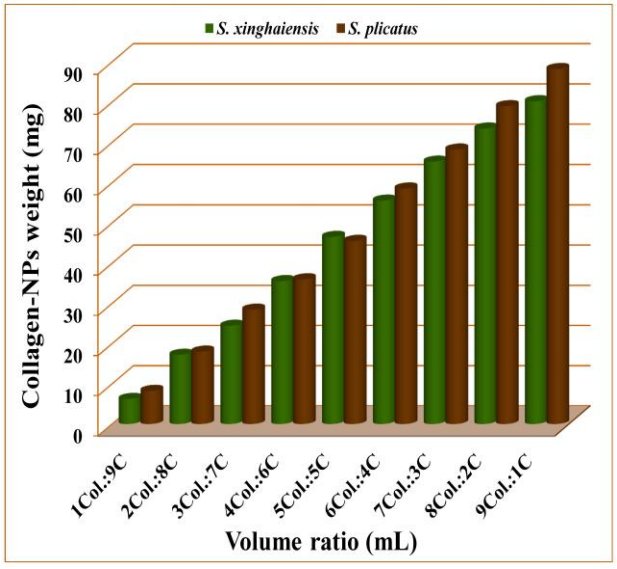

Figure 3: Effect of different volumes of collagen solution (Col.) and cell-free supernatant (C) on collagen-NPs biosynthesis [1 Col.: 9C means 1mL of collagen solution + 9 mL of cell-free supernatant]

3.3. Effect of temperature on collagen-NPs biosynthesis

This experiment was done for the detection of the optimum temperature for the biosynthesis of collagen-NPs by *Streptomyces xinghaiensis* strain NEAA-1 and *Streptomyces plicatus* strain NEAA-3. In **Figure 4**, the maximum biosynthesis of the collagen-NPs, which was 4.13 mg/mL, appeared at both 35ºC and 40ºC for *Streptomyces xinghaiensis* strain NEAA-1. Also, the maximum biosynthesis of the collagen-NPs, which was 5.16 mg/mL, also appeared at 35ºC and 40ºC for *Streptomyces plicatus* strain NEAA-3. The perfect temperature range for collagen-NP manufacturing was 35–37ºC. Collagen-like peptide proved capable of forming precise nanoparticles with diameters of around 100 nm at an equilibrium temperature of 37ºC **[21]**. According to Banerjee & Azevedo **[22]**, the triple helices of collagen nanostructures were quite stable at physiological temperature (37ºC). Collagen-like peptides tend to form spherical nanoparticles at a temperature of 37ºC, and nanoparticles are resolubilized at 80 °C using a self-assembly method **[23]**. Collagen peptides are converted to interconnected and irregularly shaped nanosized spheres (50–250 nm) at room temperature, according to Pires & Chmielewski **[24]**.

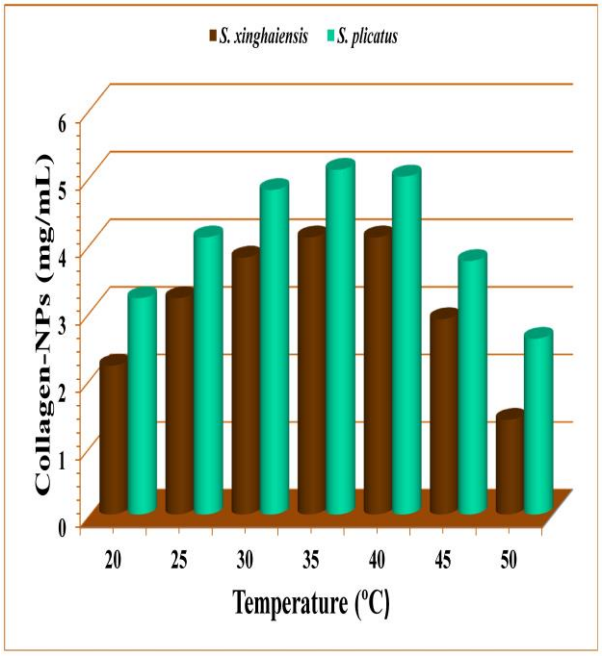

Figure 4. Effect of temperature on collagen-NPs biosynthesis using cell-free supernatant of S. xinghaiensis and S. plicatus.

3.4. Effect of pH value on collagen-NPs biosynthesis

The purpose of this experiment was to determine the optimal pH value for the biosynthesis of collagen-NPs by *Streptomyces xinghaiensis* strain NEAA-1 and *Streptomyces plicatus* strain NEAA-3. In **Figure 5**, the maximum biosynthesis of the collagen-NPs which was 5.24 mg/mL appeared at pH 7 for *Streptomyces xinghaiensis* strain NEAA-1. The maximum biosynthesis of the collagen-NPs, which was 6.33 mg/mL, also appeared at pH 7 for *Streptomyces plicatus* strain NEAA-3.

The key determining variable is the pH level of the medium, which may impact the dispersion of charges and electrostatic forces on collagen atoms, potentially altering their

capacity to assemble nanoparticles **[25]**. Many researchers prepared collagen nanostructures at pH 7.4 **[23], [24] & [26]**. Proteins are amphoteric, meaning they contain several charged functional groups. This means that by altering certain factors, like pH, they can become either cationic or anionic **[6]**. According to Sun *et al*. **[27]**, collagen tends to form nanostructures at neutral pH (7) due to the negatively charged amino acids and lanthanide ions (Ln^{3+}). The deprotonated aspartic or glutamic acids promote powerful binding of Asp/GluLn^{3+} at neutral pH, which results in the formation of nanomaterials. However, protonated amino acids lose their ability to bind Ln^{3+} at an excessively acidic pH, making it impossible for Ln^{3+} to start the nanostructures formation with a self-assembly method of preparation.

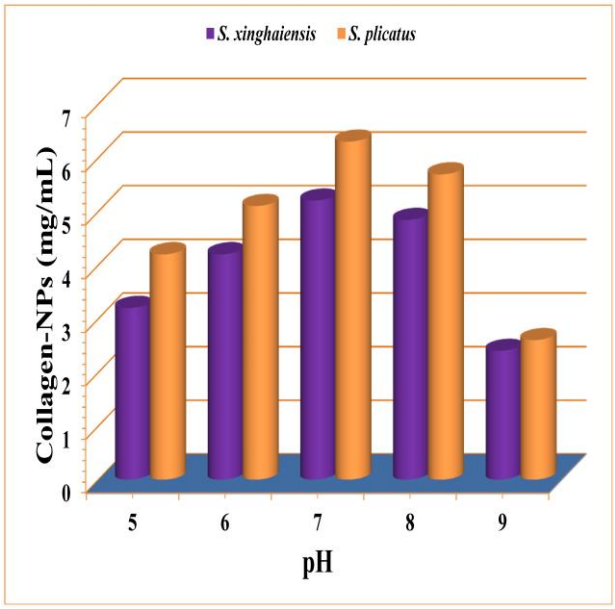

Figure 5. Effect of pH on collagen-NPs biosynthesis using cell-free supernatant of *S. xinghaiensis and S. plicatus*.

3.5. Effect of incubation time on collagen-NPs biosynthesis

The goal of this experiment was to determine the ideal incubation period for collagen-NPs biosynthesis by *Streptomyces xinghaiensis* strain NEAA-1 and *Streptomyces plicatus* strain NEAA-3. In **Figure 6,** the maximum biosynthesis of the collagen-NPs, which was 6.51 mg/mL, appeared after 48 h of incubation for *Streptomyces xinghaiensis* strain NEAA-1. Also, the maximum biosynthesis of the collagen-NPs, which was 7.42 mg/mL, also appeared at 48 h for *Streptomyces plicatus* strain NEAA-3.

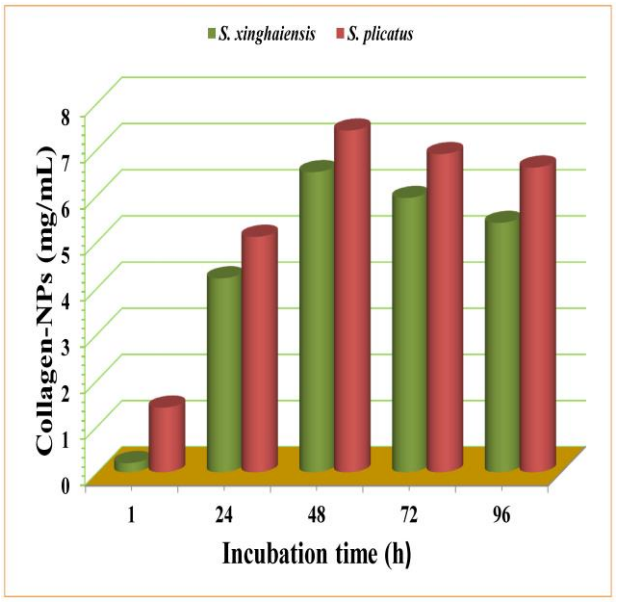

Figure 6. Effect of incubation time on collagen-NPs biosynthesis using cell-free supernatant of *S. xinghaiensis and S. plicatus*.

3.6. Anti-hemolytic activity of collagen-NPs

Hemolysis and the release of hemoglobin into the surrounding blood plasma are caused by free radicals' oxidative disruption of the erythrocytes' cell wall, which contains large amounts of hemoglobin, oxygen, and poly-unsaturated fatty acids **[28]**. When free radicals attack lipid membranes, the lipids are changed into lipid hydroperoxides, which reverse the cell wall's structure and function **[29]**. C-radicals were produced using a hydrophilic azo chemical (AAPH) in a process at a specific temperature. As a result, oxygen molecules can interact with C-radicals to produce peroxyl radicals, and if erythrocyte exposure to AAPH is prolonged, hemolysis progresses **[30]**.

When AAPH free radicals caused hemolysis, collagen-NPs for *Streptomyces plicatus* had a 96 % anti-hemolytic percentage, while conventional L-ascorbic acid (vitamin C) had a 95.8% anti-hemolytic percentage. The anti-oxidation strength of the O-H group found in complexes embedded with phenol, Ar-OH, or NH_2 in aromatic amine complexes contained in collagen-NPs and supported by FTIR results may be responsible for the anti-hemolytic efficacy. Iwatsuki *et al*. **[31]** showed that although amino-substituted phenols are thought to be more efficient than amino-substituted

ones, aromatic amines have strong anti-oxidation effectiveness.

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