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RESEARCH ARTICLE

Chemical Mutants Enable the Photoresponsive Synthesis of Hyaluronic Acid from *Streptococcus equi.*

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Abstract

Hyaluronic acid (HA) production via traditional methods is labor-intensive and time-consuming since the raw product, which is often generated from animals or microorganisms through fermentation, must be thoroughly purified. The purpose of this research is to find a new and better approach to HA stability production. In order to biosynthesize metal nanoparticles, *Streptococcus equi* (*S. equi*) bacteria are exposed to low-intensity ultraviolet (UV) light in the presence of the chemical mutagenic agent ethyl methanesulfonate (EMS) and certain amino acids (L-glutamic acid, L-aspartic acid, L-arginine, L-glycine, and L-lysine). A substantial rise in HA production is seen by the results. After four minutes of exposure to UV light (254nm), 25g/mL of EMS, and 25g/mL of amino acids (glutamic acid and lysine), the bacterial HA output increased from 0.045g/L in the control condition to 0.25g/L and 0.27g/L, respectively. The HA harvest increased by 5.55 times and by 6 times under these circumstances, respectively. The synthesized HA was very close to the reference sample in terms of molecular mass and chemical analysis. Additionally, the produced HA was used as a capping agent in the biosynthesis of silver (13.02.9nm), gold (21.81.9nm), and ultra-small zero-valent iron (4.10.3nm) nanoparticles. The process is not only useful for the synthesis of different nanomaterials, but also for the manufacture of HA, which appears to be quite successful and effective.

Keywords: *Streptococcus equi*, UV-photoproduction, Amino acids, Hyaluronic acid, Mutants, Nanomaterials.

Introduction

Hyaluronic acid and hyaluronan are two forms of anionic glycosaminoglycans that are generated naturally by the body but lack sulfate. The connective, neuronal, and epithelial tissues all contain varying amounts of hyaluronic acid. There is no chemical bond between it and the central protein of the proteoglycan (Boeriu et al., 2013). Due to its viscoelastic and pseudoplastic qualities, hyaluronan (HA) is extensively used in industrial and medicinal applications, especially in wound healing (Deangelis, 1999; Kim et al., 1996). Hyaluronic acid (HA) is produced using standard methods, and it plays an important function in the skin by allowing for tissue upkeep and elasticity (Chong & Nielsen, 2003). The synovial joint fluids, umbilical cord, hyaline cartilage, and the nucleus of the discs surrounding the eyes all contain it in significant amounts. However, its concentration rises when damage is present (Fraser et al., 1997; Laurent, 1998; Islam & Linhardt, 2002; Chong & Nielsen, 2003; Maccari et al., 2004). Hu et al. (2004), Choi et al. (2009), Saravanakumar et al. (2010), Park et al. (2015), and Vázquez et al. (2015) all point to the significance of HA in a number of different contexts, including the lubrication of arthritic joints and the promotion of the adhesion, motility, and organization of cells. In addition to its use as a therapy for arthritis and in the administration of drugs to specific areas of the body, HA has found a home in the cosmetics industry as a moisturizing and anti-aging ingredient. At almost $150 per kilogram (Suzhou Greenway Biotech Co., Ltd., 2020), HA has a higher market value than any other microbial exopolysaccharide. Bacterial fermentation and animal tissue extraction are the two main methods used in industrial production of HA. If you want to prevent contamination, fermented bacterial goods are the way to go instead of those made from animals.

On the other hand, the molecular weight of HA isolated from bacteria is often smaller than that of HA isolated from animal tissues (Islam & Linhardt, 2002; Maccari et al., 2004). Culture conditions for the production of high molecular weight HA from microbes like *S. equi* or *zooepidemicus* have been the subject of several investigations (Kim et al., 1996; Chong & Nielsen, 2003; Attia et al., 2018, 2020).

Unfortunately, natural hyaluronic acid (HA) lacks the stability required for in vivo applications in regenerative medicine and medication administration. Hydrophilicity and the presence of the enzyme hyaluronidase both contribute to HA's susceptibility to breakdown in the body's natural setting. In this research, we sought to create HA nanostructures that were both stable and efficient in entrapping hydrophobic pharmaceuticals. This was accomplished by using a technique that included fusing HA to hydrophobic polymer nanoparticles. Attempts have been made to manufacture novel HA derivatives that are more stable against chemical and enzymatic breakdown and display enhanced mechanical capabilities (Stern, 2004; Banerji et al., 2007; Rho et al., 2018) by experimenting with a wide range of chemical alterations to the HA structure. Both conjugation and crosslinking are often used to alter HA.

Hyaluronic acid (HA) chains may either have a chemical bonded to the whole chain or to specific HA chains. For use in regenerative medicine, HA molecules were modified by adding metal nanoparticles to create a stable nanoscale structure. An important target for inflammatory cells and some malignancies is CD44, which may be targeted more precisely by decorating nanomaterial surfaces with HA (Banerji et al., 2007).

This research was conducted with the hope of optimizing *S. equi's* ability to produce HA. In this study, we investigate whether UV-irradiation, EMS, and amino acids might be used as viable substitutes for more traditional, resource-intensive approaches such using animal and plant sources. In addition, the purpose of this research is to learn how useful the manufactured HA is for creating metal nanoparticles.

Materials and Methods

Microbiological Culture Media and Bacterial Strain Growth Conditions

Strains of *Streptococcus equi subsp. zooepidemicus* (ATCC® 35246™) were procured from the American Type Culture Collection and were used to produce the HA. At -80^{O}C, a complex medium with 25% glycerol was used to store the bacterial stocks. Strains were grown on nutrient-rich medium with concentrations of 50 g of glucose per liter (g/L), 5 g of yeast extract (g/L), 2 g of K_2HPO_4, 2 g of KH_2PO_4, 0.5g of $MgSO_4$, 0.5g of $(NH_4)SO_4$, and 15g of tryptone (g/L). Autoclaving and then sterilizing the medium at 121^{O}C for 15 minutes kept the pH at 7. The bacterial strains were grown in a glass bioreactor with a volume of 2 liters at 37^{O}C with 500 revolutions per minute (rpm) of agitation but no air circulation. To achieve a final concentration of 20 g/L, sterile glucose (500 g/L) was supplied every 2 hours beginning at hour 8 of the culture (Vázquez et al., 2015; Attia et al., 2018) during fed-batch cultivations.

Induction of Mutants to Increase Production of Hyaluronic Acid

Physical mutagenic agent: Ultraviolet (UV) irradiation

Within a hermetically sealed wooden container (Gardner et al., 1991), a low-power UV light with a wavelength of 254nm was placed at a distance of 25cm. After the *S. equi* cells had been cultured in a complicated medium for around 16-18 hours, they were exposed to UV light. The cells were gently stirred using a vibrating shaker for the whole exposure time. Samples of mutants were taken at times ranging from immediately after birth to five minutes later. After incubating the cultures for 24 hours at 37^{o}C, they were serially diluted using sterile 0.85% NaCl solution. Both complicated and minimal medium agar plates were used to cultivate *S. equi* mutant cells.

Chemical mutagenic agent: Ethyl methanesulphonate (EMS)

Vegative cells of *S. equi* were exposed to ultraviolet light for 4 minutes, and then grown in tryptone soybean broth (TSB) at 37^{O}Celsius for 16 hours to determine the effects of the radiation on the cells. Two milliliters of cell suspension were incubated with increasing volumes of EMS (1.17g/L) in a shaking incubator for 10, 15, 30, 45, and 60 minutes. The EMS was neutralized by adding 2 mL of filtered, sterile, 5% (w/v) sodium thiosulfate solution to the cell suspension. This was followed by 5 minutes of centrifugation at 3000 rpm. The cells were then resuspended in 2mL of sodium phosphate (50mM) in pH 6.2 buffer. As recommended by Kamal et al. (2001), the suspension was further diluted on M17 agar.

Effect of different amino acids concentrations

Amino acid levels were modified in this experiment (Armstrong et al., 1997), using a complex substance with concentrations of 0.065g/L for L-arginine, 0.130g/L for L-lysine, 0.195g/L for L-glutamic acid, and 0.265g/L for L-glycine.

Hyaluronic acid purification and recovery

After fermentation, the produced broth was centrifuged at 6000 rpm for 15 minutes to remove the cells. Hyaluronic acid (HA) was separated from the soup by adding three times as much ethanol. After dissolving the polysaccharide in 0.01M NaCl, the precipitation process was stopped by adding 5% cetylpyridinium chloride (CPC) solution. After centrifuging, the insoluble polysaccharide CPC-complex was dissolved in 10% NaCl. The acidic polysaccharide was precipitated by treating the solution with ethanol (3 volumes), and then the solution was dialyzed with distilled water. It spent 24 hours in the fridge at 4^{O}C. The acidic polysaccharides were then dissolved in distilled water and dialyzed against the water. We collected the HA that had precipitated and then dried it in a vacuum at 40^{O}C until a constant weight was attained (Kim et al., 1996).

Molecular weight determinations of HA

The molecular weight of the final product after HA purification was measured using size exclusion chromatography (Shimadzu Corporation). The mobile phase was a 0.1mol/L $NaNO_3$ solution, the injection volume was 20µL, the flow rate was 1.0mL/min, and the temperature was kept at 25^{O}C as per the column manufacturer's recommendations. The calibration curve was determined using a series of molecular weights of dextran (Pires et al., 2010) from 0^3 to 10^6 Da.

Statistical analysis

Means and standard deviations were used to describe the data. One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to analyze data from 68 samples. The statistical significance was determined using a P value of less than 0.05. Sndecor and Cochran (1980) served as inspiration for this approach.

Biosynthesis of silver, gold, and iron nanoparticles

The 1mM $AgNO_3$, 1mM $HAuCl_4$, and 1mM $FeCl_3$ solutions in 50mL were added to a solution containing 10mg of HA. After 30 minutes, 2.5 mL of a 0.1M $NaBH_4$ solution was added to the mixture while stirring continuously. The mixture was stirred for a further 30 minutes, and then placed in a refrigerator at 4^{O}C until further examination could be performed.

Results

Physical Mutagenic Agent (UV-irradiation) for the Production of Hyaluronic Acid

Suspended bacterial cells were subjected to UV radiation of varying wavelengths (254nm) for varying amounts of time. Figure-1 shows that when comparing the frequency of the samples exposed to UV irradiation and the control group, there is a statistically significant difference (p-value ≥ 0.05). This substantiates the hypothesized positive effect of UV irradiation on *S. equi* HA synthesis. After being exposed to UV light for 240 seconds, the dry weight of HA (g/L) increased dramatically, reaching a maximum of 0.143 ± 0.003g/L, as opposed to 0.045 ± 0.00325g/L immediately after exposure. At 270 seconds, the HA concentration dropped to 0.111 ± 0.007 g/L (Fig. 1), and at 300 seconds, it dropped to 0.096 ± 0.003 g/L.

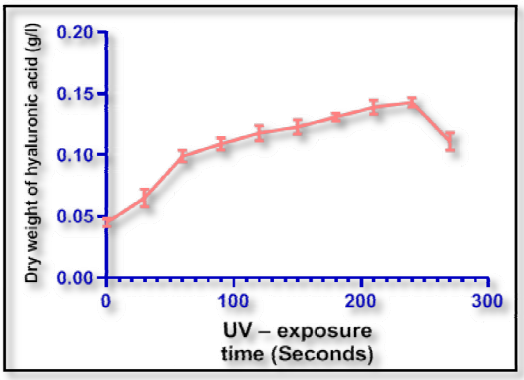

Figure 1: The synthesis of hyaluronic acid after UV-irradiation of *S. equi* at different time intervals.

Chemical Mutagenic Agent Using Ethyl Methane Sulfonate (EMS)

After UV treatment, the *S. equi* cells with the greatest hyaluronic acid (HA) synthesis were selected for mutagenesis using ethyl methanesulfonate (EMS). Figures 2 and 3 show how EMS affects HA production in *S. equi.* After irradiating vegetative cells with UV light for 240 seconds, various amounts of EMS (0, 10, 25, 50, and 100 g/mL) were added to the culture medium for varying amounts of time (15, 30, 45, and 60 minutes). As EMS concentrations rise, the data show that HA generation drops down dramatically. After 45 minutes, HA production was at its maximum with 10 g/mL EMS, whereas after 30 minutes with 25 g/mL EMS, production was at its lowest. Values of statistical significance are ranked as follows for various EMS concentrations: After treating *S. equi* (wild type) with EMS at several concentrations and durations, the best culture conditions for HA production were found to be 25 g/mL for 30 minutes > 100 g/mL for 15 minutes > 10 g/mL for 45 minutes > 50 g/mL for 30 minutes. Compared to prior results (Figure 2), the HA production reached 0.120 g/L under these growth conditions.

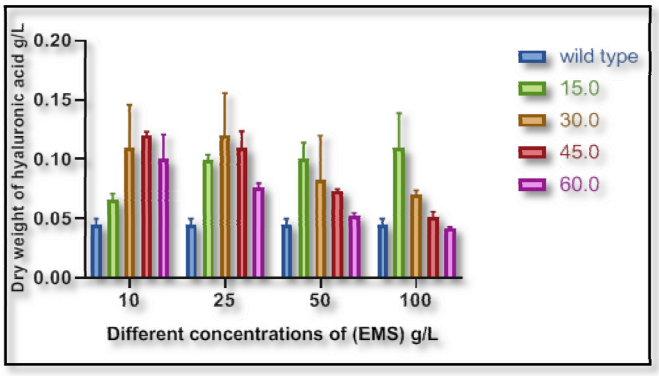

Figure 2: The production of hyaluronic acid after *S. equi* (wild type) was treated with EMS at varying concentrations and times.

To determine the optimal growing conditions for producing HA after exposure to UV-irradiation (254nm for 4 minutes) and treatment with EMS at various concentrations and durations, the significance values of various EMS concentrations can be ranked as follows: 25 µg/mL for 15 minutes > 25 µg/mL for 60 minutes > 10 µg/mL for 30 minutes > 50 µg/mL for 15 minutes. HA was produced at a rate of 0.19 g/L (Figure 3) when grown under the specified conditions.

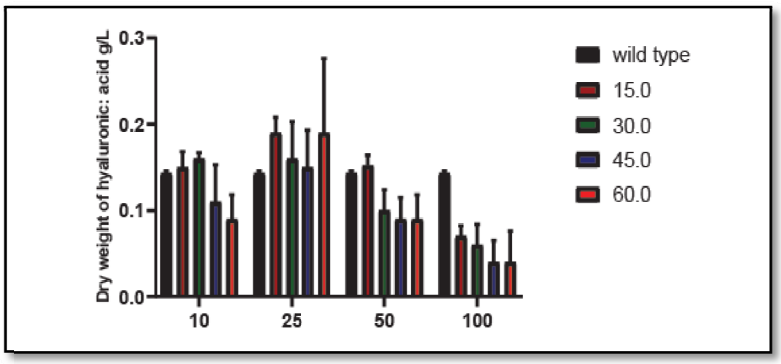

Figure 3: Hyaluronic acid synthesis following UV-irradiation of *S. equi* (254nm for 4 minutes) and subsequent treatment with varying concentrations and durations of EMS.

Effect of Various Amino Acid Concentrations on the Production of HA

The synthesis of hyaluronic acid (HA) seems to rely heavily on amino acids. We conducted an experiment in which we examined the effects of varying the amino acid concentration used to produce HA from 0.065g/L to 0.260g/L. After UV irradiation and EMS treatment (25µg/mL) for 15 minutes, a variety of amino acids were introduced to vegetative *S. equi* cells. These amino acids included L-arginine, L-lysine, L-aspartic acid, L-glutamic acid, and L-glycine. Figure 4 shows that the maximum dry weight of hyaluronic acid (0.25 ± 0.002g/L) was achieved in the presence of 0.130g/l L-arginine. However, a considerable drop in HA output was seen when the L-arginine concentration was raised from 0.195g/L to 0.260g/L.

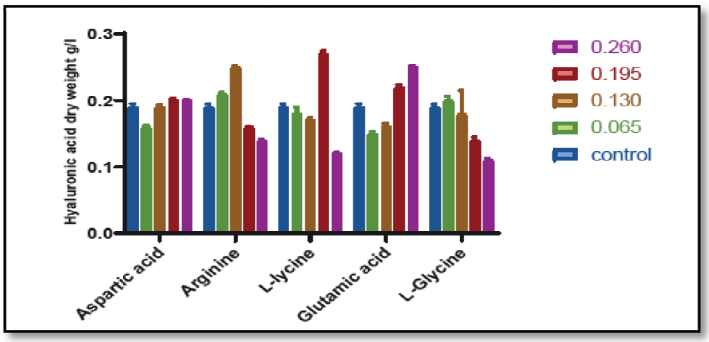

Figure 4: The concentration of hyaluronic acid in a mutant strain of *S. equi* was measured while it was being grown in variable concentrations of amino acids under shaking conditions.

Aspartic acid, arginine, glutamic acid, L-glycine, and L-lysine are some of the essential amino acids used in the laboratory as the principal components of the fermentation medium. In Figure 4, we see the range of amino acid concentrations that, when added to a fermenting slurry of *S. equi* mutants, result in the maximum output of HA (hyaluronic acid). L-lysine showed the best results for encouraging HA synthesis by the *S. equi* mutant out of the five primary amino acids employed in the medium. Similar amounts of HA were produced from a mixture of 0.25g/L arginine and 0.25g/L glutamic acid. The arginine concentration was 0.130g/L, while the glutamic acid concentration was 0.195g/L. In contrast, 0.20g/L of aspartic acid was produced at a concentration of 0.195g/L.

The significant values at various amino acid concentrations are ranked as follows. When compared to other amino acids, L-lysine at 0.195g/L is superior than Arginine at 0.130g/L, Glutamic Acid at 0.260g/L, Aspartic Acid at 0.195g/L, and L-glycine at 0.65g/L. Referring to Fig. 4, the concentration of HA produced in this culture was 0.27g/L.

UV-irradiation (254nm for 240sec) increases hyaluronic acid (HA) content, and Figure 5 shows the ideal concentration of each amino acid to achieve this. The fermentation medium used to grow the strain has different amounts of amino acids. The most effective concentration of these for HA synthesis was L-lysine, at 0.195g/L. Moreover, at the same dose, aspartic acid and glutamic acid showed outcomes that were equivalent to L-lysine. When 0.195g/L of arginine was used, a concentration of 0.29g/L was attained. L-lysine at 0.195g/L > aspartic acid at 0.195g/L > glutamic acid at 0.195g/L > arginine at 0.195g/L > L-glycine at 0.65g/L; this is the order of relative relevance for various medium doses of amino acids. Figure 5 shows that production of HA reached 0.35g/L under these growing conditions.

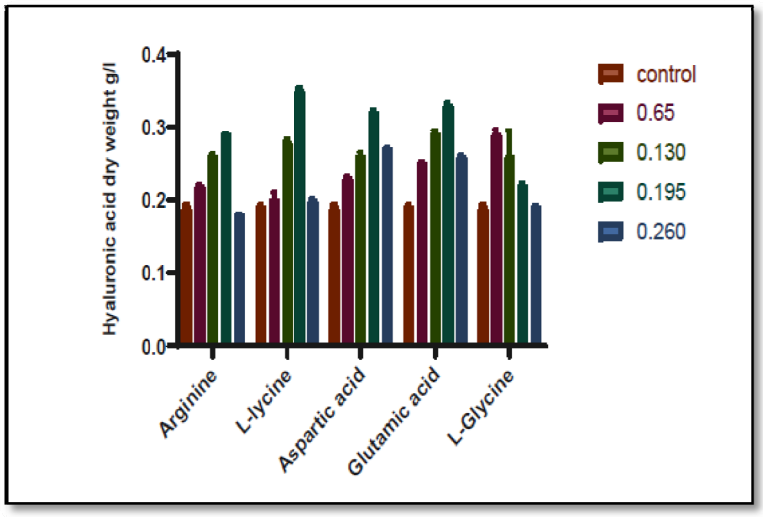

Figure 5: The level of hyaluronic acid in a mutated strain of *S. equi* was measured following exposure to UV radiation (254nm for 4 minutes). The bacteria were cultivated in shaken cultures with varying concentrations of amino acids.

Chemical Structure and Molecular Weight of HA

Ethanol precipitation was used to remove proteins from a *S. equi* culture and get a polysaccharide. Upon chemical examination, the precipitate of bacterial mucopolysaccharides was found to be composed of glucuronic acid (47.1%), N-acetyl glucosamine (36.57%), nitrogen (4.7%), and water (10.74%) on a dry weight basis. By employing the buriet technique, we found no protein or sulfate. Hydrolysis with 1N HCl was carried out in its entirety, and subsequent HPLC analysis revealed that the resultant molar ratio of HA to the sigma standard sample (1.038) was 1.044.

HA Capped Silver, Gold, and Iron Nanoparticles

Using $NaBH_4$ as a reductant, silver, gold, and iron salts were reduced to nanoparticles, with the resultant HA acting as a protective coating. In addition to a large plasmon band with a maximum wavelength of 459nm, the average size of a nanosilver particle is just 13,02.9±nm. Contrarily, the average size of gold nanoparticles is 21.81.9±nm, and they have a maximal absorption plasmon band at 580 nm. The zero-valent iron nanoparticles are so tiny that they don't show any absorption band, despite having an average size of 4.10.3±nm. In addition, the 9-month storage duration demonstrates that the produced particles are quite stable.

Discussion

According to the findings, UV-induced mutants are more resistant to changes in their environment throughout time (Thoma, 1971). UV radiation exposure has also been shown to promote protein synthesis and enzyme activity (Hartke et al., 1995), which all contribute to improved resistance to environmental challenges. UV irradiation induced mutation increased hyaluronic acid synthesis in *S. pyogens*, according to research by Saranraj et al. (2011). This was accomplished by targeting the HAS gene (hyaluronate synthase genes) in the microbes. The cis-syn thymine cyclobutane dimer lesion, also known as the thymine dimer, is one kind of DNA damage that may result from DNA being exposed to UV irradiation for an extended period of time. This thymine dimer prevents proteins from being synthesized because it induces a frameshift. Due to DNA damage, microorganisms experience cell death and HA synthesis drops with prolonged irradiation (Rumora et al., 2008; Attia et al., 2020).

Researchers found that EMS, at its greatest concentrations, was harmful to cells. In contrast, UV-light mutagenic treatment of *S. equi* has been shown to successfully increase HA production. These results are consistent with those found by Kim et al. (1996), who exposed *S. equi* (ATCC 6580) to 100 mg/ml N-methyl-N-nitro-N-nitroso-guanidine for 40 minutes to induce mutations and acquire a mutant strain, S. equi KFCC 10830. Non-hemolytic, hyaluronidase-negative, and kanamycin-resistant HA was produced at a large quantity, but that wasn't all. Reducing EMS-related mutagenesis due to direct mispairing may be possible with effective elimination of O_6-ethylguanine through de-ethylation. Accordingly, EMS-induced noncoding base lesions may function as premutagenic lesions that need error-prone repair to become apparent. Because EMS is a direct-acting mutagen in bacteria, it may induce lesions that prevent regular replication and need repair before normal replication can begin (Drake et al., 1977). Directly induced base mispairing routes and misrepair pathways are the two most common types of mutagenic pathways. However, only O_4-alkylthymine and O_6-alkylguanine, two of the several DNA reaction products produced by alkylating agents like EMS, are expected to contribute to direct mispairing. The conversion of cytosine to uracil and guanine to a cytosine analog by these lesions is a major problem for big genomes. The presence of additional alkylation products, which cause DNA lesions that prevent DNA chain elongation, is also found. Also, DNA polymerase activity in the incorrect removal of inserted nucleotides and in the selection of deoxynucleoside triphosphates are controlled by strong genetic factors (Mohmoud et al., 2013).

Armstrong et al. (1997) found that *S. zooepidemicus* has a unique dietary requirement for four amino acids (not including glutamine). *Streptococci* often have a high need for amino acids. Both Milligan et al. (1978) and Rijn & Kessler (1980) state that *Streptococci* strains need these amino acids for development. When *S. equi zooepidemicus* (ATCC39920) was cultured in P10 medium with 0.2g/L of L-arginine, Aroskar et al. (2012) found that the amount of HA (hyaluronic acid) produced by the bacteria increased dramatically under shaking flask conditions. L-arginine hydrochloride was shown to be a necessary amino acid for the development and HA synthesis by *S. zooepidemicus* (ATCC 35246), as described by Armstrong et al. (1997). As a carbon and nitrogen donor in purine and pyrimidine synthesis, arginine plays an important role in these metabolic processes that are essential for microbial growth, multiplication, and energy consumption (Gao et al., 2006). When glutamine was present at a concentration of 0.2g/L, HA synthesis was significantly boosted, as was previously found by Aroskar et al. (2012). This is due to glutamine's direct involvement in the HA synthesis pathway, in which it provides an amine group for the conversion of fructose 6-phosphate into glucosamine-6-phosphate, a crucial precursor for HA synthesis that ultimately yields N-acetylglucosamine (Chong et al., 2005). Fig. 5 shows that exposing the microbe to UV light for 4 minutes while it was growing in different amino acid concentrations under shaken culture conditions resulted in enhanced HA synthesis.

S. equi's acidic polysaccharide found in the extracellular space has a sugar-to-acid molar ratio of 1.04. After extracting polysaccharides from the vitreous humor of cow eyes and umbilical cords, Meyer and Plamer (1934) found that the polysaccharides possessed large molecular weights. The polysaccharide was made up of glucuronic acid and N-acetyl glucosamine residues in equal parts (20.5% each). Hyaluronic acid isolated from group A *Streptococci* was shown to have a similar composition.

When compared to the molecular weight of 1.45 x 10^6 daltons found in the reference HA sample, the molecular weight of the purified HA sample is 1.47 x 10^6. Polymers of 2.91 × 10^6, 5.01 × 10^6, and 1.19 × 10^5 daltons were produced using a mutant strain previously reported to be capable of generating HA with extraordinarily high molecular weights (Kim et al., 1996; Kakizaki et al., 2002).

The relevance of these metal nanoparticles' medicinal uses is greatly increased by using HA as a covering ingredient. In addition, a nanoscale HA structure that may be used in regenerative medicine is obtained.

Conclusion

In this study, hyaluronic acid was used as a capping material in the synthesis of silver (Ag), gold (Au), and iron (Fe) nanoparticles. Using EMS and amino acids to create chemical mutants, we were able to extract hyaluronic acid from *S. equi* using UV photoproduction. Increased hyaluronic acid synthesis was the outcome of a mutation in *S. equi* cells produced by UV-irradiation and EMS treatments. When compared to the control group (wild type), the mutant cells' output increased even more after being supplemented with the amino acids arginine, glutamic acid, and lysine. With a molar ratio of 1: 1.04 between glucuronic acid and N-acetyl glucosamine, the chemical structure of the *S. equi* photoproduced hyaluronic acid was very similar to that of the reference sample (which had a molar ratio of 1: 1.03). The biological benefits of silver, gold, and iron nanoparticles may be improved with the use of hyaluronic acid as a capping agent. Using this technique, hyaluronic acid with a stable nanoscale structure is successfully created for use in regenerative medicine.

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CONFLICTS OF INTEREST

The author does not have conflicts of interest to declare.

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