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Photoresponsive Production of Hyaluronic Acid from *Streptococcus equi* under Chemical Mutants

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THE TRADITIONAL methods for hyaluronic acid (HA) production are costly and require extensive purification of the basic product. These techniques are based on animal extractions and the fermentation from micro-organisms. This research aims to develop a new, improved method for the stable HA production by irradiation *Streptococcus equi* (*S. equi*) to low-power UV light under the ethyl methanesulfonate (EMS) influence as a chemical mutagenic agent as well as certain amino acids (L-glutamic acid, L-aspartic acid, L-arginine, L-glycine, and L-lysine) for biosynthesizing metal nanoparticles. The results clearly show the steady development of the control HA (0.045g/L) to 0.25, and 0.27g/L during UV light (254nm) exposure for four minutes and treatments of 25µg/mL (EMS) and amino acids (glutamic acid and lysine). Under these conditions, the incubation of *S. equi* resulted in an increase in the HA yield by factors of 5.55 and 6, respectively. The molecular mass and chemical analysis of the obtained HA showed considerable similarities compared to the standard sample. The obtained HA was utilized as a capping agent for the biosynthesis of silver (13.0±2.9nm), gold (21.8±1.9nm), and ultra-small zero-valent iron (4.1±0.3nm) nanoparticles. This method provided ample achievement and is effective in HA production.

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

Introduction:

Nonsulfated anion glycosaminoglycans that are naturally formed by the body are hyaluronic acid or hyaluronan. In connective, neural, and epithelial tissues, HA is widely distributed. The proteoglycan core protein is not covalently linked with it (Boeriu et al., 2013). Hyaluronan (HA), because of its viscoelastic and pseudoplastic properties, plays a major role in industrial and biomedical purposes, especially wound healing (Deangelis, 1999; Kim et al., 1996). The traditional methods for hyaluronic acid (HA) production. It is a key structural element within the skin and fulfills important functions such as structural maintenance of tissues and flexibility (Chong & Nielsen, 2003). It can be detected at a high concentration inside the eye's humor, disc nucleus, synovial joint fluids, umbilical cord, and hyaline cartilage. However, its concentration increases when there is damage (Fraser et al., 1997; Laurent, 1998; Islam & Linhardt, 2002; Chong & Nielsen, 2003; Maccari et al., 2004). In different functionalities, HA plays an important role, such

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as arthritis joint lubrication. HA participates in important cell functions such as adhesion to the cell-matrix, motility, and organization of the cells (Hu et al., 2004; Choi et al., 2009; Saravanakumar et al., 2010; Park et al., 2015; Vázquez et al., 2015). HA is used in cosmetics as the hydrate, anti-aging material, arthritis treatment, and the targeted delivery of drugs because of its properties and functions. The market value of HA is much greater than that of other microbial exopolysaccharides ($ US 150 per kilogram, Suzhou Greenway Biotech Co., Ltd., 2020). HA is commercially produced by either extracting from bacterial fermentation or animal tissues. The bacterial fermentation procedure is the most desirable production system due to the contamination of animal-derived products. But, HA with a lower molecular weight was obtained from bacterial sources compared with that extracted from animal tissues (Islam & Linhardt, 2002; Maccari et al., 2004). The culture conditions for production of HA with a high molecular weight from microorganisms such as *S. equi* or *zooepidemicus* have been developed by several studies (Kim et al., 1996; Chong & Nielsen, 2003; Attia et al., 2018, 2020).

Unfortunately, native HA exhibits properties incompatible to make stable structures to be utilized in vivo for regenerative medicine and drug delivery applications. Indeed, HA is degraded due to high hydrophilicity and the influence of an enzyme known as the hyaluronidase when injecting it in the physiological environment. For these reasons in this study in order to obtain HA stable nanostructures, able to incorporate hydrophobic drugs. This strategy was to synthesize nanostructured systems in which HA was anchored onto nanoparticles, based on hydrophobic polymers. Various chemical modifications of the HA structure have been developed to synthesize new HA derivatives which are less susceptible to chemical and enzymatic hydrolysis and show enhanced mechanical properties (Stern, 2004; Banerji et al., 2007; Rho et al., 2018). Two main ways can be used to modify HA, conjugation or crosslinking.

A compound is grafted onto the HA chain in the former case, while the latter is connected with separate HA chains. Moreover, to obtain a HA stable structure at nanoscale for regenerative medicine application, HA molecules were modified by capping metal nanoparticles. HA decoration of nanomaterial surfaces has the benefit of targeting cells that overexpress HA receptors, for example, CD44, a strategic target for inflammatory cells or certain forms of tumors (Banerji et al., 2007).

Herein, this work aims to enhance the HA production by the microorganism, *S. equi*, using UV-irradiation, EMS, and amino acids, as a substitute thereto found within the animal and plant sources, which are very expensive and produced in small quantities. In addition, one of the objectives of this work is to identify the role of the produced HA within the synthesis of metal nanoparticles.

Materials and Methods

Microbiological culture media and growth conditions of bacterial strains

The production of HA was performed using the purchased bacteria-strains of *Streptococcus equi subsp. zooepidemicus* (ATCC® 35246™, selection American Type Culture Collection, Manassas, VA, USA). Bacterium Stocks have been stored at −80°C in a complex medium with 25% glycerol. Nutrients, glucose (50g/L), yeast extract (5g/L), K_2HPO_4 2g/L), KH_2PO_4 (2g/L), $MgSO_4$ (0.5g/L), $(NH_4)SO_4$ (0.5g/L), and tryptone (15g/L) were located on the growth media for the strains. In all cultures, after autoclaving and sterilized at 121°C/15min, the pH was maintained at 7. Glass bioreactor (2-liter capacity) was used to cultivate the bacteria-strains at 37°C, 500rpm of agitation, and without aeration. In the fed-batch cultivations, sterile glucose (500g/L) was added every 2hrs. (from 8 to 10hrs. of culture) up to 20g/L (Vázquez et al., 2015; Attia et al., 2018).

Induction of mutants for increasing hyaluronic acid production

Physical mutagenic agent: Ultraviolet (UV) irradiation

Low power ultraviolet lamp (254nm) was fixed at 25cm in a tightly closed wooden chamber (Gardner et al., 1991). The UV-irradiation was triggered by an aliquot of *S. equi* cells cultivated in a complex medium between 16-18hrs. During the exposure time, it was gently agitated on a vibratory shaker. The mutants were isolated at different times (zero-5min). After that, the cultures have been diluted in a serial way into sterile 0.85% NaCl and incubated for 24hrs. at 37ºC, the mutant cells of *S. equi* were plated into complex and minimum medium agar plates.

Chemical mutagenic agent: Ethyl methanesulphonate (EMS)

After exposing vegetative cells of *S. equi* to UV irradiation for 4min, those and the wild type were grown in tryptone soybean broth (TSB) for 16hrs. at 37ºC. In a shaking incubator, the EMS (1.17g/L) was then added to 2mL cell suspension in various quantities (0, 10, 25, 50, and 100µL) and incubated in different cycles (0, 10, 15, 30, 45, and 60min). Afterwards, the cell suspension was applied with a fresh 5% (w/v) filter-sterilized solution of sodium thiosulfate (2mL) for quenching the EMS and then centrifugation was performed at 3000 rpm for 5min. The treated cells were subsequently suspended with a buffer of 2mL of sodium phosphate (50mM) at pH 6.2. On M17 agar, the suspension was diluted. (Kamal et al., 2001).

Effect of different amino acids concentrations

A complex medium was used in this experiment with certain changes concerning the concentration of amino acid source (L-arginine, L-lysine, L-glutamic acid, L-aspartic acid, and L-glycine) 0.065, 0.130, 0.195, and 0.265g/L (Armstrong et al., 1997).

HA purification and recovery

The cultivated broth was centrifugated for 15min at 6000rpm to remove the cells after fermentation. By adding three volumes of ethanol, HA was precipitated from the broth. The above polysaccharide was dissolved into 0.01M NaCl and added to the purification 5% aqueous cetylpyridinium chloride (CPC) until no precipitation was observed again in purification. Centrifuging and dissolving the insoluble polysaccharide CPC-complex into 10 percent NaCl. The solution was dialyzed against distilled water to precipitate the acidic polysaccharide by treating with ethanol (3 volumes) and refrigerated at 4ºC for 24hrs. The acidic polysaccharides were then dissolved and dialyzed against distilled water. The HA precipitation was recovered and vacuum dried at 40°C to a constant amount of weight (Kim et al., 1996).

Molecular weight determinations of HA

The average molecular weight of the purified HA was calculated by using the size exclusion chromatography (Shimadzu Corporation, Kyoto, Japan). The analytical conditions were: 20µL of the injected sample, 0.1mol/L $NaNO_3$ solution as the mobile phase, flow rate of 1.0mL/min, and 25ºC temperature, as suggested by the manufacturer of the column. Dextran (American Polymer Standards, Mentor, OH, USA) in the range of 0^3 to 10^6 Da molecular weight was Tab used as a standard for the calibration curve (Pires et al., 2010).

Statistical analysis

Descriptive values of data were represented as means ± standard errors. Statistical analysis was performed for 68 samples using one way variance analysis (ANOVA) followed by Duncan's Multiple Range Test with $P \leq 0.05$ being considered statistically significant (Sndecor & Cochran, 1980).

Biosynthesis of silver, gold, and iron nanoparticles

10mg of the produced HA were added to 50mL of 1mM $AgNO_3$, 1mM $HAuCl_4$, and 1mM of $FeCl_3$ solution. Then, stirring was for 30min and 2.5mL of 0.1M $NaBH_4$ were added. Stirring followed for 30min and then storage at 4ºC for further characterizations.

Results

Physical mutagenic agent (UV-irradiation) for hyaluronic acid production

The suspension of the bacterial cells was irradiated for various periods with UV light (254nm). From the results in figure 1, the p-value more than or equal to 0.05 which means that the difference in frequency between the samples exposed to UV- irradiation and the control is significant. This confirms the impacts of UV-irradiation on increasing HA production by *S. equi*. The dry weight of HA (g/L) grew rapidly to a maximum level at 240sec. of UV-irradiation 0.143± 0.003g/L compared to 0.045± 0.00325g/L at zero seconds but begins to degrade at 270 and 300sec. to 0.111± 0.007g/L and 0.096± 0.003g/L, respectively (Fig. 1).

Chemical mutagenic agent using ethyl methane sulfonate (EMS)

S. equi cells which generated the highest HA production after UV treatment were mutagenized via EMS. The influence of EMS on the HA production by *S. equi* is shown in Fig. 2 and 3. Different concentrations of EMS (0, 10, 25, 50, and 100µg/mL) were added to vegetative cells for different periods (15, 30, 45, and 60min) and after UV irradiation for 240 seconds. Results clearly show that HA production was decreased sharply

by increasing EMS concentration. The highest HA production was obtained after 45 and 30min with 10 and 25µg/mL of EMS, respectively but other treatments induced little HA production. The significance values of different EMS concentrations are in the following order: 25 (µgmL) for 30min > 100 (µgmL) for 15min > 10 (µgmL) for 45min > 50 (µgmL) for 30min in order to control the optimal cultivation conditions of HA production after treatments of *S. equi* (wild type) with EMS with different concentrations for different times. Under such culture conditions, the HA output reached 0.120g/L. These values were significantly higher than those previously obtained (Fig. 2).

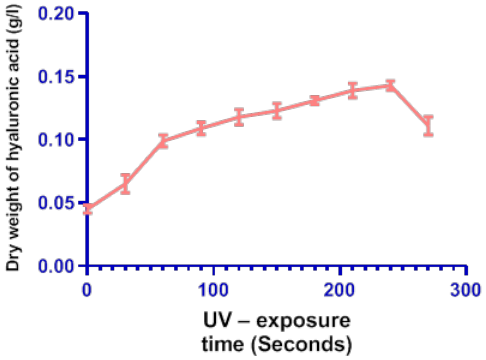

Fig. 1. Hyaluronic acid production after exposure of *S. equi* to UV-irradiation at different times

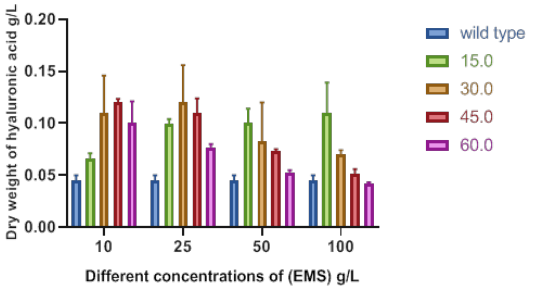

Fig. 2. Hyaluronic acid production after treatments of *S. equi* (wild type) with EMS with different concentrations for different times

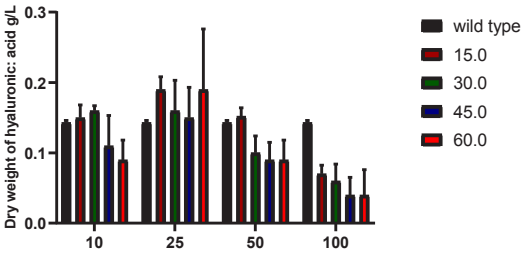

Fig.3. Production of hyaluronic acid after exposure of *S. equi* to UV-irradiation (254nm for 4min) and treatment with different concentrations of EMS for different times

To determine the optimal cultivation conditions for HA production after exposure to UV-irradiation (254nm for 4min) and treatment of EMS with different concentrations for different times, the significance values of different EMS concentrations are in the following order: 25 (µgmL) for 15min > 25 (µgmL) for 60min > 10 (µgmL) for 30min > 50(µgmL) for 15min. Under such culture conditions, the HA output reached 0.19g/L (Fig. 3).

Influence of different concentrations of amino acid on HA production

Amino acids seem to be an important factor for HA production. In our experiment, different amino acids concentrations (0.065g/L to 0.260g/L) were tested for production of HA such as L-arginine, L-lysine, L-aspartic acid, L-glutamic acid, and L-glycine which added to vegetative cells of *S. equi* after UV irradiation and treatment with EMS (25µg/mL) for 15min. Figure 4 states that the maximum dry weight of hyaluronic acid (0.25± 0.002g/L) is recorded by the presence of 0.130 g/l L-arginine. While increasing L-arginine concentration from 0.195g/L to 0.260g/L, a high decrease in HA yield production is observed.

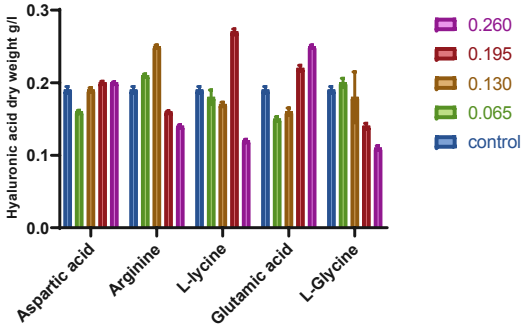

Fig. 4. Hyaluronic acid content of *S. equi* mutant grown on different concentrations of amino acids under shaken culture condition.

The essential amino acids used for the laboratory as the origins of the fermentation media are aspartic acid, arginine, glutamic, L-glycine, and L-lysine acid. Figure 4 represents the best concentration of each amino acid which provides the highest HA content of *S. equi* mutant strain cultivated in the fermentation medium with different amino acid concentrations. L-lysine was the best amino acid for *S. equi* mutant for HA production development among the five main amino acids used in the medium. Arginine and glutamic acid contributed similar output of HA,

arginine gave 0.25g/L at concentration 0.130g/L, glutamic acid gave 0.25g/L at concentration 0.195g/L, respectively. When aspartic acid gave 0.20g/L at 0.195g/L. The significance values at different amino acids concentrations of are in the following order: L lysine at conc. 0.195 g/l > Arginine at conc. 0.130g/L > glutamic acid at conc.0.260g/L > aspartic acid at conc 0.195g/L > L glycine at 0.65g/L. Under such culture conditions, the HA output reached 0.27g/L (Fig. 4).

Figure 5 shows the best concentration of each amino acid which gives the highest HA content after UV-irradiation (254nm for 240sec). The strain was submersion-cultured in a fermentation medium with different amino acid concentrations. L-lysine also gave the highest result for HA production that was generated at the concentration 0.195g/L, while the results of aspartic acid and glutamic acid were very close to L-lycine at the same concentration. When arginine gave 0.29 (g/L) at 0.195g/L. The significance values of different medium concentrations of amino acids are in the following order: L lysine at conc. 0.195g/l > aspartic acid at conc. 0.195g/L > glutamic acid at conc.0.195g/L > arginine at conc 0.195g/L > L glycine at 0.65g/L. Under such culture conditions, the HA output reached 0.35g/L (Fig. 5).

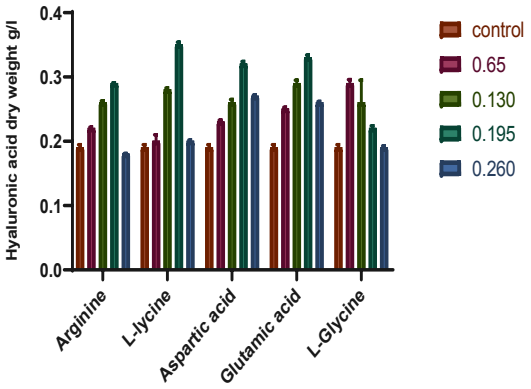

Fig. 5. Hyaluronic acid content of *S. equi* mutant after exposure to UV-irradiation (254nm for 4min) grown on different concentrations of amino acids under shaken culture condition

Chemical structure and molecular weight of HA

Polysaccharide derived from *S. equi* was isolated from protein-free culture by ethanol precipitation. The bacterial mucopolysaccharide precipitate was subjected to chemical analysis that calculated on a dry weight basis of HA as glucuronic acid (47.99%), N-acetyl glucosamine (36.57%), nitrogen (4.7%), and moisture (10.74%). No protein or sulfate has been detected by the buriet method. After complete hydrolysis with 1N HCl and determination by using HPLC, the molar ratio of produced HA was 1.044 in comparison with the standard sample (1.038) from sigma.

HA capped silver, gold, and iron nanoparticles

Nanosilver, gold, and iron nanoparticles were formed by reduction of their salts using $NaBH_4$ and the generated HA as capping material. Figure 6 shows the absorption spectra and TEM images for the formed nanoparticles. The size of the formed nanoparticles is 13.02.9±nm for nanosilver with broad plasmon band at maximum wavelength 459nm, 21.81.9±nm for Au nanoparticles with maximum absorption plasmon band at 580nm, and ultra-small zero-valent iron 4.10.3±nm with no absorption band. The formed particles also show high stability with time (stored for 9 months).

Figure 7 displays the patterns of XRD of synthesized HA capped gold nanoparticles. Intense XRD peaks were observed with respect to the crystalline nature of the Ag NPs corresponding to the (111), (200), (220), (311) planes at 2θ angles of 38.29°, 44.38°,64.56°, and 77.64°, respectively (Li et al., 2012; Attia et al. 2016a; Arisha et al., 2019) while the diffraction peaks at 2θ values of 38.30°, 44.43°, 64.67°, and 77.67°, corresponding to the (111), (200), (220), and (311) reflection of the crystalline metallic gold, respectively (Manivasagan & Oh, 2015; Attia et al., 2016b). Iron nanoparticles show XRD peak at 2θ angles of 53.35°corresponding to (422) planes (Li et al., 2008; Abdelsalam et al., 2017).

Discussion

From the results, the changes due to UV induced mutants that were more stable through a long term of generations and subculturing (Thoma, 1971). In addition, UV exposure stimulates tolerance to multiple environmental stresses, improvements in the synthesis of proteins and increasing biosynthesis enzyme activities (Hartke et al., 1995). This has led to the study (Saranraj et al., 2011) that *S. pyogens* have irradiated with UV light for 10min, resulting in an efficient UV mutation effect on the HAS gene (hyaluronate synthase genes) and a UV irradiation mutation enhances the development of hyaluronic acid.

DNA exposure to UV irradiation for a long time also contributes to the formation of various kinds of DNA damage, including the cis-syn thymine cyclobutane dimer lesion, hereafter called the thymine dimer which causes a frameshift and blocking for protein synthesis. The DNA damage causes death to the microbial cells and decreases HA production at long irradiation time (Rumora et al., 2008; Attia et al., 2020).

The highest levels of EMS were found to be cell-toxic. However, UV-light mutagenic treatment has proved effective in enhancing HA production by *S. equi*. through EMS. The above findings agree with Kim et al., (1996) who mutated *S. equi* (ATCC 6580) by 100 mg/ml N-methyl-N—nitro-N-nitroso-guanidine during 40min and obtained a mutant *S. equi* KFCC 10830. The development of HA with a high molecular weight at a high level of production, was not only non-hemolytic, hyaluronidasis-negative or kanamycin-resistant. In the case of EMS, the efficient de-ethylation of O_6- ethylguanine might reduce mutagenesis by direct mispairing. Thus, noncoding base lesions residual which induced by EMS might constitute premutagenic lesions requiring error-prone repair for realization. In addition, EMS that has been considered a direct-acting mutagen in bacteria may induce lesions that block normal replication and that must be repaired before normal replication resumes (Drake et al., 1977). Mutagenic pathways in this regard have two main classes directly induced base mispairing and misrepair. Alkylating agents such as EMS for example produce a wide range of different DNA reaction products, but only two of them are likely to candidate for directly induced mispairing (O4-alkylthymine and O6-alkylguanine). One that presents a particularly serious challenge to large genomes and cytosine converted to uracil and guanine to an analog of cytosine. DNA lesions that break DNA chain elongation, including many other products of alkylation. Furthermore, mutagenic mechanisms are subjected to powerful genetic controls which include DNA polymerase activity in the improper removal of inserted nucleotides and in the selection of deoxynucleoside triphosphates (Mohmoud et al., 2013).

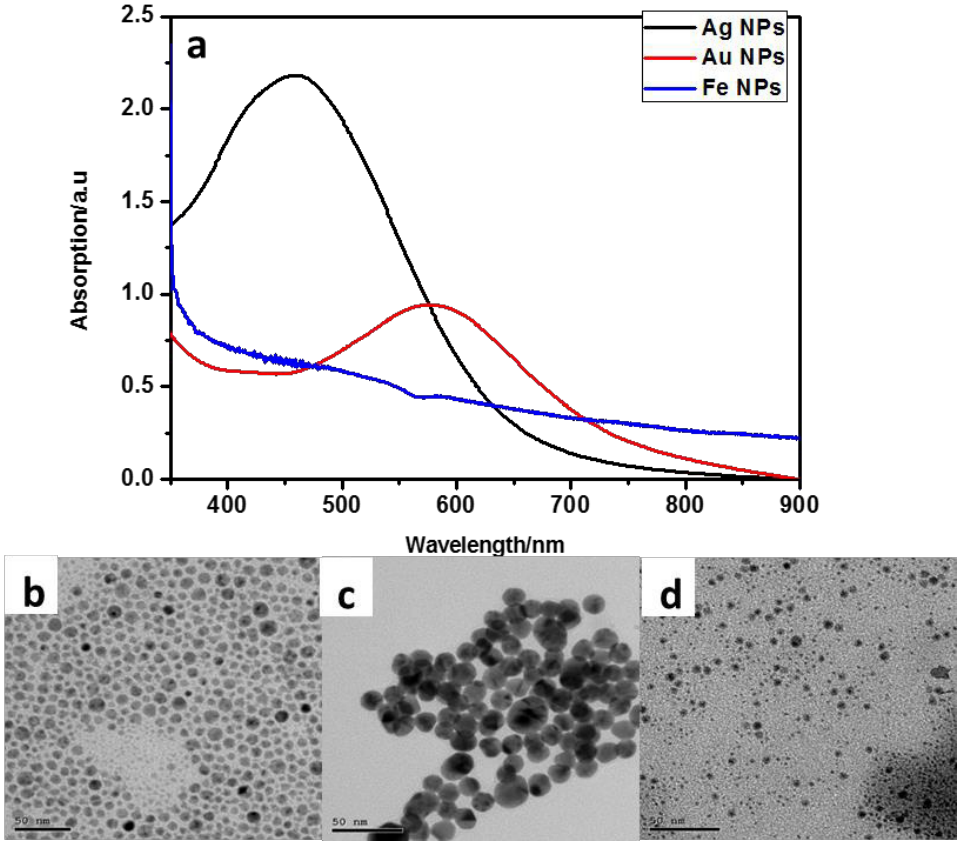

Fig. 6. Absorption spectra of Ag, Au and Fe nanoparticles (a). TEM images of the formed particles Ag (b), Au (c) and Fe (d) nanoparticles

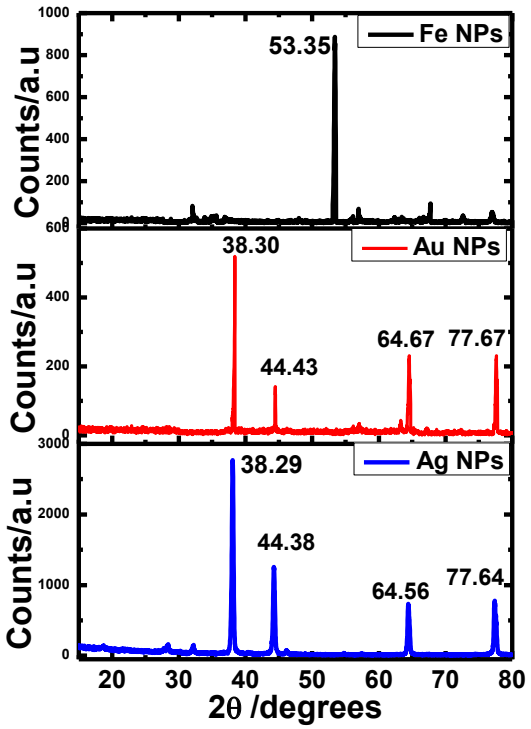

Fig. 7. XRD pattern of HA capped iron (a), gold (b) and silver (c) nanoparticles

S. zooepidemicus was found to have an essential growth requirement for 4 amino acids (excluding glutamine), according to Armstrong et al. (1997). This exacting nutritional requirement for amino acids is not uncommon in *Streptococci*. It is reported that the amino acids are essential for the growth of *Streptococci* strains (Milligan et al., 1978; Rijn & Kessler, 1980). Aroskar et al. (2012) showed that there was a significant increase in the yield of HA when L- arginine at 0.2g/L was added in P10 medium under shake flask conditions by *S. equi zooepidemicus* (ATCC39920). In addition, it was reported that the essential amino acids that are necessary for the growth and production of HA from *S. zooepidemicus* (ATCC 35246) are L-arginine hydrochloride (Armstrong et al., 1997). The role of arginine is that it acts as a carbon and nitrogen donor in the purine, and pyrimidine synthesis, which are the basic needs for the growth, multiplication and the energy-consuming pathway for the synthesis by the microbes (Gao et al., 2006). Aroskar et al. (2012) showed that there was a significant increase in HA production observed at 0.2g/L concentration of glutamine. That due to glutamine is a component that involves directly in the pathway for HA synthesis and donates the amine group to the conversion of fructose 6- phosphate into glucosamine- 6- phosphate which is an important precursor for HA synthesis that produces N- acetylglucosamine in further reactions (Chong et al., 2005). The production of HA increased after exposure to UV light for 4min and grown in different concentrations of amino acids under shaken culture condition as shown in Fig. 5.

The molar ratio of the sugars from extracellular acidic polysaccharide elaborated from *S. equi* was 1.044. Meyer & Plamer (1934) noted that the polysaccharides had highly molecular weights from the vitreous humor of cattle eyes and umbilical cord. It was composed of an equal number of N-acetyl glucosamine (20.5%) and glucuronic acid residues (20.5%). It was similar to the hyaluronic acid isolated from group A Hemolytic *Streptococci.*

The molecular weight of the obtained HA was calculated to be 1.47×10^6 da for the purified sample compared with the standard sample of HA that gave the value of the molecular weight 1.45×10^6 da. It is reported that HA-producing mutant of very high molecular weight gave polymers ($2.9x10^6$, $5x10^6$ and $1.19x10^5$ da) (Kim et al., 1996; Kakizaki et al., 2002) and the obtained results are in agreement with that.

The presence of HA as a capping material for these metal nanoparticles increases the importance of their biomedical applications. In addition, a robust HA nanoscale structure is obtained for use in regenerative medicine.

Conclusion

In this study, Ag, Au, and Fe nanoparticles were formed by using hyaluronic acid as a capping material that was UV photoproduced from *S. equi* under chemical mutants of EMS and amino acids. UV-irradiation and EMS treatments induced mutation to the cells of *S. equi* and increased hyaluronic acid production. Also, the addition of arginine, glutamic acid, and lysine to mutant cells increased production compared with wild type (control). The chemical structure of the photoproduced hyaluronic acid from *S. equi* was similar to the chemical structure of the standard sample and glucuronic acid and N–acetyl glucosamine has the molar ratio 1: 1.04 compared with the standard sample (1:1.03). HA capping of Ag, Au, and Fe nanoparticles could improve the

HA biomedical applications. This ample method is very efficient to obtain a HA stable structure at nanoscale for regenerative medicine application.

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Competing interests: The authors declare no conflicts of interest.

Authors' contributions: All authors contributed equally in all parts of this study.

Ethical approval: Not applicable

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إنتاج مستجيب ضوئيًا لحمض الهيالورونيك من المكورات العقدية *Streptococcus equi* تحت الطفرات الكيميائية

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الطرق التقليدية لإنتاج حمض الهيالورونيك (HA) مكلفة وتتطلب تنقية واسعة النطاق للمنتج الأساسي. تعتمد هذه التقنيات على عمليات الاستخراج من الحيوانات والتخمير من الكائنات الحية الدقيقة. يهدف هذا البحث إلى تطويرطريقة محسنة لإنتاج HA الثابت بواسطة تشعيع المكورات العقدية (*Streptococcus equi*) إلى ضوء الأشعة فوق البنفسجية منخفض الطاقة تحت تأثير إيثيل ميثان سلفونات (EMS) كمادة كيميائية وعامل مطفر وكذلك بعض الأحماض الأمينية (حمض الجلوتاميك، حمض الأسبارتيك، حمض أرجينين، حمض جلايثين وحمض لايسين) وذلك للتحضير الحيوي للجسيمات النانوية المعدنية. تظهر النتائج بوضوح التحسين المستمر للانتاج حمض الهيالورنيك من (0.045 جم / لتر) إلى 0.25 و 0.27 جم / لتر بعد التشعيع إلى ضوء الأشعة فوق البنفسجية (254 نانومتر) لمدة أربع دقائق وعلاجات 25 ميكروغرام/ مل (EMS) والأحماض الأمينية (حمض الجلوتاميك والليسين). في ظل هذه الظروف، أدى حضانة *S. equi* إلى زيادة في عائد HA بعوامل 5.55 و 6 على التوالي. الكتلة الجزيئية والتحليل الكيميائي من HA الذي تم الحصول عليه أظهر أوجه تشابه كبيرة مقارنة بالعينة القياسية. تم استخدام HA الذي تم الحصول عليه كعامل تغطية للتحضير الحيوي لجسيمات الفضة النانوية (13.0 ± 2.9 نانومتر)، جسيمات الذهب النانوية (21.8 ± 1.9 نانومتر)، وجسيمات الحديد النانوية فائقة الصغر (4.1 ± 0.3 نانومتر). هذه الطريقة قدمت إنجازات وافرة وفعالة في إنتاج HA.