ENVIRONMENTAL FRIENDLINESS OF THE PRODUCTION OF GLYCOSAMINOGLYCANS BY BIOTECHNOLOGICAL MEANS

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Glycosaminoglycans (GAGs) are an important biomolecule with wide applications in pharmaceuticals, cosmetics, and medicine. Traditionally obtained through extraction from animal tissues, this method poses contamination risks and quality control issues. Microbial engineering, or chemical synthesis, emerges as a promising eco-friendly, controlled, and cost-effective alternative for producing GAG. Animal tissue extraction has significant environmental drawbacks due to high energy and water consumption, as well as toxic waste emissions during raw material processing. It also risks transmitting zoonotic diseases from close animal contact and tissue processing. The microbiological method ensures high product purity and quality. Unlike animal sources, biotechnological production eliminates contamination risks and allows obtaining a pure product under controlled conditions. It is less costly and more environmentally friendly than traditional extraction methods. Moreover, it aligns with the growing cruelty-free and vegan cosmetics trend, as mandated by EU Regulation No. 1223/2009 on cosmetic products. However, natural GAG producers are zoonotic pathogens grown on media with sheep's blood and brain heart infusion. Their synthesis product contains endotoxins, requiring costly additional cleaning and isolation steps. This issue is addressed by developing new non-pathogenic producer strains capable of growing on eco-friendly media. Thus, it was possible to create Bacillus subtilis 3NA*, which allows obtaining 7 g/l of hyaluronic acid, using technical glycerin as a carbon source. Also, it was possible to create Pichia pastoris capable of producing heparin with a concentration of 2.08 g/l in a medium with methanol.*

Keywords: glycosaminoglycans, hyaluronic acid, microbial synthesis, chondroitin sulfate, heparin

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Introduction

Trends in the skincare sector have changed significantly. The growing consumer awareness about the importance of skincare, including the desire to prolong youth, has led to a demand for products that not only improve appearance, but also have a positive impact on skin health. New and effective ingredients that improve skin condition are being discovered, with glycosaminoglycans (GAGs) (Rathod, Mali, Shinde, Aloorkar, 2020) becoming extremely popular as new anti-aging components, especially hyaluronic acid, chondroitin sulfate, and dermatan sulfate. They provide water retention, elasticity and suppleness to the skin, and also have antioxidant properties, making them an integral part of health and youth.

Materials and Methods

Literature sources of foreign and domestic scientists in leading periodicals and specialized world publications devoted to various methods of obtaining hyaluronic acid and selecting the most optimal method from an environmental point of view were analyzed. The search for scientific articles was carried out using global scientometric databases such as Google Scholar and PubMed/

Results and Discussion

Previously (until the second half of the 20th century), these compounds were obtained by extraction from animal tissues - cockscombs, cartilage and cattle hides, etc. This method has many disadvantages, in particular, that this production leads to significant environmental burden due to high energy and water consumption, as well as producing emissions of toxic waste during raw material processing (Cristiano, Guagni, 2022). Additional environmental risks include the potential transmission of zoonotic diseases, prions, and exotoxins due to close contact with animals and processing of their tissues.

Animal wastes from agricultural operations have had a severely negative impact on the environment. The shift towards more efficient and concentrated animal production methods has led to the accumulation of immense amounts of animal waste being concentrated in relatively small areas. This concentration of liquid and solid wastes has resulted in significant water and air pollution problems. Runoff from open livestock facilities like cattle feedlots and improper land application of untreated manure have been major causes of water pollution. Runoff from cattle feedlots in particular contains extremely high levels of fecal bacteria indicative of poor sanitary conditions, as well as high concentrations of ammonia nitrogen, suspended solids, chemical oxygen demand, and volatile acids. Cases of fish kills and degradation of recreational water bodies have occurred due to this polluted runoff entering surface waters.

The uncontrolled land disposal of animal wastes as a fertilizer can also lead to leaching of nutrients and nitrates into groundwater supplies. Odor emissions from the facilities themselves and from land application of the malodorous fresh wastes have been an ongoing nuisance issue, with animal operations facing complaints and possible legal action from neighboring residential areas impacted by the offensive odors. On a national scale in the United States, the production of animal wastes vastly exceeds the quantity of human waste generated, with animal wastes being 5 times greater in biochemical oxygen demand, 10 times higher in total solids, and 7 times more in total nitrogen content. Agriculture in general, and the animal production industry in particular, can be considered a prime contributor to overall pollution levels in some regions, especially at local watershed levels.

In certain areas, the insoluble solid fractions of animal wastes have caused long-term damage, forcing the closure of recreational water bodies, shellfish harvesting areas, and disrupting other aquatic life due to the excessive nutrient and waste loadings. Addressing the environmental impacts of the staggering volumes of animal wastes being produced has become a critical issue facing agriculture. Implementing proper waste management and treatment systems is crucial to minimizing the detrimental effects of these concentrated animal farming operations on water resources, air quality, and preserving environmental quality for the general public (Parihar, et al. 2019).

The microbiological method of obtaining GAGs ensures high purity and quality of the product. Compared to animal sources, biotechnological production effectively eliminates contamination risks and allows obtaining a pure product under controlled conditions. This method is

also less expensive and more environmentally friendly compared to the traditional extraction of these compounds (Balbinot-Alfaro, Rocha, Alfaro, Martins, 2021). And the main advantage of the microbial method of obtaining GAGs is that currently there is a major trend towards cruelty-free and vegan cosmetics, with the EU even defining the unacceptable method of testing cosmetic products on animals in accordance with EU Regulation No. 1223/2009 on cosmetic products. Some consumers consciously refuse cosmetics containing animal-derived ingredients, so in order not to lose a huge portion of potential buyers, new biotechnological methods of obtaining GAGs are increasingly being explored.

Hyaluronic acid is one of the most popular representatives of glycosaminoglycans. Its natural producers are *Streptococcus zooepidemicus*, the classic media for the growth of this microorganism are media containing sheep blood and spinal cord infusion and the average yield of HA is 7 g/L, but given this composition, these media raise safety concerns. The use of animal sera, particularly fetal bovine serum (FBS), as a component of cell culture media has several significant drawbacks that can have serious implications for the quality and safety of cell cultures.

The first and most significant issue is the risk of microbial contamination. Despite the fact that suppliers sterilize sera by filtration, this process does not guarantee the complete absence of pathogenic microorganisms. Sera may contain mycoplasmas, viruses, bacteria, and fungi, which can enter the cell culture and cause contamination. The potential presence of mycoplasmas is particularly problematic, as they can evade detection during routine sterility testing. In general, six species of mycoplasmas, including *Acholeplasma laidlawii, Mycoplasma arginini, M. hyorhinis, M. orale, M. fermentens, and M. hominis*, account for 90-95% of cell culture contamination cases.

The second major problem is the variability between different batches of sera and even within a single batch. The composition and quality of sera can vary significantly, affecting the growth, metabolism, and gene expression of cultured cells. This complicates the reproducibility of experiments and biopharmaceutical manufacturing processes.

Furthermore, animal-derived sera may be contaminated with extraneous animal viruses, such as the Cache Valley virus, epizootic hemorrhagic disease virus, vesivirus, reovirus, and bluetongue virus. The introduction of these viruses into cell cultures can have serious consequences for the safety and quality of final biological products.

Finally, failure to follow proper laboratory practices, such as mouth pipetting or cross-contamination between cultures, can lead to the introduction of human-derived mycoplasmas, such as M. fermentens, M. orale, and M. hominis, into cultures (Nims, & Harbell, 2017). That is why, in many studies, scientists are trying to find the optimal composition of the nutrient medium, which would not contain these components (Table 1).

However, another problem with natural producers is that these are rather pathogenic strains, and their endotoxins can pose a significant risk to human life and health. Therefore, new non-pathogenic synthetic strains are now being created with the help of genetic engineering (Table 2).

Although hyaluronic acid is one of the most popular GAGs (glycosaminoglycans) for cosmetic procedures, it is not the only one with beneficial properties for human skin. Alginate in cosmetics has tightening and modeling properties, improves blood microcirculation, tones and refreshes the skin, and smooths wrinkles. It is usually obtained from brown algae. However, with changes in the environment, such as rising ocean temperatures and an increase in biotechnological applications of alginates with specific properties, there is a need for more reliable sources of alginate

that can be modified. Therefore, biotechnological methods for obtaining this substance are currently being actively developed.

Table 1. Influence of the carbon source on the yield of hyaluronic acid in *Streptococcus zooepidemicu*

Strain	Carbon Source	Cultivation Conditions	HA Yield (g/L)	Reference
ATCC 35246	Whey	T = 37°C, 1 vvm, 500 rpm, pH = 6.7	3.2	(Amado, Vázquez, Pastrana, & Teixeira, 2016)
	Locust bean extract	T = 37°C, pH = 6.7; 0.1 vvm; 200 rpm; 12 hours	2.6	(Ozcan, Germec, & Turhan, 2022)
	Fishery waste	T = 37°C, 500 rpm, no aeration, pH = 6.7, glucose feeding every 2 hours up to 20 g/L, up to 10 hours	2.3	(Vázquez et al., 2015)
ATCC 39920	Sugarcane molasses	T = 37°C, pH = 8.0, 100 rpm, 24 hours	2.8	(Pan, Pereira, da Silva, Vasconcelos, & Celligoi, 2017)
	Cashew apple juice	T = 37°C, pH = 7.0; 150 rpm, 24 hours	1.76	(Oliveira, Ogrodowski, de Macedo, Santana, & Gonçalves, 2014)
	Soy peptone	T = 37°C, 150 rpm, 24 hours	0.3	(Benedini, & Santana, 2013)

For example, *Pseudomonas aeruginosa* is known for its ability to produce alginate, a valuable compound with various industrial applications. However, it's important to note that *P. aeruginosa* is inherently pathogenic, posing significant risks when cultivated on a large scale. In light of this, a study conducted by (Valentine et al., 2020) aimed to develop a non-pathogenic strain of P. aeruginosa. This involved the targeted removal of five key pathogenicity genes from the bacterium's chromosome, resulting in the creation of a marker-free strain known as PGN5.

Remarkably, when administered intraperitoneally to mice, PGN5 exhibited a mortality rate of 0%, in stark contrast to the 95% mortality observed with the wild-type P. aeruginosa strain. This outcome underscores the remarkable attenuation of systemic virulence in PGN5. Notably, despite its reduced pathogenicity, PGN5 retained its ability to produce significant amounts of alginate. This alginate, synthesized in response to the overexpression of MucE, a key activator of alginate biosynthesis, exhibited structural similarities to alginate produced by its pathogenic counterpart.

Other valuable representatives of GAGs are heparin and chondroitin sulfate. Heparin has anti-inflammatory properties, and chondroitin promotes skin hydration and reduces external signs of aging. For heparin synthesis, used recombinant *Pichia pastoris* yeast, which can produce heparin from methanol. This allowed for the production of 2.08 g/L of bioengineered heparin in fed-batch cultures (Zhang et al., 2022).

Table 2. Genetic modifications to create non-pathogenic hyaluronic acid synthetics

Micro-organism	Genetic Modifications	Cultivation Conditions	Carbon Source	HA Yield (g/L)	Reference
Lactococcus lactis MKG6	Transformation of L. lactis NZ9020 with a combination of hasABD genes. Simultaneous expression of hasA and hasB genes	No aeration, impeller speed 200 rpm, pH 7, 30°C, 8% inoculum. HA production induced with 2 ng/ml nisin when culture reached OD 600 = 0.6.	Glucose	3.03	Kaur, M., & Jayaraman, G. (2016)
Bacillus subtilis RBSFA	B. subtilis 168 harboring pDG148-hasA	37°C, 200 rpm, 40 hours	Lactose	0.602 ± 0.0166	Amjad Zanjani et al. (2022)
Corynebac-terium glutamicum 183.2	Parent strain - Corynebacterium glutamicum ATCC 13032 Plasmid 183.2. Genes: 11355 bp hasA, hasB, hasC	Fermentation time 35 hours in a 2 L fermenter at pH 7, 200 rpm.	Urea	2.15	Karami et al. (2020)
Kluyvero-myces lactis	pm hasA gene from Pasteurella multocida	24 hours, 1.3 L New Brunswick BioFlo 115 bioreactor. 30°C and 200 rpm, 2 vvm, pH 6.0 maintained with 2 M NaOH	Glucose	1.89	Gomes, Netto, Carvalho, & Parachin (2019)
Escherichia coli HA03GlcA	Genetic modifications to enhance biosynthesis of UDP-glucuronic acid	37°C and 200 rpm.	Galactose, Glucose	0.029	Woo, Seong, Lee, & Jang (2019)
Streptomyces albulus CRM003	hasA gene from S. zooepidemicus	30°C, 72 hours, 500 rpm. Aeration maintained at 3.5 VVM, pH 4.2. During fermentation, a mixture of glucose and ammonium sulfate (mass ratio 10:1) was continuously fed to maintain glucose concentration at approximately 5% (w/v).	Glucose	6.2	Yoshimura, Shibata, Hamano, & Yamanaka (2015)
Bacillus subtilis 3NA	hasA, tuaD, gtaB, and gcaB genes from S. zooepidemicus	37°C, 11 hours, agitation 1200 rpm, pH 7.0 and aeration 1 vvm. Cultivation with supplementation (2 hours after start adding 9 g/L·h glycerol, initial concentration 2.44 g/L.) (	Glycerol	7	Cerminati et al. (2021)

Alternative approaches to heparin production extend to mammalian cells, with Chinese hamster ovary (CHO) cells emerging as a prominent candidate. Renowned for their robustness and

resistance to viral contamination, CHO cells have become a cornerstone in biopharmaceutical manufacturing. Leveraging their intrinsic enzymatic machinery, which encompasses most of the heparin biosynthesis pathway, except for key enzymes HS3ST1 and NDST2 crucial for heparin's anticoagulant properties, CHO cells offer a promising platform for heparin bioengineering.

Moreover, CHO cells naturally synthesize heparan sulfate (HS), a glycosaminoglycan (GAG) closely related to heparin in its biosynthetic pathway but devoid of anticoagulant effects. This intrinsic capability aligns with the demand for heparin-related compounds in cosmetic applications, where the anticoagulant properties are negligible. Consequently, the precursor molecule heparosan, synthesized by CHO cells, emerges as a viable alternative for topical heparin utilization in cosmetic formulations. This paradigm underscores the versatility of CHO cells in meeting diverse industrial demands, ranging from traditional pharmaceuticals to emerging cosmetic applications, thus positioning them as a versatile platform for heparin and related compound production (Oduah, Linhardt, & Sharfstein, 2016).

To boost the production of heparosan, a naturally occurring compound synthesized by *Escherichia coli* Nissle 1917 (EcN), conducted a study employing metabolic engineering techniques. This involved optimizing the biosynthesis of precursors essential for heparosan synthesis, such as UDP-GlcA and UDP-GlcNAc. Additionally, they introduced genes encoding enzymes crucial for heparosan synthesis, such as bsGalU and ecKfiD for UDP-GlcA, and ecGlmM for UDP-GlcNAc. Furthermore, they enhanced the expression of endogenous genes responsible for heparosan synthesis. These collective genetic manipulations resulted in a significant increase in heparosan yield, rising from 0.15 g/L to 1.29 g/L (Hu et al., 2022).

Another recombinant producer of heparosan can be *Bacillus megaterium*. Researchers Williams et al. performed genetic transformation of B. megaterium MS941, introducing two plasmids: one containing the T7 RNA Polymerase gene (pT7-RNAP), and another containing the PmHS2 gene (obtained from *Pasteurella multocida* and used for heparosan synthesis). As a result of these manipulations, the product yield was 2.74 g/L (Williams et al., 2019).

In a study by (Zhou et al., 2018) using the same *Bacillus subtilis* showed that a chondroitin yield of up to 7.15 g/L could be obtained. Such results were achieved through genetic engineering. The genes tuaD, glmU, gtaB, glmM, glmS, and kfoA were amplified from the genomic DNA of B. subtilis E168C. Another study (Jin et al., 2016), *Bacillus subtilis* was used as a producer of heparosan by integrating synthase genes obtained from Escherichia coli. The resulting strain was able to produce up to 5.82 g/L of heparosan. Additionally, this microorganism was capable of producing 5.22 g/L of chondroitin

In general, for the production of chondroitin, *E. coli* K4 is predominantly used, as it is the first and most well-studied microorganism involved in the industrial production of this glycosaminoglycan (Couto, Rodrigues, Rodrigues, 2022). However, researchers noted that the use of this bacterium has its limitations and problems, which include the need for chemical treatment to obtain chondroitin from *E. coli* K4, complicating the production process and potentially affecting product quality. Moreover, *E. coli* K4 is a pathogenic bacterium that causes disease, posing a risk of product contamination with virulence factors and toxins. Additionally, in most cases, the yields of chondroitin obtained from *E. coli* K4 are low. Due to these limitations, researchers are trying to find alternative microorganisms for chondroitin production. Some alternative producers include E. *coli* K5*, S. zooepidemicus, E. coli* BL21 *(DE3), B. subtilis, Corynebacterium glutamicum, and E. coli* K-12 (Badri et al., 2021; Badri, Williams, Linhardt & Koffas, 2018).

Conclusions

Thanks to genetic engineering, highly productive strains of microorganisms capable of synthesizing glycosaminoglycan have been successfully created. The best productivity was observed in *Bacillus subtilis* 3NA (7 g/L) of producing HK and *Pichia pastoris* capable of producing heparin with a concentration of 2.08 g/l in a medium with methanol. This achievement positively impacts the environment by eliminating the need for brain-heart infusion medium and using a non-pathogenic microorganism.

Conflict of interest

The authors state no conflict of interest.

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