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TESTING OF BACTERIAL FILTERS AND PRESERVATIVES FOR QUALITY ASSURANCE OF LYOPHILIZED SNAIL MUCUS AS A COSMETIC COMPONENT

Snail mucus is the popular cosmetic ingredient that is rapidly taking over the modern market of beauty industry. Conducted microbiological tests using differential diagnostic agar media showed that mucus produced by snail farms had the hazard risks of contamination with coliform bacteria and staphylococci. In order to comply with biological safety requirements and extend the shelf life of products, it is advisable to carry out fine filtration and add antiseptic substances to the mucus filtrate. Microbiological analysis of two batches of Helix aspersa lyophilized mucus was carried out after the addition of preservatives such as Sharomix 300, Lysozyme, Natamycin with Nisin and the using of microporous acetyl cellulose filters (pore diameter of 2 μm). The best results of purification were demonstrated by 1% preservative Sharomix 300 or the fine-pore bacterial filters using in combination with 0.5% Sharomix 300

Keywords: HelixComplex, lyophilized mucus, microbiological hazard risks, cosmetic product

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ВИПРОБОВУВАННЯ БАКТЕРІАЛЬНИХ ФІЛЬТРІВ ТА КОНСЕРВАНТІВ ДЛЯ ЗАБЕЗПЕЧЕННЯ ЯКОСТІ ЛІОФІЛІЗОВАНОГО СЛИЗУ РАВЛИКА КОСМЕТИЧНОГО ПРИЗНАЧЕННЯ

Слиз равлика є одним з популярних інгредієнтів, що стрімко захоплює сучасний ринок косметичних засобів. Застосування слизу в косметології обумовлено високим вмістом в ньому біологічно активних сполук: специфічні протеїди, глікопептиди, гіалуронова кислота, алантоїн, забезпечують зволоження, профілактичну та лікувальну дію на пошкоджену і піддану віковім змінам шкіру. Склад та властивості слизу залежать від виду равликів-продуцентів, умов їх вирощування на фермі та технологічних рішень для отримання кінцевого продукту. Нами були проведені мікробіологічні тести щодо аналізу чистоти та біобезпеки слизу равлика з використанням диференційно-діагностичних агарових середовищ, який продемонстрував ризики зараження слизу, який виробляють равликові ферми, бактеріями групи кишкової палички та стафілококами. З метою дотримання вимог біологічної безпеки та продовження строку придатності доцільно проводити тонку фільтрацію та додавати антисептичні речовини до фільтрату слизу. Був проведений мікробіологічний аналіз двох партій ліофілізованого слизу равлика Helix aspersa після внесення консервантів, таких як Шаромікс 300, лізоцим, натаміцин у поєднанні з нізином і використання ацетилцелюлозних мікропористих фільтрів з діаметром пор 2 мкм. Найкращі результати щодо очищення від небажаних мікроорганізмів показало внесення 1% консерванту Шаромікс 300 та застосування бактеріальних фільтрів в комбінації з 0,5% Шаромікс 300.

Ключові слова: HelixComplex, ліофілізований слиз, ризики мікробіологічної небезпеки, косметичний продукт.

Issue statement and its connection with research and practical tasks

Snail mucus as ingredient for skincare cosmetic products has long been popular in South Korea and has already made its way as innovative one for beauty industry all over the world. Actually, the Global Snail Cosmetic Products Market is a huge business. The most popular product types with snail mucus are such as cell renewal cream, multi-functional cream, anti-acne cream, face skin mask, anti-aging eye patches, anti-wrinkle serum. According to expert estimations, Snail Beauty Market was valued at US $ 555.9 million in 2022 and is expected to grow up to US $ 1,232.7 million by 2030 [1].

Research of snail mucus content are often limited by access and complexity of investigation based on purification and identification of complicated organic compounds. The properties of mucus and content biological active compounds in it are strongly depend on snail species-producers, conditions of their growing at the farm and technological decisions for final product obtaining. Molluscs mucus is a complex matrix, highly influenced by biological and environmental factors. It is fundamental to understand how these factors influence the quality of the raw material in order to ensure effective, standardized cosmetic or pharmaceutical products. So, the points of quality, hazards analysis of snail mucus raw material as future cosmetic ingredient remain relevant. Another issue, that has to be solved, is low shelf life of product due to high protein content.

Review of latest research

In general, snail mucus demonstrates the high potential in cosmetic industry, medicine, pharmacy, and biotechnology. Different molluscs species have been applied as mucus producers in various sectors for biomedical or biotechnology applications. Terrestrial widespread garden snail *Helix aspersa* has been used for mucus and mucin production for commercially available cosmetic products proposed by company such as Benton, Mizon, Cos Rx, Biopelle, Missha. Mucus of terrestrial tropical snails *Archachatina marginata* and *Achatina fulica* also has been applied in medicine and pharmacology for antimicrobial wound care and drug delivery [2].

Wide application of mucus in cosmetology is assured due to rich content of biological active compounds. Specific proteins, glycopeptides, hyaluronic acid, allantoin, that are included in native snail mucus, provide moisture and treatment effect on irritated, damaged, and aged human skin. Some authors also reported about its antimicrobial, antioxidant, anti-tyrosinase and antitumoral activities [3]. Biochemical analysis of *H. aspersa* mucus *(further in text*

HelixComplex) demonstrated the presence of mucopolysaccharides which create a lot of hydrogen bonding with surrounded water molecules and effectively lead to tissue hydration. In addition, it stimulates endogenous hyaluronate synthesis, resulting in an increase in water-binding capacity and viscoelasticity of human skin [4, 5]. Microbiological analysis provides scientific support in the field of predictive microbiology for the estimation of the shelf-life and the quantitative microbiological risk assessment in snail mucus as ingredient of cosmetic products.

It is reported that HelixComplex demonstrated bio-adhesive effect and defensive properties against the ozone in concentration 0.5 ppm for 2 hours exposure in human keratinocytes [6]. In mentioned research cytotoxicity, tissue morphology and cytokine levels were determined. In addition, HelixComplex was able to protect from O_3 exposure by preventing oxidative damage and the consequent pro-inflammatory action in both 2D and 3D skin models. Moreover, the presence of mucopolysaccharide could improve mucus adhesion to skin and act as a barrier to prevent epithelial cell destruction from toxic agents. The presence of polyphenols could give to the mucus the ability to prevent and counteract the pollution induced cutaneous oxidative damage [6].

HelixComplex showed antibacterial effect against some pathogenic bacteria as strong action on several strains of *Pseudomonas aeruginosa* and a weak action on *Staphylococcus aureus* [7, 9]. Size separation experiments indicated that the antimicrobial substances were two proteins with molecular mass of 30-40 kDa and 50-60kDa [7]. Despite on weak or middle antimicrobial effect of snail mucus, this product isn't sterile and could be inhabited by bacteria and fungi [8, 10].

The aim of current research

In Ukraine there are a lot of farms which are breeding *H. aspersa* for meat and caviar as gastronomy diet products and for mucus obtaining. Our working group collaborated with snail farm in Kyiv region for estimation the quality and hazard control of produced mucus. Usually we get samples of native filtrated mucus and concentrated lyophilized ones. Estimation of physical and chemical properties such as moisture, pH, water soluble proteins content [11, 12] and mineral ash elements [12] had been made. Next important points that have to be studied were estimation of microbiological purity of native and lyophilized mucus. After getting original results of initial microbiological tests on snail mucus and finding a lot of saprophytic bacteria and in some samples the contamination by bacteria of *E. coly* group and *Staphylococcus* sp. we decided to conduct product purification from microorganisms.

Therefore, the main aim of current research was the method screening for purification of mucus from undesirable microorganisms via using preservatives or/and special bacterial filters. Biological safety is valuable parameter of product quality and optimization of its conservation technology and storage.

Material and methods

The preservatives were added to the snail mucus directly at the manufacture site after rough filtering and centrifugation but before lyophilisation. In particular cases (samples 5 and 6), the preservatives addition was partially or completely replaced by using of fine-pored bacterial filters with pore diameter of 2 μm. Concentrations of preservatives were selected in a range recommended by producers. Preservatives have been tested at the beginning of research, and then the most effective one was tested in a complex with filtration. Thus, the samples were tested in the following variants (table 1).

Table 1

Samples of lyophilized snail mucus with addition of preservative and subjected by micro-pored filtration

Sample No	Type and concentration of preservative or filtration
1	1% Sharomix 300
2	0.5 % Sharomix 3000
3	0.5 % Lysozyme
4	12% Natamycin + 0.08% Nisin
5	fine-pore filtration (Membrane filter CA, 2 μm)
6	fine-pore filtration (Membrane filter CA, 2 μm) and 0.5 % Sharomix 300

Microbial purity determination in mucus samples was made by agar-plate method on common nutrient agar and several standard differential media. The 2 ml aliquot of a 5% aqueous solution of lyophilized snail mucus were covered by melted sterile medium and cultivated in each Petri dish. Five nutrient media had been used in experiment for obtained data on quantitative microbiological risk assessment, and all tests were carried out in three repetitions:

1. Nutrient agar (NA) for estimation the Total Microbial Count (TMC)
2. Endo agar for detection Enterobacteria of coliform group
3. Egg yolk-salt agar for detection of pathogenic Staphylococci
4. Cephaloridine fucidin cetrimide agar (CFC) for detection of *Pseudomonas aeruginosa*.
5. Sabouraud dextrose agar (SDA) with chloramphenicol for detection of mold fungi

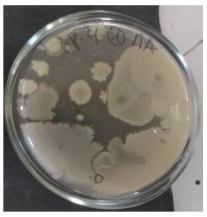
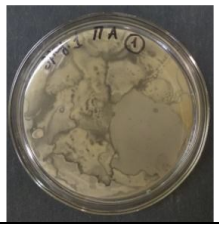
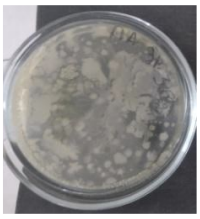
Inoculated Petri dishes had been being incubated at 37^0C during 3 days. Visual observations had been made on every day of cultivation. For the NA and CFC data of first day cultivation was valued. If bacterial growth is observed on the differential media, the calculation and description of colony forming units (CFU) are provided.

Results and discussion

First, action of preservatives were tested, and among them Sharomix 300 was used in two concentrations. Results of experiment are presented in table 2.

Table 2

Tested samples of 5 % water solution of lyophilized mucus with preceding preservatives addition

Observation day	Media	SAMPLE 1 1% Sharomix 300	SAMPLE 2 0.5% Sharomix 300	SAMPLE 3 Lysozyme	SAMPLE 4 Natamycin + Nisin
1st day	NA	65 % space of medium surface is overgrown with white colonies 	85% space of surface is overgrown with white colonies 	80% of the surface is overgrown with white colonies 	90 % space of medium surface is overgrown with white colonies 
	Endo	Bacterial growth is absent	Bacterial growth is absent	Bacterial growth is absent	Bacterial growth is absent
	Yolk-salt	Growth is absent	Growth is absent	Growth is absent	Growth is absent
	CFC	Blue colonies are absent	Blue colonies are absent	Blue colonies are absent	Blue colonies are absent
	SDA	Growth is absent	Growth is absent	Growth is absent	Growth is absent
2nd day	Endo	Growth is absent	Growth is absent	Growth is absent	Growth is absent
	Yolk-salt	Growth is absent	Growth is absent	**6 small white CFU**	Growth is absent
	SDA	Growth is absent	Growth is absent	Growth is absent	Growth is absent
3rd day	Endo	Growth is absent	Growth is absent	Growth is absent	Growth is absent
	Yolk-salt	Growth is absent	**2 small yellow CFU**	**20 white and 10 yellow CFU**	**7 yellow CFU**
	SDA	Growth is absent	Growth is absent	**One CFU with Ø 4 mm grew in one sample**	Growth is absent

The conducted analysis showed that all samples had satisfactory quality, because bacteria of *E. coli* group and *Pseudomonas aeruginosa* were completely absent. With the exception of Lysozyme, all other preservatives destroyed mold fungi. However, almost all samples of this series had been infected with *Staphylococcus*, especially suspicious were yellow colonies on yolk-salt agar detected on the third day of cultivation. Among used preservatives, Sharomix 300 was the best one for destroying bacteria. So, the second experiment was conducted on selected preservative Sharomix 300 and fine filtration. Results were presented on the table 3.

Table 3

Tested samples of 5 % water solution of lyophilized mucus with preceding fine filtration and preservative addition

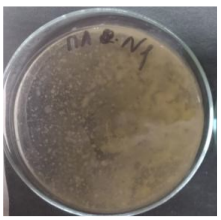
Observation day	Media	SAMPLE 5 fine-pore filtration (Membrane filter CA, 2 µm)	SAMPLE 6 fine-pore filtration (Membrane filter CA, 2 µm) and 0.5 % Sharomix 300
1	2	3	4
1st day	NA	60 % of surface is overgrown with white colonies 	20 % space of surface is overgrown with white colonies 
	Endo	Growth is absent	Growth is absent
	Yolk-salt	Growth is absent	Growth is absent
	CFC	Blue colonies are absent	Blue colonies are absent
	SDA	Growth is absent	Growth is absent

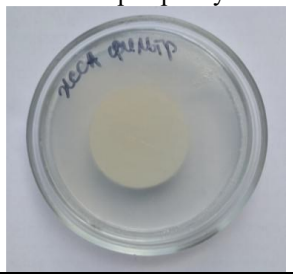
Table 3 – continues

1	2	3	4
2nd day	Endo	**Growth is registered, 10 CFU**	Growth is absent
	Yolk-salt	Growth is absent	Growth is absent
	SDA	Growth is absent	Growth is absent
3rd day	Endo	**More than 50 CFU** 	Growth is absent 
	Yolk-salt	Growth is absent	Growth is absent
	SDA	Growth is absent	Growth is absent

The ability of the filters to hold up various groups of improper microorganisms was also analyzed. For this purpose, the filters after uses were placed on the surface of differential media and the visual examination was carried out after 3 days of cultivation at 37^0C (table 4).

Table 4

Analysis of trap capacity of bacterial membrane filter CA, 2 µm

Endo agar	SDA	Yolk-salt agar
The filter substantially doesn't hold up bacteria of coliform group. Acid fuchsine reaction was present, but weren't colony growth 	The filter effectively traped spores and vegetative forms of mold fungi 	*Staphylococcus* wasn't detected on the filter. However, considering fact that samples didn't contain staphylococci at all, it's difficult to evaluate the trap capacity of filter 

Conclusions

Two batches of lyophilized mucus produced on a snail farm were examined. Microbiological analysis showed that there are risks of contamination with coliforms and staphylococci at that snail farm. As the main recommendation, it is proposed to carry out the disinfection of technological lines on the farm. To ensure the quality and biosafety standards of the mucus lyophilizate and to extend the product's shelf life, we recommend adding 1% of the preservative Sharomix 300 before lyophilization of native mucus or using fine-pore bacterial filters with 0.5% of Sharomix 300. The study of the trap capacity of acetylcellulose fine pore 2 µm filters showed that they effectively trap spores and vegetative forms of mold fungi.

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