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OPTIMIZATION OF CONDITIONS THE CULTIVATION FOR HYALURONIDASE PRODUCTION BY STREPTOCOCCUS PYOGENES

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Hyaluronidase (HAase) is used in biotechnology processes and therapy due to its therapeutic, pathophysiological, physiological and biological importance. A lot of the preparations of HAase produce from animal source (bovine and ovine testicular sources) with limited sources of microbial origin. The enzyme is used as a factor of spread in several medical fields. There are ophthalmology, orthopaedia, surgery, gynecology, dermatology, oncology and other. The enzyme acts as an adjuvant, accelerate and increase absorption and dispersion of injected drugs, e.g. antibiotics, to promote resorption of excess fluids and improve the effectiveness of local anaesthesia and to diminish pain due to subcutaneous or intramuscular injection of fluids [1], for hypodermoclysis and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents [2]. HAase was first found in the vitreous humor of bovine eyes by Meyer and his coworkers in the year 1949 to describe enzymes that are able to break down primarily hyaluronic acid (HAc). Bacterial HAase were reported to be virulence factors that facilitate the spreading of bacteria in host tissues by degradation of HAc. These bacterial HAase reduce hyaluronic acid to the same disaccharide whereas a tetrasaccharide is the end product results from testicular activity (diffusing factor). The substrate of a HAase is hyaluronic acid (hyaluronan), a nonsulfated glucosaminoglycan that consists of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine and which is a major structural component of the interstitial matrix of connective tissue. There is the group third, the bacterial hyaluronidase or hyaluronate lyases (EC 4.2.99.1).

Bacterial hyaluronate lyases were reported to be virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronan. Group A streptococci (GAS) appear to have of interest their production of HAase. These streptococci produce an anti-phagocytic capsule. HA is the component of capsule. It therefore seems reasonable to hypothesize that the HAase production is going to deplete this protective capsule leaving the organisms potentially susceptible to phagocytosis, as the capsule is susceptible to degradation by the hyaluronidase. However, continued production of HAase establishes a means for the organism to degrade host connective tissues allowing for bacterial spread. Perhaps other antiphagocytic factors such as M-protein provide protection for S. pyogenes during HAase production [3-5].

Typically, during an infection of any type, the ground substance secreted by connective tissues provides a mechanism of defense against infectious agents. The viscous consistency of ground substance usually provides resistance to penetration of infectious agents and their extracellular products; however, some bacteria have adapted ways to weaken the restraints of connective tissues. Many pathogenic bacteria able to establish infections at the mucosal or skin surface produce the enzyme hyaluronidase. Since hyaluronate is a major constituent of the ground substance of most connective tissues, particularly the skin, hyaluronidase may be an essential component in enabling the spread of the pathogens from an initial site of infection. The ultimate products of hyaluronidase degradation of hyaluronate are disaccharides. These disaccharides can be transported and metabolized intracellularly to supply needed nutrients for a pathogen as it replicates and spreads. The role of providing nutrients for the cell may be the main function of hyaluronidases in Gram-negative organisms [3-5]. HAase facilitates diffusion of antiviral drugs, dyes and toxin. Production by S. pyogenes of both a HAc capsule and HAase enzymatic activity capable of destroying the capsule is an interesting, yet-unexplained, phenomenon [6]. Production of HAase by GAS has been suggested to aid the organism in its spread through the connective tissue. HAase has been designated as one of the spreading factors of microbial origin.

Based upon the medical, physiological, biological and commercial importance of hyases, authors have screened and isolated a newly promising bacterial strain with higher yield followed by its characterization employing detailed taxonomic studies [7-8].

The aim of this study is to investigate the influence at the choice of optimum conditions of the cultivation S. pyogenes strains were give the HAase.

Materials and methods

In order to determine prevalence of hyaluronidase activity, clinical strains from the Centr urgent of psychiatry of asylum regional Kharkiv's from 2008 to 2011 were collected and tested. None of these strains was associated with alcogohicum delirium, and they were derived from a diverse group of patients varying by age and place of residence. We tested the culture conditions of S. pyogenes ATCC 2432 and 110 clinical isolates S. pyogenes. The newly isolated strain was identified based on their micro- and macromorphological, cultural, physiological and biochemical parameters by the requirements in normative document [9]. It preparated suspension of microorganisms with definite concentration. The measured values are displayed directly in McFarland units Densi-La-Meter (PLI-VA-Lachema a.s., Chechia). The instrument can perform measurement of turbidity at the range of 0.0 to 15.0 McFarland according to instruction of instrument and circular of innovation in health service № 163-2006 "The standardization of preparation the suspension of microorganisms", city Kyiv. The synchronization of culture carried by action low temperature [10]. The test-strains preserved in semisolid medium. The growing power of cells sustained by method replanting on the stiff medium. The cultural characters were studied by inoculating the organisms in different media viz. Sabouraud's peptone agar, peptone broth (PB), blood agar (BA) and other by microscopy. The mediums standard prepared satisfy the requirements of maker. The mediums stan-

dard in our research were BA, PB with 1 % glucose, PB with 0,5 % hyaluronic acid, with 0,4 ml serum, with quaternary ammonium compound (QAC) (1-10 mg/ml), with magnesium (1-10 mg/ml), with calcium gluconate (1-10 mg/ml). Incubation at 37° C for 18-20 h [11-12]. There is teste by 30 isolates at every strain. HA was measured spectrophotometrically by turbidity reduction assay using HAc sodium salt (Sigma Aldrich, USA) as a substrate. The enzymatic assay is based on McClean method in which the enzymatic reduction in turbidity, resulting when 1 ml of HAc at 70 µg/ml was incubated with 1 ml of enzyme sample in the presence of 0.05 M sodium phosphate buffer with 0.05 M NaCl (pH 7.0). After incubation of the mixture for 30 min., 2.5 ml of acidified protein solution (1% w/v) bovine serum albumin fraction-V (BSA) in 0.5 M sodium acetate buffer (pH 3.1), was added and incubated at 37° C for 10 min. and reduction in turbidity was read by measuring the absorbance at 600 nm. One unit of enzyme activity was defined as the amount of enzyme that causes a reduction in turbidity, measured spectrophotometrically at 600 nm (A600) in 30 min. at 37 C, at pH 7.0 under specified assay conditions similar to that caused by one unit of an international standard [13-14].

To one ml of substrate [containing 0.25 ml (0.04 %) hyaluronic acid, 0.5 ml distilled water and 0.25 ml of acidified bovine serum albumin (BSA) fraction V (1% w/v) in 0.5 M sodium acetate buffer (pH 3.1)] 0.5 ml of the supernatant (diluted 1:2 in saline) of an 18-24 h broth culture of isolated microorganisms was added, mixed and incubated at 37° C for 30 min. At the end of incubation time the tubes were cooled in ice bath. To the above mixture 0.1 ml of acetic acid (2 N) was added to precipitate the remaining HA. Tubes containing sterile broth or broth from inactive cultures became turbid while tube containing broth from hyaluronidase producing organism remained clear on addition of the acid [15-17].

The test-control was the reaction with reference strain with HA Staphylococcus aureus ATCC 25923, reference strain hyaluronidase inactivity Escherichia coli ATCC 25922 and left one test tube with broth and hyaluronic acid non seeding.

We have got calculate by formula:

$$HA = \frac{Ec - Ee}{Ec} \times n,$$ where

HA – hyaluronidase activity put into arbitrary unit (a.u.);

Ec – optical density of the test-control;

Ee – optical density of the test-strains;

n – dilution of the substrate [13, 18].

Statistical data manipulation analyzed according to the rules of the statistics [19-21]. Credibillity in divergence sized up by Student's test and calculated the mean M, standard error S, mean error m, reliability of test p. The datas analyzed on grouped by predicable and variational parameters. We have got the research result, wich treated data by computer program Statistika-6, Microsoft Office Excel 2003.

Experimental resoults: Of the 110 tested, 91 produced detectable levels of hyaluronidase activity (HA) (83 %) (fig. 1).

The higher HA detected from the strains from the patients with pneumonia (HA - 1,0±0,001 a. u., the value from 0,05±0,05 to 1,0±0,1 a. u. have got 33 test-strains). The higher HA have got from the strains with colony forming unit from 10^5, from the patients with the inflammatory complications - pneumonia (fig. 2). At the first phase of research we determineted that clinical strains S. pyogenes have got higher HA at next parametres: pH 7,4±0,2, a parcial specific volume of protein 1,0±0,1 %, temperature of cultivation 24±0,2º C, interval of cultivation 48 hours. (figure 3).

We studied an influence of component nutrient medium on enzyme production. Assay of HA of GAS shows the increase in enzyme activity of medium containing glucose, hyaluronic acid, serum and them combination (tab. 1).

The effect of different salts on HA was studied. Figure 4 shows the effect of divalent cations Ca^{2+}, Mg^{2+} and quaternary ammonium compound (QAC) on HA. A marked increase of enzymatic activity was observed with calcium gluconate at the concentration 4-5 mg/ml. Magnesium doesn't the effect. A marked increase of enzymatic activity was observed with QAC at the concentration 5 mg/ml.

Conclusion

Study of HA of GAS shows the increase in enzyme activity in medium containing glucose, hyaluronic acid, serum and their combination. For increase in activity we recommended calcium gluconate and QAC at the concentration 5 mg/ml.

The future studies will be at the optimization of growth conditions of the isolate by selecting a suitable production medium with its bioparametric studies including nutritional and physical parameters, perform strain improvement studies and then subjected to purification and characterization of the enzyme from the newly isolated strain.

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ОПТИМИЗАЦИЯ УСЛОВИЙ КУЛЬТИВИРОВАНИЯ ДЛЯ ПРОДУЦИРОВАНИЯ ГИАЛУРОНИДАЗЫ S. PYOGENES

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Гиалуронидаза используется в биотехнологии и медицине. В основном препараты гиалуронидазы получают из животного сырья, ограниченное количество – микробного происхождения. Бактериальная гиалуронидаза - фактор вирулентности, который способствует проникновению бактерии в соединительную ткань путем деградации гиалуроновой кислоты. В статье приведены результаты исследований по выбору оптимальных условий культивирования для продуцирования гиалуронидазы тест-штаммами S. pyogenes. Более высокий уровень гиалуронидазной активности определен для штаммов, изолированных у больных пневмонией. Определены оптимальные значения pH, температуры и продолжительности культивирования микроорганизмов, исследовано влияние компонентов питательных сред на продуцирование фермента. Изучение гиалуронидазной активности бактерий стрептококков группы А показало повышение ее при культивировании на среде с глюкозой, гиалуроновой кислотой, сывороткой и их комбинацией. Для повышения продуцирования гиалуронидазы можно рекомендовать кальция глюконат и четвертичное аммониевое соединение

Ключевые слова: гиалуронидаза, S. pyogenes, условия культивирования.

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ОПТИМІЗАЦІЯ УМОВ КУЛЬТИВУВАННЯ ДЛЯ ПРОДУКУВАННЯ ГІАЛУРОНІДАЗИ S. PYOGENES

Лук'яненко Т. В.

Гіалуронідаза використовується в біотехнології та медицині. Взагалом, препарати гіалуронідази отримують із тваринної сировини, обмежену кількість – мікробного походження. Бактеріальна гіалуронідаза - фактор вірулентності, який сприяє проникненню бактерії сполучною тканиною шляхом деградації гіалуронової кислоти. У статті наведено результати досліджень із вибору оптимальних умов культивування для продукування гіалуронідази тест-штамами S. pyogenes. Вищий рівень гіалуронідазної активності визначено у штамів, ізольованих від хворих на пневмонію. Визначено оптимальні показники pH, температури та тривалості росту мікроорганізмів, досліджено вплив компонентів живильних середовищ на продукування ферменту. Вивчення гіалуронідазної активності бактерій стрептококів групи А показало збільшення її при культивуванні на середовищі з глюкозою, гіалуроновою кислотою, сироваткою крові та їх комбінацією. Для збільщенн продукування гіалуронідази можна рекомендувати кальцію глюконат і четвертинну амонієву сполуку.

Ключові слова: гіалуронідаза, S. pyogenes, умови культивування.

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Hyaluronidase is used in biotechnology processes and medicine. A lot of the preparations of hyaluronidase produce from animal source with limited sources of microbial origin. Bacterial hyaluronidase were reported to be virulence factors that facilitate the spreading of bacteria in host tissues by degradation of hyaluronic acid. This article included the results of studies by the choice of optimal conditions of the cultivation for hyaluronidase production by test-strains S. pyogenes. The higher HA detected from the strains from the patients with pneumonia. Optimum values of pH, temperature and cultivation term of microorganisms determined, an influence of components of nutrient mediums on enzyme production was studied. Study of HA of GAS shows the increase in enzyme activity in medium containing glucose, hyaluronic acid, serum and their combination. For increase in activity we recommended calcium gluconate and quaternary ammonium compound at the concentration.

Key worlds: hyaluronidase, S. pyogenes, conditions cultivation.

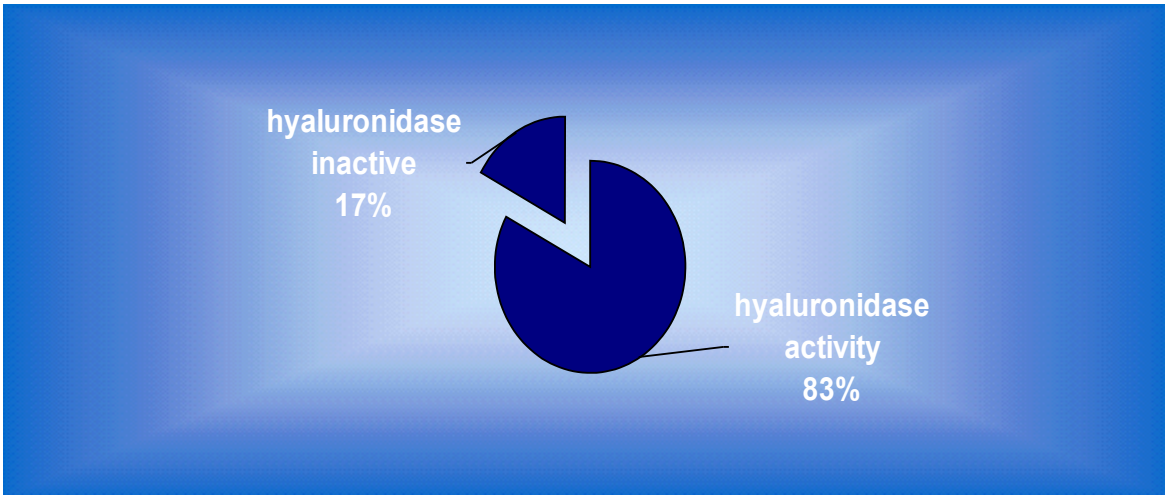

Fig. 1 – Hyaluronidase activity of clinical strains collected from the Centr urgent of psychiatry of asylum regional Kharkiv's from 2008 to 2011

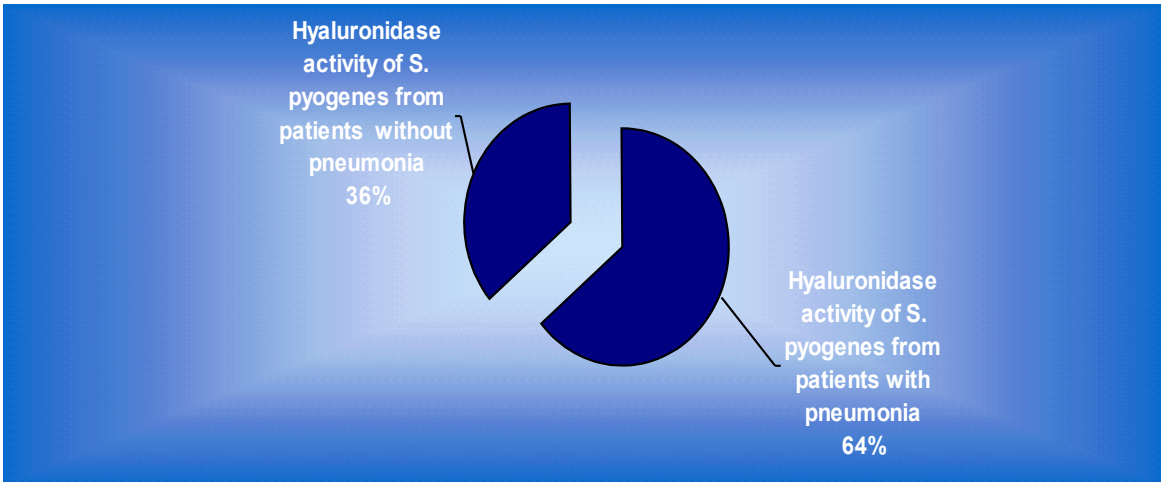

Fig. 2 – Hyaluronidase activity of strains S. pyogenes isolated from patients with alcoholicum delirium complicated and noncomplicated by pneumonia.

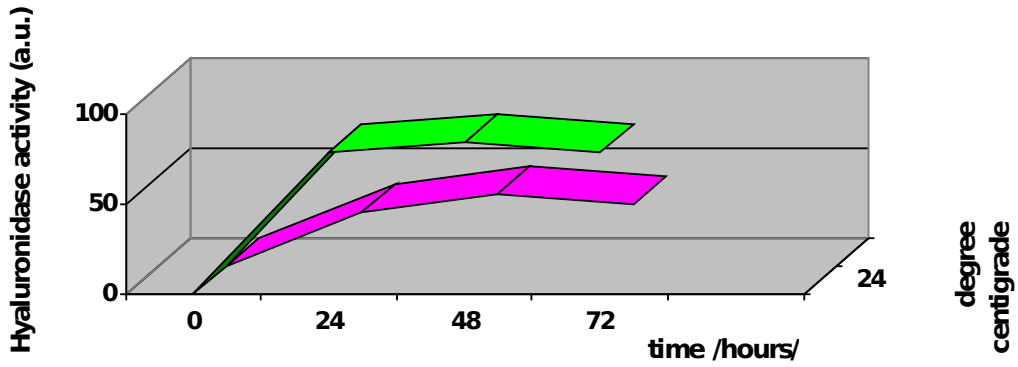

Fig. 3 – The influence of incubation temperature and time on hyaluronidase activity of S. pyogenes

Table 1 – The effect of some components in nutrient medium on the production of streptococcal hyaluronidase

№	Medium	Hyaluronidase activity (a.u.), (M±m)
1	Peptone broth	1,0±0,31
2	Peptone broth + 1 % glucose	1,32±0,23*
3	Peptone broth + 0,5 % hyaluronic acid	1,59±0,42*
4	Peptone broth + 0,4 ml serum	1,71±0,14
5	Peptone broth + 1 % glucose + 0,5 % hyaluronic acid + 0,4 ml serum	1,83±0,27*

*p≤0,05

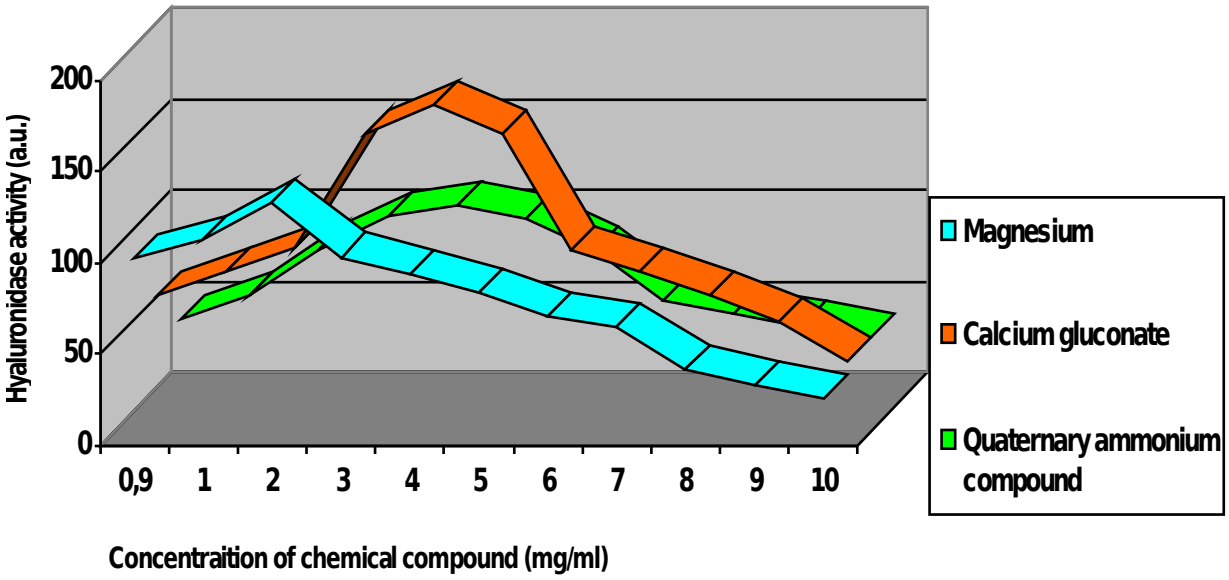

Fig. 4 – The influence of on chemical compound in nutrient medium on the production of streptococcal hyaluronidase