Sensitivity of *Janthinobacterium Lividum* to Low Concentrations of Hydrogen Peroxide and the Effect of Mild Oxidative Stress on Pigment Yield

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Abstract. In the course of assessing the sensitivity of the *Janthinobacterium lividum* VKM B-3515 strain to low concentrations of hydrogen peroxide, it was found that at a content of 0.003% H_2O_2, the growth properties of the bacterium during submerged cultivation without pigmentation differed statistically insignificantly relative to the control variant at 16 hours of incubation and beyond. Whereas in the presence of peroxide at 12 hours the optical density was lower than in the control by 97%. When cultivating by the surface method, the respiration intensity did not significantly differ between the control and experimental variants. However, during the extraction of the pigment, it was found that in the presence of hydrogen peroxide, the optical density of the acetone extract significantly exceeds the control variant by 28%. It can be assumed that, at the same growth parameters of the culture, the biosynthesis of violacein is stimulated and the population can adapt to the peroxide content, and the peroxide concentration itself decreases due to the cost of catalytic reactions. Further studies of the sensitivity of *J. lividum* VKM B-3515 to various oxidizing agents will allow us to consider the effect of weak oxidative stress on the biosynthesis of violacein.

1 Introduction

Reactive oxygen species (ROS) are due to the formation of a free radical and are able to oxidize organic and inorganic molecules. An example of such an effect is the oxidation of polyunsaturated fatty acids in the cell membrane under the influence of ROS. Oxygen attacks on double bonds of acids leads to the initiation of a chain reaction in which further oxidation occurs through the formation of lipoperoxyl radicals and other active compounds. Such processes can cause critical biochemical damage to cell membranes, which can eventually lead to rupture of cell membranes and their death [1]. Impact ROS on amino acids leads to functional and structural damage, which can lead to disruption of signaling proteins, membrane receptors, transporters, and cytoskeletal elements.

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An example of the effect of hydrogen peroxide on organic molecules involved in the life process can be the oxidation of Fe-S molecular clusters (Fenton's reaction with iron) with the formation of a hydroxide radical. Such damage leads to metabolic defects due to enzyme inactivation and protein carbonylation. Violations in the structure of DNA can lead to mutations or violations of the implementation of genetic information. In addition, there is information about the negative effect of ROS on the redox processes of the cell, and in particular on the processes of respiration [2].

Reactive oxygen species are used by organisms in the processes of signal transmission and as an immune response to a number of pathogenic processes, including infections [3]. However, it is known that microorganisms, including phytopathogenic representatives, are capable of adaptive responses under conditions of oxidative stress. Thus, it is known that bacteria treated with low concentrations of hydrogen peroxide and other oxidants adapt to subsequent high doses by inducing the expression of many functionally related genes [4].

One of the adaptive responses to adverse environmental conditions (oxidative stress, ultraviolet radiation, etc.) in microorganisms is the biosynthesis of pigments [5]. For example, it is known that *Ralstonia solanecearum*, a common phytopathogen that affects more than 200 plant species from 50 different families (for example, brown rot of potatoes, wilting of tomatoes, tobacco and eggplant, etc.) forms melanins in a nutrient medium with a minimum substrate content in a stationary growth phase, and it has been shown that pigments are involved in the development of bacterial resistance to oxidative stress [6].

Pigments of biological origin (mainly microbial) can be classified as [7]:1. yellow riboflavin; 2. carotenoids (beta-carotene, canthaxanthins, astaxanthins); 3. prodigiosin; 4. pyocyanin; 5. phenazines; 6. violacein.

Thus, violacein, formed by a number of producer strains, for example, *Chromobacterium violaceum* and *Janthinobacterium lividum*, can protect cell membranes from peroxidation [8]. However, bacterial pigments are known to be capable of increasing oxidative stress: pyocyanin, produced by *Pseudomonas aeruginosa*, is capable of accepting electrons from biological reducing agents such as NADPH and glutathione to form ROS and increase oxidative stress. Perhaps this is one of the main mechanisms of *Pseudomonas aeruginosa* virulence since the source provides information that mutants incapable of pyocyanin biosynthesis exhibit significantly less infectious activity [8].

Thus, pigments formed by microorganisms are capable of both protecting against oxidative stress and stimulating an increase in its intensity for microorganisms. It can be assumed that in order to implement the protective function, bacteria increase the degree of pigment biosynthesis under conditions of weak oxidative stress. At the same time, the strain may show resistance to the effects of pyocyanin produced by *Pseudomonas aeruginosa* due to adaptation to ROS.

The purpose of the study is to assess the sensitivity of the *Janthinobacterium lividum* test culture to hydrogen peroxide and to evaluate the possible role of the violacein pigment in adaptation to mild oxidative stress.

2 Materials and methods

The objects of the study were the pigment-forming strain of the bacterium *Janthinobacterium lividum* VKM B-3515, and the resulting violacein (3-(1,2-dihydro-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrole-3-ylidene)-1,3-dihydro-2H-indol-2-one). The pigment is blue-violet crystals, insoluble in water, but soluble in ethanol, methanol, acetone, ethyl acetate and DMSO. The melting temperature of the pigment is more than 290°C, and the absorption maximum is observed at wavelengths (λ) 258, 372, 575 nm in ethanol. The molar mass is 343.3 g/mol, and the molar extinction coefficient is 0.05601 ml $mM^{-1}cm^{-1}$ [9].

The selection of the effective concentration of hydrogen peroxide was carried out by the method of wells in agar [10]. To do this, in Petri dishes with Sabouraud's agar nutrient medium (composition (g/l): enzymatic meat peptone - 10.0; sucrose - 20.0; microbiological agar - 20.0), the test culture of *Janthinobacterium lividum* VKM B-3515 was passaged with a "lawn". Holes cut with a sterile cork drill (diameter 9 mm) were filled with 80 µl of a solution (at concentrations of 3%, 0.3%, 0.03%) of hydrogen peroxide. The repetition of each variant was 2 Petri dishes (with 9 wells per dish).

In addition, we used the method of serial dilution of 3% hydrogen peroxide to the 10^{-n} degree, while the volume of the applied producer cell suspension did not change.

To do this, 1 ml of a 3% hydrogen peroxide solution was transferred into a test tube with 9 ml of water, diluting the initial concentration by 10^{-1} degrees, from which 1 ml was taken and diluted in another 9 ml, obtaining 10^{-2}. The following dilutions were made in the same way (up to 10^{-6} or 10^{-n}). 500 µl of *J. lividum* VKM B-3515 cell suspension was added to each of the tubes containing hydrogen peroxide solution with different concentrations.

From test tubes containing various concentrations of hydrogen peroxide and 500 µl of a suspension of bacteria, pass the resulting mixture (in a volume of 100 µl) with a "lawn" onto Petri dishes. A test tube containing no hydrogen peroxide is used as a control. The cultures are incubated at 25°C for 2 days. The intensity of pigmentation is used to judge the appropriate concentration of hydrogen peroxide for oxidative stress in the biosynthesis of violacein.

The effect of hydrogen peroxide on the growth properties of the population during submerged cultivation was assessed by passing a suspension of a “fresh” culture into a Sabouraud liquid nutrient medium (in a volume of 200 ml) containing 0.003% H_2O_2. Crops that did not contain hydrogen peroxide acted as a control. Variants were incubated at 25°C with stirring (300 rpm) and aerating for 24 hours. Optical density (OD) was measured every 4 hours at a wavelength of λ = 600 nm using a UV-1900i spectrophotometer, Shimadzu, Japan. The average OD values were used to calculate the culture growth parameters.

The logarithmic phase of growth was detected using the specific growth (R) when the strain was cultivated in a liquid nutrient medium (Saburo) for 24 hours according to the formula:

$$R = \frac{OD_1 - OD_0}{OD_0}, \quad (1)$$

where OD_0 are the optical units (OU) of the culture liquid (CL) containing microbial cells at the zero hour of incubation, and OD_1 is the OU at the end of incubation.

The largest significant increase was taken as the logarithmic growth phase. Further minor differences are for the deceleration or stationary phase.

The growth rate constant was calculated using the formula [11]:

$$\mu = \frac{(lg\, x_1 - lg\, x_0)}{lg\, e(t_1 - t_0)}, \quad (2)$$

where, x_0 and x_1 are the OD values of the cell suspension corresponding to the growth time t_0 and t_1.

The fission rate constant was calculated using the formula [11]:

$$v = \frac{(lg\, x_1 - lg\, x_0)}{lg\, 2(t_1 - t_0)}, \quad (3)$$

where, x_0 and x_1 are the OD values of the cell suspension corresponding to the growth time t_0 and t_1.

The generation time was calculated using the formula [12]:

$$g = \frac{1}{v}, \quad (4)$$

where, *g* is the generation time, *v* is the fission rate constant.

The formula [13] was used to calculate the degree of inhibition:

$$IR = \left(\frac{OD_c - OD_g}{OD_c}\right) \cdot 100\%, \quad (5)$$

where OD_c is the optical density of the culture in the control variant at the end of incubation, and OD_e is in the experimental variant.

Confidence intervals (Δ) were calculated using formula (6) [14]:

$$\Delta = t_{st} \cdot m, \quad (6)$$

where t_{st} is the standard value of the Student's test of significance, m is the error of the representativeness of the arithmetic mean and is calculated by formula (7) [14]:

$$m = \frac{\sigma}{\sqrt{n}}, \quad (7)$$

where σ is the standard deviation and is calculated as [14]:

$$\sigma = \sqrt{\frac{\sum(V-M)^2}{n-1}}, \quad (8)$$

where *V* is the date, *M* is the arithmetic mean value of the optical density, n is the sample size.

The significance of the difference between the averaged values was calculated statistically using the difference method [10].

Catalase activity was determined for cultures at 12 and 24 hours of incubation. The culture was pelleted by centrifugation at 13800 rpm for 90 min (Neofuge 1600R, Heal Force, China). The supernatant was discarded and the pellet was washed with phosphate buffer (pH 7.4). Cells were suspended in buffer (pH 7.4) and disrupted with an ultrasonic homogenizer at 98% power. The suspension was removed by centrifugation for 90 min at 13800 rpm.

To measure catalase activity, the samples were added to cuvettes containing 90 µL of hydrogen peroxide and phosphate buffer (1 M, pH = 7). The rate of decomposition of hydrogen peroxide was recorded as a decrease in absorbance at 240 nm. [17]. The specific activity of the enzyme was calculated using the Lambert-Beer formula:

$$E = \frac{\Delta A/min \cdot V}{\varepsilon \cdot l \cdot v}, \quad (9)$$

where *E* is the activity of enzymes, E/l; *ΔA/min* - change in the optical density of the reaction mixture for 1 min, opt. density/min; V is the volume of the reaction mixture, ml; *ε*-millimolar extinction coefficient, l/mmol cm; *l* is the length of the optical path, cm; *v*-sample volume, ml; *1000* is the conversion factor for activity in µmol/(min ml).

Protein determination in solution was carried out by the Lowry method [17, 18]. 450 µl NaCl, 2 ml biuret reagent, and 50 µl homogenizate cell free extract were added to the tube. After 30 min, the optical density was measured with respect to distilled water at a wavelength of λ = 570 nm using a UV-1900i spectrophotometer, Shimadzu, Japan. The protein concentration was calculated by the formula:

$$c = \frac{A_1}{A_0} \cdot 100\ [g/l], \quad (10)$$

where A_0 is the optical density of the control variant (biuret reagent without homogenizate), and A_1 is in the presence of the test sample.

The influence of hydrogen peroxide on the intensity of respiration of the culture in the biofilm and the concentration of the pigment was assessed during surface cultivation in liquid Sabouraud nutrient medium in the presence of the detected concentration of

hydrogen peroxide for 7 days in two repetitions and without access to light. The variant without H_2O_2 acted as a control. The inoculations were incubated at 25°C with air aeration at a rate of 600 ml/min using an air filter. Exhaust air from the culture vessel was used to saturate the 1% NaOH solution. The intensity of respiration was judged by the change in the pH of sodium hydroxide.

After incubation, the variant biofilm was pelleted by centrifugation at 13800 rpm for 90 min. The supernatant was discarded and the precipitate was suspended in acetone (extractant). The suspension was collected in a container and stirred for 1 hour and re-centrifuged. The extraction was repeated and evaporated to 10 ml using a rotary evaporator. The extract was taken and photometered at a wavelength of λ = 575 nm. The effect of hydrogen peroxide on the intensity of the pigment yield was judged by the difference in the optical density of the pigment extracts according to formula (5). Reliability was calculated by statistical difference method.

3 Results and discussion

Using the agar well method, it was found that the most suitable concentration of hydrogen peroxide for the study of oxidative stress in relation to *J. lividum* VKM B-3515 is 0.003%. In the course of the dilution study, the effective concentration of hydrogen peroxide showed a stimulating effect on the biosynthesis of violacein - 10^{-3} (0.003%), since in this variant the strain is characterized by a greater intensity of pigmentation, relative to the control.

During submerged cultivation of *J. lividum* VKM B-3515 in the presence of H_2O_2, the change in optical density did not statistically significantly differ from the control variant. It can be assumed that the presence of hydrogen peroxide in the medium has little effect on the growth properties of the population.

In the course of the analysis of the specific growth of the culture, it was revealed that there was no statistically significant difference between the variants, except for the values at 12 hours, where in the presence of hydrogen peroxide, the specific growth was significantly lower than in the control group by 97%, while at 16 hours after incubation, the difference in optical density is insignificant: in the presence of H_2O_2, the specific increase was 4.7% higher than in the control variant.

Presumably, a sharp increase in the specific growth at the 16th hour of cultivation may be due to the development of resistance to a low concentration of hydrogen peroxide. One of the adaptive responses of the population during deep cultivation is an increase in the intensity of the biosynthesis of catalytic enzymes. In this case, as the population develops, the peroxide concentration may decrease due in the course of reaction.

In the course of determining the specific activity of catalytic enzymes, it was shown that at 24 hours of incubation in the culture variant with H_2O_2, this indicator is statistically significantly higher than the control one by 50%, and the Student's significance test was t = 2.78, which is higher than the tabular one at the error level $p < 0.05$ ($t_{st} = 2.306$).

In the course of assessing the effect of oxidative stress on the growth properties of the test culture, it was found that the intensity of respiration, based on changes in the pH of the solution, does not differ on day 5 from day 4.

The study revealed that the optical density of the acetone extract in the variant with H_2O_2 was statistically significantly higher than the control variant by 28%, and the calculated Student's reliability criterion was 74.79, which exceeds the tabular one at an error level of $p < 0.001$ ($t_{st} = 4.587$).

Thus, it can be assumed that under conditions of weak oxidative stress, the intensity of pigment formation increases, while the intensity of respiration differs insignificantly between the variants.

Examples of the antioxidant activity of violacein are described in the literature [19]. Violacein and its complex with *β*-cyclodextrin may be involved in the processes of protection against peroxidative damage and stimulation of mucosal defense mechanisms.

In addition, a number of examples of the isolation of strains from glacial melt waters that form carotenoids, prodigiosin, melanins, indigoidin, scytonemin, and others have been described. Their pigments can be involved in shielding and protecting cells from the effects of ROS and UV radiation [20].

4 Conclusions

Thus, in the course of assessing the sensitivity of the *Janthinobacterium lividum* BKM B-3515 strain to low concentrations of hydrogen peroxide, it was found that at a content of 0.003%, the growth properties of the bacterium during deep cultivation without pigmentation differed statistically insignificantly relative to the control variant at 16 hours of incubation and beyond. Whereas in the presence of peroxide at 12 hours the optical density was lower than in the control by 97%. It can be assumed that the population can adapt to the peroxide content and the peroxide concentration itself decreases due to the cost of catalytic reactions. In turn, in the course of measuring the total specific catalytic activity, it was shown that the experimental group was characterized by a significant excess of the reaction rate relative to the control variant, which may be one of the factors in the population response to the stressor.

When cultivating by the surface method, the respiration intensity did not significantly differ between the control and experimental variants. However, during the extraction of the pigment, it was found that in the presence of hydrogen peroxide, the optical density of the acetone extract significantly exceeds the control variant by 28%. It can be assumed that violacein biosynthesis is stimulated at the same growth parameters of the culture. This is consistent with data reported in the scientific literature. It is known that pigments are involved in adaptation to the presence of reactive oxygen species [19]. Thus, the cultivation of *J. lividum* BKM B-3515 in the presence of hydrogen peroxide, presumably, increases the pigment yield, which may be biotechnologically promising in the field of obtaining a preparation of microbial origin.

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