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Tracing the Development of *Bacillus Subtilis* in Broth with Added Micelles and Extracts From Medicinal Mushrooms Higher

Kamelia Loginovska[1], Maria Doneva[1], Nikolay Solakov[1], Alexandar Valchkov[1], Iliana Nacheva[1]

[1]Institute of Cryobiology and Food Technologies, 1407 Sofia, Bulgaria 53 Cherni Vrah Blvd.

Abstract

The growth of cell culture of strain *Bacillus subtilis* NBIMCC 2353 has been traced by two experiments. At 48th hour the number of cells reaches its maximum, which is above 8,00 log units (experient 1), after included on the 24th hour of enrichment agents in native form - mushroom body and micelles of the high medical mushrooms *Ganoderma lucidum* and *Cordyceps sinensis*. Cell culture basically fails to develop in its maximum capabilities due to difficulty absorbing macro-, micro-elements and protein compounds, contained in the high mushrooms. Positive effects have been found in experiment 2, such as prolonging the propagation time and doubling the number of cells, upon added into the nutrient medium of easily accessible components, such as *Cordyceps sinensis* and *Ganoderma lucidum* powder extracts. The largest increase in cell amount was observed at the 54th hour from the start of incubation, which was over 11,50 log units, for variants with added 2 g extract.

Key words: *Cell culture, Bacillus subtilis, increase, extracts, high mushrooms*

1.Introduction

Increasing interest represent so-called biologically active foods in the last decades, which are enriched with various beneficial components as well as probiotic bacteria that regulate the functions of the body and improve the health status of the consumers [1, 2, 3, 4]. The probiotic bacteria, producing biologically active substances with high health potential, are of great interest. *Bacillus subtilis*, which produces a set of proteolytic enzymes and, in particular, those with a fibrinolytic effect, such as the enzyme nattokinase, provokes especially attitude.

Bacillus subtilis is a gram-positive bacterium, found in the soil and the gastrointestinal tract of ruminants and humans. Its cells are stick with a length of about 4-10 μm and a diameter of 0,25-1,0 μm. The bacterium has excellent fermentation properties, high yields of products, that produce and complete absence of undesirable by-products, in biotechnological processes [5, 6, 7, 8, 9]. *B. subtilis* has been intensive studied for many years and is currently the most well-characterized safe bacteria, as withstands of unfavorable cultivation conditions [10, 11].

High mushrooms *Ganoderma lucidum* and *Cordyceps sinensis*, used for medical application, represent a rich biological food matrix, containing a huge amount of biologically active substances and are suitable for the production of functional foods for regular use and dietary regimens. *Ganoderma lucidum* widely has been used for health benefits, such as prophylaxis and treatment of hepatitis, hypertension, cancer, etc. At present, this sponge is one of the most sought-after medicinal mushrooms in the Oriental countries. There are even some food products on the market, containing extract thereof [10, 12, 13, 14, 15]. *Cordyceps sinensis* has been used for a long time in Chinese medicine and as a food supplement. It is believed that this sponge can improve liver function, treat chronic fatigue and cough, "cure" anemia and heart arrhythmias, reduce cholesterol, and more [16, 17, 18, 19].

Mushroom residues as feed additives for cultivation of *Bacillus subtilis* have been experimented, such as oyster mushroom residues, shiitake mushroom residues, residues of needle-like mushrooms, etc. to produce poly-γ-glutamic acid [20]. However, there have still been no studies for the cultivation of *Bacillus subtilis* in culture medium with added micelles and extracts of the high fungi *Cordiceps sinensis* and *Ganoderma lucidum*.

The aim of the present study is to trace growth of the number of live cells of *Bacillus subtilis* NBIMCC 2353 strain in a culture medium in which: 1) mushroom body and mycelium from the high mushrooms *Ganoderma lucidum* and *Cordyceps sinensis* have been added (experiment 1); 2) extracts from these fungi have been added (experiment 2). The idea is to determine in which of the two environments growth is greater over the 72 hour period.

2. Materials and Methods

For experiments was used depth cultivation. The main nutrient medium for the propagation and maintenance of the strain, and as a control, was a composition (g/l) in the experiments: Meat extract – 10,0; Peptone – 10,0; NaCl – 5,0; Agar – 20,0; distilled water - 1 l. The components were dissolved in distilled water and sterilized in an autoclave for 20 minutes at 121°C and 1 atm. To prepare vegetative sowing material, cultivation was carried out in 500 ml Erlenmeyer flasks containing 100 ml of seed medium for 24 hours at 30°C on a BS/4 circular shaker apparatus at 100 turnover / min.

Experiment 1:

Erlenmeyer flasks with a volume of 250 ml with an amount of nutrient medium of 50 ml were used to perform the experimental work. Cultivation of the strain was proceeded in a BS/4 circular shaker apparatus with the following parameters: Amplitude 100 turnover / min and temperature 30°C.

Experiment 2:

Erlenmeyer flasks of 250 ml volume were used with an amount of nutrient medium 50 ml. Cultivation of the strain here also was occured on a BS/4 circular shaker apparatus under the same parameters as described at experiment 1. Determination of cell culture density, calculating the number of strain cells in different nutrient media were determined using Spekol 11. A computer program for graphical data expression was used to construct the strain growth curve. The results were processed using software product MS Office Excel 2007.

Experimental part:

Biotechnological studies were carried out on *Bacillus subtilis* strain NBIMCC 2353 in two experiments - the first one to which micelles of the higher fungi *Cordyceps sinensis* and *Ganoderma lucidum* have been added to the culture medium and a second one, where powdered extracts of these mushrooms have been imported into the medium. The cell growth of the strain was traced for 72 hours in both media and the cell culture density was determined at certain times, by which the number of cells for these periods was calculated. For the experiments the strain was provided by the National Bank for Industrial Microorganisms and Cell Cultures.

Experiment 1:

Culturing the strain was occurred on at a temperature of 30°C for a period of 72 hours. The inoculum was an initial cell concentration of $1,5x10^6$ CFU/ml. At 24th hour from the beginning of

cultivation, the mushroom body and mycelium of the two mushrooms were imported into concentrations of 2% and 4%. The mushroom body and the micelles, under sterile conditions, were measured in the corresponding amounts and crushed maximum finely prior to being added to the primary culture medium.

Experiment 2:

Cultivation of the strain was carried out for 72 hours at 30°C. The extracts of the two types of medicinal mushrooms have been added in powder form in two different concentrations: 1 and 2g, for enrichment of the medium and tracking the multiplication of the cells. The inoculum was an initial cell concentration of $1{,}6x10^7$ CFU/ml in all variants.

3. Results and Discussion

Table 1 shows the variants of the one used nutritional medium with added mushroom body and the micelles of the high fungi *Ganoderma lucidum (Red reishi)* and *Cordyceps sinensis* at the corresponding concentrations of experiment 1.

Table 1: Variants of the used nutrient medium with added mushroom body and micelles of two types of mushrooms

Variant sample	Composition	Concentration %
1.	Mushroom body	2
2.	Mushroom body	4
3.	*Ganoderma Lucidum*	2
4.	*Ganoderma Lucidum*	4
5.	*Cordyceps Sinensis*	2
6.	*Cordyceps Sinensis*	4

Figure 1 reflects the increase in the number of cells of the test strain for the 72 hour period.

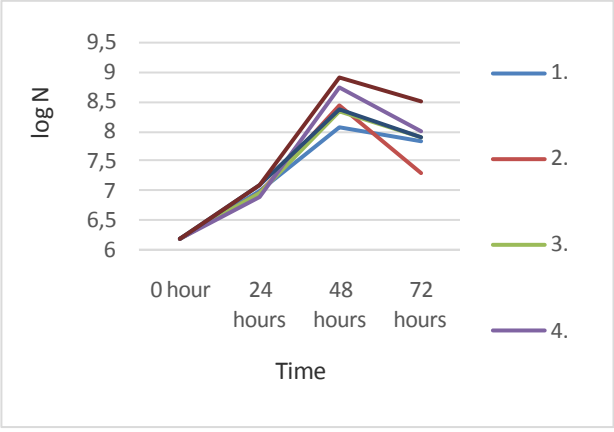

Figure 1: Number of cells of *B. subtilis* NBIMCC 2353 strain in logarithmic units for a time range of 0 to 72 hours (log N)

It was seen that the increase in live cell count averaged 0,7 – 0,8 log units in the first 24 hours of cultivation in pure nutrient medium. The cells were multiplied more intensively and their number was increased up to 2 log units, after the 24th hour, after adding the specified percent micelle and mushroom body, as at the 48th hour was reached its maximum, which is above 8,00 log units. The largest amount of cells for this hour, of all variant samples, was recorded in sample 6 – 8,90 logN, followed by sample 4, where the amount was 8,75 logN. The smallest number of live cells for the same hour was noted for variant sample 1 – 8,08 log units, correspondingly. The weakest propagation after the 24th hour was observed in variant 1 with 2% mushroom body and variant 5 with *Cordyceps sinensis* 2% where the growth of cells was barely about 1,0-1,2 log units. The breeding intensity, in these variants, is maintained the same as in the first 24 hours prior to the enrichment of the medium. Better results in cell suspension increase after 24th hour were achieved in variants 4 and 6, where the growth was 1,85 and 1,82 log units, respectively. There was a slight decrease in the amount of cells in all variant samples between 7,30 and 8,20 logN by the 72th hour, but their number was higher than these recorded at the 24th hour.

Nutritional environments and the respective amounts of the extracts of the two types of high mushrooms distributed in 10 experimental variants are presented in Table 2.

Table 2: Nutrient media and quantities of extracts of two types of high mushrooms.

Variant sample	Composition	Amount of extract /g/	Total quantity /ml/
1	Nutrient medium /control 1/		80
2	Nutrient medium + cells		80
3	Nutrient medium + *C.Sinensis* /control 2/	1	80
4	Nutrient medium + *C. sinensis* + cells	1	80
5	Nutrient medium + *C.Sinensis* /control 3/	2	80
6	Nutrient medium + *C. sinensis* + cells	2	80
7	Nutrient medium + *G. Lucidum* /control 4/	1	80
8	Nutrient medium + *G. Lucidum* + cells	1	80
9	Nutrient medium + *G. Lucidum* /control 5/	2	80
10	Nutrient medium + *G. Lucidum* + cells	2	80

Figure 2 shows the increase in the number of *Bacillus subtilis* cells NBIMCC 2353 in logarithmic units for a time range of up to 72 hours.

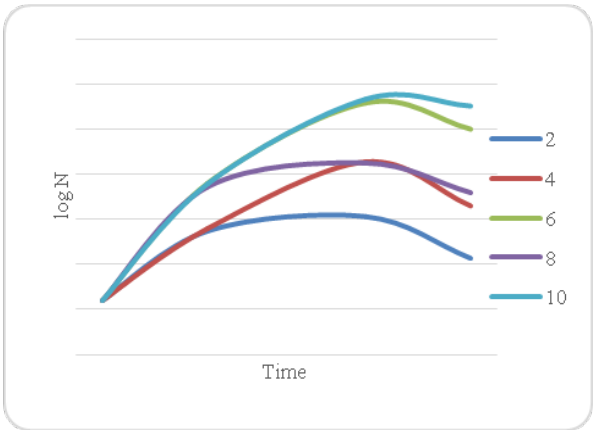

Figure 2: Growth the number of cells *of Bacillus subtilis* NBIMCC 2353 in logarithmic units for 72 hours period (log N)

Peak of an increase of cell count in control variant 2, where the cell culture was propagated without added enrichment agents, was at 40-48 hours after inoculation, which was increased to 1,87 log units. After this period, began dying of the cell culture and at 72th hour was observed reducing the cells by almost 1 log unit. A certain similarity was observed in the breeding time range of culture at variant samples 4 and 8 with added 1 g extract of *Cordyceps sinensis* and *Ganoderma lucidum*. The peak of breeding was also at 48th hour from the beginning of the experiment and the increase was slightly above 3 log units (10,28 logN for variant 4 and 10,26 logN for variant 8). Was observed sharper reduction in variant sample 4 with 1 log unit to 9,30 logN, and in variant 8 cells decreased by 0,66 to 9,6 logN, to 72th hour. A greater number of cells was found, almost 4,5 log units in variants 6 and 10 with added 2 g of extract of the two high mushrooms. The largest number was reported for a variant sample of 10 - 11,65 logN (at 48th hour). The cellular extinction after the peak of reproduction up to 72th hour was also labeled with a smaller number (about 0,5 log units), respectively at variant 6 - up to 11 logN, and at variant 10 - up to 11,50 log units, in which it can be seen, that up to 72th hour, a very small number of cells died and the number of living cells remained almost the same as that recorded at 48th hour.

4. Conclusions

It can be said from experiment 1, that in the growth of cell culture, after following the importation of the determined percentages of mycelia and mushroom body, the best results were obtained in the variants with added 4% micelles from the *Ganoderma lucidum* and *Cordyceps sinensis* mushrooms, where the number of cells from 24th to 48th hour was increased by 1,85 and 1,82 log units, respectively. The higher percentage concentration of the imported micelles contributes to a more intense development of the cell population of *Bacillus subtilis* NBIMCC 2353 strain. The lowest population growth in the 2% mushroom body variant was observed after addition of the additional components. A slightly larger cell growth was seen in the variant sample with *Cordyceps sinensis* 2%, but in both variants it can be seen that the cell propagation intensity was preserved the same as in the first 24 hours before enrichment of the medium.

It can be concluded from experiment 2, that in all variant samples the growth of the cell population was reached to 48th hour. However, at the variants where extracts of the two medicinal

mushrooms were added, higher maximum cell quantities were reported, than in the control variant. Of these, the largest number was found, that it was in the variant samples with 2 g of extracts added, where to 48th hour the number of cells increased by almost 4,5 log units, and by 72th hour it was observed that a small amount of cells died. Especially clear this has underlined for variant 10.

It can be concluded from both experiments, that a higher maximum number of living cells was recorded in the culture medium with 2 g extracts of the high mushrooms (experiment 2), 11,65 and 11,58 log units, respectively. Maximum amounts of cells were registered in the experiment 1 culture medium, at the samples where 4% micelles of the two mushrooms were added, 8,90 and 8,75 logN, respectively. From this it can be seen, that in experiment 2 the maximum number of registered living cells per strain *Bacillus subtilis* NBIMCC 2353 was with about 3 log units greater than that at experiment 1. When added to the nutrition media easily accessible for absorption components, as they are powdered extracts of *Cordyceps sinensis* and *Ganoderma lucidum*, positive effects have been found: prolonging the propagation time and doubling the number of cells.

5. Inferences

In the primary nutrient medium, where enrichment agents were added in the native form of mushroom body and mycelium, for multiply of the cell culture *Bacillus subtilis* NBIMCC 2353, the number of cells was smaller. Cell culture generally failed to develop to in its maximum capabilities, due to difficulty in absorbing macro-, micro- and protein compounds contained in high mushrooms.

An increase in the number of cells of the cell culture was established, when importing the powdered extracts of *Cordyceps sinensis* and *Ganoderma lucidum* to the nutrient medium. With this, we can say, that at using a nutrient medium to cultivate *Bacillus subtilis* NBIMCC 2353 strain with imported powdered extracts from the two high mushrooms a higher population was achieved. It is thus established, that this approach was suitable for use in the production of biologically active extracts for incorporation into functional foods.

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