International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-6, Issue-11, November 2020
ISSN: 2395-3470
www.ijseas.com

Optimization of the biosynthesis of proteolytic enzymes from the producer strain *Bacillus subtilis* NBIMCC 2353 by enriching the nutrient medium with natural components

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Abstract

Four fermentation experiments have been performed to optimize the composition of the nutrient medium of the strain *Bacillus subtilis* NBIMCC 2353 in order to achieve high yields of proteolytic enzymes. Natural substrates have been added for the development of cell culture in the nutrient medium. It was established that the intensity of the enzyme production is parallel to the growth curve and the peak is around the 48th hour. The results obtained for a proteolytic activity at the 24th, 48th and the 72nd hour, with the inclusion of powdered extracts from medical mushrooms, are low due to the presence of a large amount of amino acids in the nutrient medium that inhibit enzymatic biosynthesis. With the addition of mushroom body and micelles from higher mushrooms to the cell culture, a higher yield of produced proteases is observed. The highest proteolytic activity is reported after the inclusion in the nutrient medium of natural substrates (native collagen and fresh mycelium from medicinal mushrooms).

Key words: *Bacillus subtilis*, biosynthesis, nutrient medium, proteolytic activity, medical mushrooms

1.Introduction

The biosynthesis of proteolytic enzymes depends both on the biochemical characteristics of the producers and on the conditions for their cultivation. The selection of a suitable nutrient medium is of particular importance and ensures the development of producing microorganisms and the achievement of the maximum proteolytic activity.

Bacterial proteases are characterized by a wide variety of catalytic properties, high yield, stability, regular supply due to the lack of seasonal fluctuations, rapid growth of microorganisms in an accessible environment, the possibility of genetic manipulation etc., compared to those derived from plants, animals and fungi [1, 2, 3].

Microbial proteases are produced by strains of microbial species such as *Bacillus sp., Alcaligenes faecalis, Pseudomonas fluorescens, Aeromonas hydrophilia* and others. *Bacillus sp*. are the main producers of extracellular proteases and industrial sectors often use this species for the production of various enzymes [4]. They are found mainly in the soil, have a rod-shaped shape, can form a rough protective endospore. They are resistant to adverse environmental conditions [5, 6]. One of the largest producers of proteases from this species is *Bacillus subtilis*. It is gram-positive and has excellent fermentation properties. Although this bacterium can grow in the gastrointestinal tract of animals, it is not considered as a human pathogen [7, 8, 9].

Studies on proteolytic enzymes by *Bacillus sp*. have proved that nutritional factors, including carbon and nitrogen sources, can effectively influence the production of protease enzymes [10, 11].

Yeast extract is one of the sources of nitrogen that is used as a natural substrate for the biosynthesis of proteolytic enzymes. Boominadhan and Rajakumar, (2009) [10] in their experiment

characterized that the yeast extract is the optimal source of nitrogen for the production of protease enzymes from *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus megaterium* and *Bacillus licheniformis*, isolated from various wastes. Yeast extract, tryptone and peptone, as nitrogen sources, have positive effects on the yield of keratinase from *Bacillus subtilis* [12]. Moharam et al., (2019) [13] reported that with 0.71% yeast extract, the enzymatic activity of a fibrinolytic enzyme derived from *Bacillus subtilis* Egy was 16.6 U / ml. Yeast extract (0.5%) is also used in the basic medium to produce protease from *Bacillus subtilis* AKAL7 [3].

Collagen protein can be used to increase the activity of proteolytic enzymes derived from various microorganisms. Dreisbach and Merkel, (1978) [14] confirmed this by an experiment where they observed an increase in extracellular collagenase activity after the addition of collagen to cell cultures in the last part of the exponential growth phase.

One of the important components that must be present in the nutrient medium of strains producers, for a better yield of a proteolytic enzyme, are minerals. The presence of magnesium is of particular importance. It is an activator of peptidases and magnesium ions affect protein synthesis [3, 15]. In their study, Hakim et al., (2018) [3] found that Mg^{2+}, K^{+} + и Ca^{2+} ions stimulate the protease activity from a producer strain *Bacillus subtilis* AKAL 7.

Inulin is a mixture of linear fructose polymers with different chain lengths. It belongs to the fructan group of polysaccharides and serves to store carbohydrates in many plant species. Compounds such as inulin reduce the energy density of food and are used to enrich it with dietary fiber or to replace sugar and fat [16].

Fructooligosaccharides (FOS) are breakdown products from the hydrolysis of inulin or polyfructose with a chain length between 10 and 60 residues. They are fragments of oligopolymers obtained by the binding of Beta-D-fructose (2 → 1) and Alpha-D-glucose (1 → 2) glycosidic linkages. Fructooligosaccharides are one of the most widely offered prebiotics on the market with many health benefits - immune modulation, improvement of the gastrointestinal tract, absorption of minerals, protection against colon cancer and reduced risk of obesity-associated disorders [17]. Fructoligosaccharides, as a prebiotic, are also used by probiotic bacteria. The effects of FOS, used from *Bacillus subtilis*, on growth efficiency, immunity, intestinal microflora and the resistance to sea cucumber (*Apostichopus japonicus*) have been experimentally determinated [18]. Venil and Lakshmanaperumalsamy, (2009) [2] report that when using food sources - dextrose (1%), $(NH_4)_2$ PO_4 (0,5%), as well as with the following parameters - temperature (30°C) and with stirring (100 rpm), the yield of protease obtained from Bacillus subtilis HB04 has an activity of 290 U/ ml.

Peptone is one of the reducing agents actively involved in the formation of a proteolytic enzyme. Kembhavi et al., (1993) [19], in their research of the enzymatic activity of a protease derived from *Bacillus subtilis*, isolated from a hot spring, have identified bacteriological peptone as the best inducer for a maximum production of protease. Uchida et al., (2004) [20] show that *Bacillus subtilis* CN2, isolated from Vietnamese fish sauce, produces a large amount of alkaline protease when grown on soy peptone medium.

There is a lack of data on the use of mushrooms as a nutrient medium for microorganisms to optimize the conditions of product production. Tang et al., (2015) [21] experimented in their study, using various fungal residues as feed additives to the nutrient medium for the cultivation of *Bacillus subtilis* in order to obtain poly-γ-glutamic acid. The higher medical mushrooms *Ganoderma lucidum, Cordyceps sinensis, Ganoderma neo-japonicum, Cordyceps militaris* have a rich set of biologically active substances that stimulate the living organism and have healing properties [22, 23, 24]. In previous experiments, non-

hydrolyzed fragments of living mycelium and dry extract of the medicinal fungi *Ganoderma lucidum* and *Cordyceps sinensis* were used to monitor the increase in the number of live cells of *Bacillus subtilis* NBIMCC 2353 over a period of 72 hours [25].

In recent years, the interest in new substrates of different origins has deepened, as well as the combinations between them in order to optimize the biosynthesis of proteolytic enzymes from different microorganisms.

The purpose of the present study is to optimize the composition of the nutrient medium of the producer strain *Bacillus subtilis* NBIMCC 2353 in order to achieve a high yield of proteolytic enzymes by adding natural components to the nutrient medium.

2.Materials and methods

For experiments has been used, a starter culture of a producer strain of *Bacillus subtilis* NBIMCC 2353, purchased from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC), Meat extract, Pepton, Agar and yeast extract, bone broth extract and native bovine collagen, purchased from Pic and Co OOD - Sofia, laboratory-grown fresh mycelium of *Ganoderma lucidum, Ganoderma neo-japonicum, Cordyceps sinensis* and *Cordyceps millitaris*, grown in The Institute of Cryobiology and Food Technology – Sofia, Bulgaria and hydrolyzed extracts of *Ganoderma lucidum* and *Cordyceps sinensis*, purchased from Cangzhou Fungi Extracts Co, Ltd – China.

The fermentation experiments were performed in triplicate repetition.

The experiments are performed with the use of the following methods:

The starter culture medium for the propagation and maintenance of the strain *Bacillus subtilis* NBIMCC 2353.

Parameters for cultivation of the producer strain *Bacillus subtilis* NBIMCC 2353. The nutrient medium is with the following composition (g / l): Meat extract - 10.0; Peptone - 10.0; NaCl - 5.0; Agar - 20.0; Distilled water - 1l. The components were dissolved in distilled water and sterilized in an autoclave for 20 minutes at 121°C and a pressure of 1 atm.

Cultivation conditions. In order to obtain vegetative reproduction, cultivation was performed in 500 ml Erlenmeyer flasks, containing 100 ml of growing medium for 24 hours at 30°C on a BS /4 circular shaker apparatus at 100 rpm.

To improve the biosynthesis of proteolytic enzymes, natural enrichments were added to the culture fluid of *Bacillus subtilis*:

1. Inclusion of dry hydrolyzed extract from medical mushrooms *Ganoderma lucidum* and *Cordyceps sinensis* to the starter culture medium

Erlenmeyer flasks with a volume of 250 ml with a nutrient medium of 50 ml were used.

The total operating amount of enriched and inoculated medium for propagating and tracing of the strain is 80 ml. To enrich the medium and monitor cell proliferation, extracts of two species of higher mushrooms *Cordyceps sinensis* and *Ganoderma lucidum* were added in a powder form in two different concentrations: 1 and 2 g. In all variations, the inoculum has an initial cell concentration of $1{,}6x10^7$ CFU/ml.

2.Improvement of the biosynthesis of proteolytic enzymes by adding non-hydrolyzed fragments of live mycelium of four species of medical mushrooms *Ganoderma lucidum, Ganoderma neo-japonicum, Cordyceps sinensis, Cordyceps militaris* to the culture fluid of *Bacillus subtilis*.

In this experiment, live, laboratory-grown micelles of medical mushrooms was used that were separated in the required quantities directly from the cultured mycelium and placed in a pre-prepared nutrient medium for the propagation of *Bacillus subtilis* NBIMCC 2353.

3. Inclusion in the nutrient medium of Fructooligosaccharides (FOS), yeast extract, inulin and fresh mycelium from *Ganoderma lucidum*.

Composition of the nutrient medium (per 1000 ml of distilled H_2O):

8 g FOS (Fructooligosaccharides);
8 g Fresh mycelium of the fungus *Ganoderma lucidum*;
1.5 g Yeast extract;
1 g Inulin;
1.3 g K_2HPO_4;
0.3 g $MgSO_4$.

The active acidity (pH) of the nutrient medium is 8.

Erlenmeyer flasks of 250 ml were used, the amount of the culture medium together with the inoculum of *Bacillus subtilis* NBIMCC 2353 cells is 100 ml. Initial cell concentration is $0{,}4x10^7$ CFU/ml.

4. Inclusion of native bovine collagen protein in the nutrient medium

Composition of the nutrient medium (per 1000 ml of distilled H2O):

20 g Dextrose;
1 g FOS (Fructooligosaccharides);
10 g Beef bone broth extract;
8 g Fresh mycelium of Ganoderma lucidum mushroom;
20 g Beef collagen;
2 g Peptone;
10 g Yeast extract;
5 g $CaCl_2$;
5 g K_2HPO_3;
5 g $MgSO_4$.

The active acidity (pH) of the nutrient medium is 8.

Erlenmeyer flasks of 250 ml were used, the amount of the culture medium together with the inoculum of *Bacillus subtilis* NBIMCC 2353 cells is 100 ml. Initial cell concentration is $0{,}4x10^7$ CFU/ml.

3.Results and discussion

A monitoring was performed of the proteolytic enzyme activity in the culture fluid variations of *Bacillus subtilis* with a duration of up to 72 hours from the start of the experiment. The results for the proteolytic activity at the 24th, 48th and 72nd hours with the inclusion of powdered extracts of medical mushrooms *Ganoderma lucidum* and *Cordyceps sinensis* are presented in Figure 1.

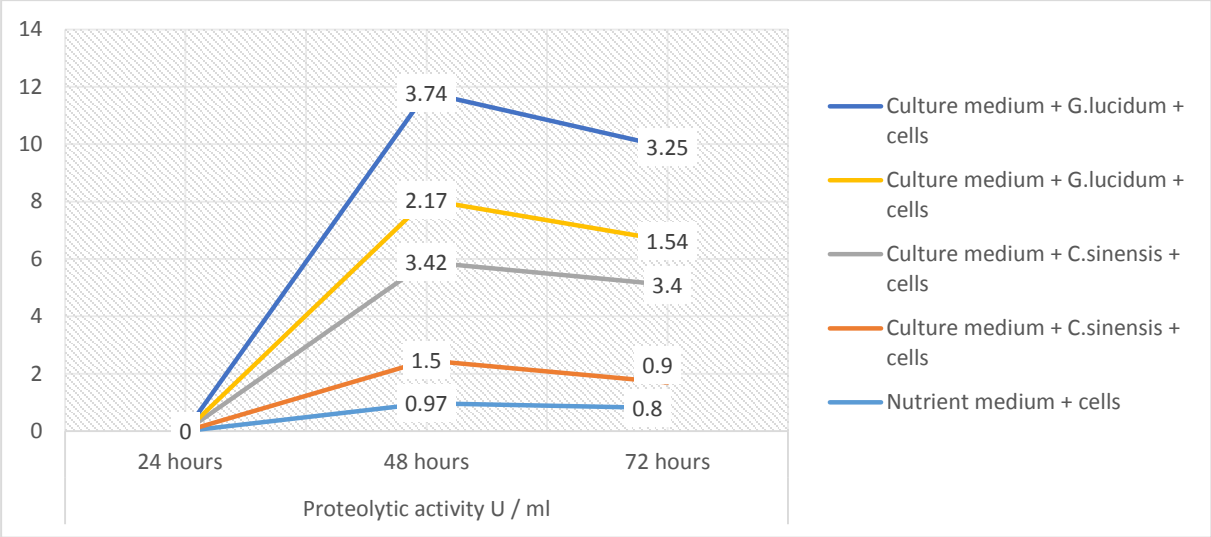

Figure 1: The change in the proteolytic activity (U/ml) in the nutrient medium with added extracts of two species of higher mushrooms *Cordyceps sinensis* and *Ganoderma lucidum* in the time range from 0 to 72 hours

The values obtained are the highest at the 48th hour, which coincides with the peak increase in the number of cells reported in a previous study of the team [25]. After this period, cell culture begins to die and by the 72nd hour, a decrease in the units for proteolytic enzymes is observed. The values at all three reporting points are low, most likely due to the presence of a large amount of amino acids in the nutrient medium, which under certain conditions inhibit enzymatic biosynthesis. In such cases, this leads to an increase in the number of cells but without the accumulation of proteolytic enzymes. The data completely coincides with the results of our previous experiment [25], where with an increase of the number of live cells of *Bacillus subtilis* NBIMCC 2353, in addition to powdered extracts of these higher mushrooms, an increase in the number of cell culture cells was found. This proves that when using a nutrient medium for culturing a strain with imported powder extracts of both fungi, a higher population is achieved, but not increased enzymatic biosynthesis.

Table 1 presents the variations of the nutrient medium used with added mushroom body and micelles of higher mushrooms *Ganoderma lucidum (Red Reishi), Ganoderma neo-japonicum (Black Reishi), Cordyceps sinensis* and *Cordyceps militaris* in the respective concentrations. The dynamics of the change of this activity for a period of up to 72 hours is presented in Figure 2.

Table 1: Variations of the nutrient medium used with added mushroom body and micelles of higher mushrooms (% concentration)

Variant №	Composition	Concentration %
1.	*Ganoderma lucidum*	2
2.	*Ganoderma lucidum*	4
3.	*Ganoderma neo-japanicum*	2
4.	*Ganoderma neo-japanicum*	4
5.	*Cordyceps sinensis*	2
6.	*Cordyceps sinensis*	4
7.	*Cordyceps militaris*	2
8.	*Cordyceps militaris*	4

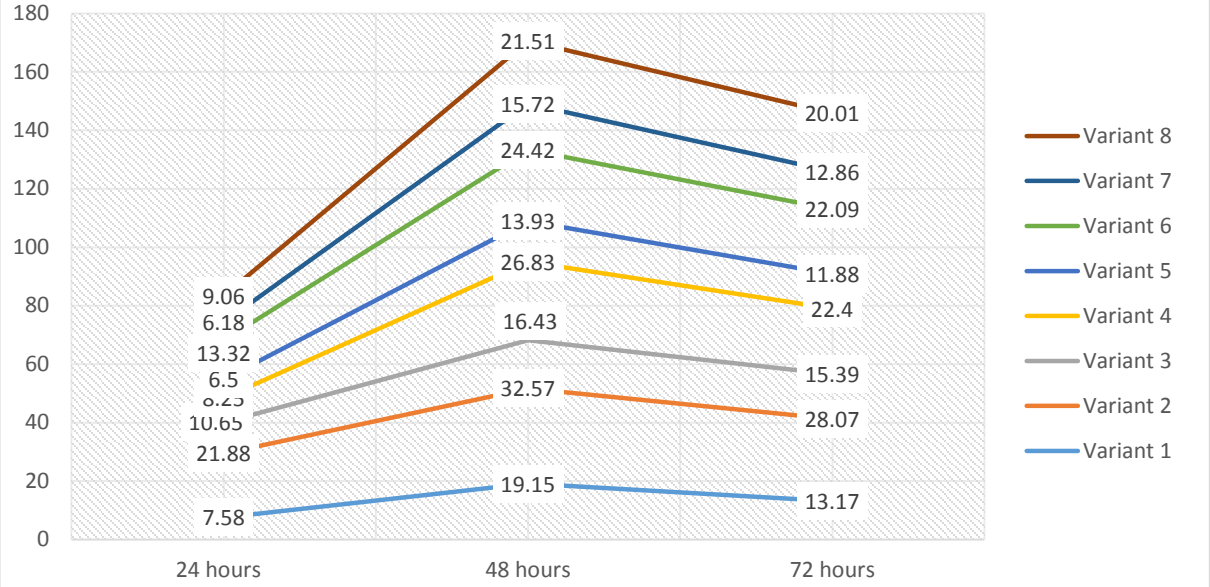

Figure 2: Change in the proteolytic activity (U / ml) in the nutrient medium of the variations of the used nutrient medium with added mushroom body and micelles of higher mushrooms (% concentration) in the time range from 0 to 72 hours

With the addition of enrichments (mushroom body and micelles of higher fungi) to the cell culture, higher values of proteolytic activity are reported. The variation with added enrichment (mushroom body and mycelium) from *Ganoderma lucidum* in the concentration of 4% has the highest enzymatic activity. The peak of the reported values is at the 48th hour in all 8 variations. At the 72th hour from the start of the experiment, a decline was observed again associated with cell death after the peak of their proliferation and enzymatic autolysis.

The obtained data coincide with a previous experiment [25] where with the addition of mushroom body and micelles from higher mushrooms *Ganoderma lucidum* and *Cordyceps sinensis*, the increase in the number of live cells of *Bacillus subtilis* NBIMCC 2353 for a period of 72 hours was observed and it

was established that the cell culture fails to develop to its maximum capacity due to the difficult absorption of macro-, micro- and protein compounds contained in higher mushrooms. However, this is the reason why cells stimulate increased enzyme biosynthesis.

To achieve a higher yield of produced proteolytic enzymes, the composition of the culture medium for culturing a strain *Bacillus subtilis* was changed. The composition includes FOS, yeast extract and inulin. Also fresh mycelium from *Ganoderma lucidum*, the use of which in the previous experiment showed the highest proteolytic activity.

Figure 3 shows the proteolytic activity at the 24th, 48th and 72nd hour from the inclusion of Fructooligosaccharides (FOS), yeast extract, inulin and fresh *Ganoderma lucid* mycelium in the nutrient medium of the producer strain *Bacillus subtilis* NBIMCC 2353.

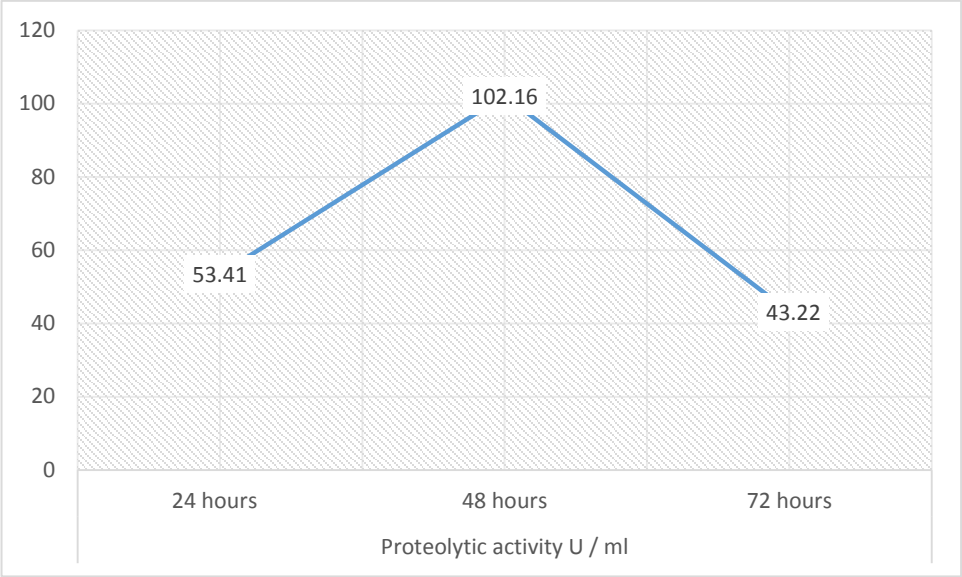

Figure 3: Change in the proteolytic activity (U/ml) in the nutrient medium with the inclusion of Fructooligosaccharides (FOS), yeast extract, inulin and fresh mycelium from *Ganoderma lucidum*

The highest enzyme activity is observed at the 48th hour. It is noticed that the enzyme activity from the 24th to the 48th hour increases with 48.75 U/ml and by the 72nd hour it decreases more - with 58.94 U/ml. The enzyme activity obtained at the 48th hour is about 80 U/ml higher than that recorded with added mushroom body and mycelium from *Ganoderma lucidum* at a concentration of 4%.

Figure 4 shows the proteolytic activity at the 24th, 48th and 72nd hour of native bovine collagen protein included in the nutrient medium of the producer strain *Bacillus subtilis* NBIMCC 2353.

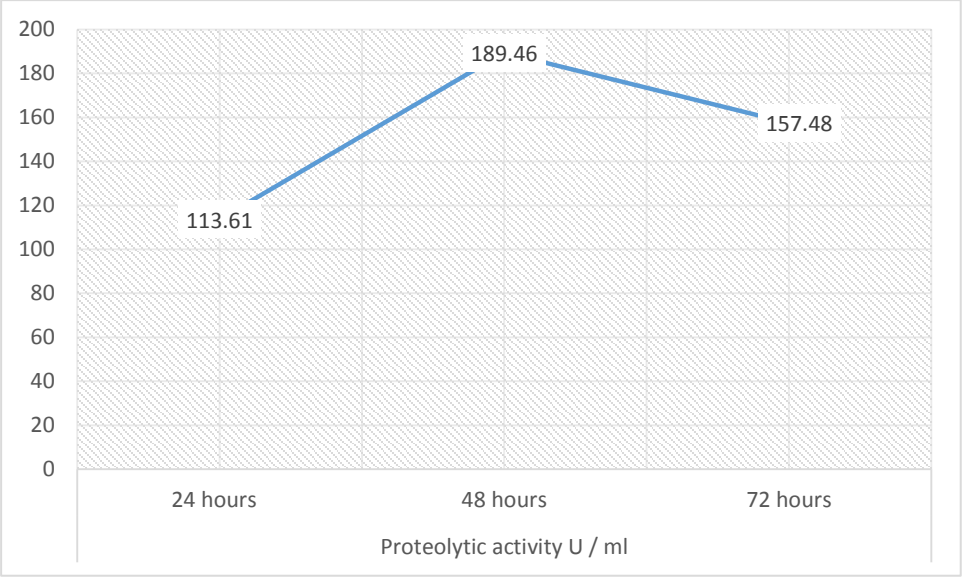

Figure 4: Change in the proteolytic activity (U/ml) in the nutrient medium with bovine collagen protein

In this case, the highest proteolytic activity was established at the 48th hour - 189.46 U/ml, from all experiments performed. In all three time intervals, the values are above 100 U/ml.

In all four fermentation experiments, the intensity of the enzyme formation is parallel to the growth curve and the peak is around the 48th hour.

With the optimization of the composition of the nutrient medium in the last two experiments with the inclusion of fresh mycelium of *Ganoderma lucidum* and native collagen, higher values of total proteolytic activity were monitored. The highest amount of enzyme is in the sample with native collagen.

Proteinases belong to the inducible enzymes and their biosynthesis is associated with the presence of an inducer actively involved in the formation of the enzyme protein. In its absence, microorganisms produce proteinases but only in very small quantities. Organic nitrogen sources are often used as an inducer. Accurate determination of the concentration of the inducer leads to increased production of proteolytic enzymes. To optimize the fermentation process in the nutrient medium, peptone and native collagen were included as an inducer of enzymatic biosynthesis. Also in the nutrient medium of *Bacillus subtilis* NBIMCC 2353 yeast extract and fresh mycelium of medical mushrooms was added as a natural substrate for the biosynthesis of proteolytic enzymes.

The mineral composition of the nutrient medium is also important for the biosynthesis of proteinases by microorganisms. The microelements participate in the synthesis of the enzyme protein and activate the peptidases because of this in their absence the proteolytic activity is significantly reduced.

With the purpose of more efficient production of proteases, potassium phosphate is added to the composition of the nutrient medium. It contributes to the buffering properties of the nutrient medium and is involved in the absorption of carbon compounds and other vital processes for the cells.

Sulfur, which is part of the enzyme protein in the form of sulfhydryl groups, is also required for the biosynthesis of proteolytic enzymes. Magnesium sulphate is used as a source of sulfur. Sulfates are subjected to reduction in the cells and are used in the synthesis of sulfur-containing amino acids [3, 26, 27, 28, 29].

A higher yield of produced proteolytic enzymes (189.46 U/ml) was achieved with the added additives (native collagen, yeast autolysate and fresh mycelium of medicinal mushrooms) for the development of the cell culture colony of *Bacillus subtilis* strain.

4.Conclusion

The biosynthesis of proteinases from *Bacillus subtilis* strain is parallel to the growth curve until the start of the stationary phase after which the proteolytic activity decreases.

The excess of hydrolyzed nitrogen source in the nutrient medium leads to the accumulation of a large amount of biomass, but with reduced production capacity.

After the optimization of the composition of the nutrient medium, by adding natural substrates (native collagen, yeast extract and fresh mycelium of medicinal mushrooms), a higher yield of produced proteases was achieved (189.46 U/ml).

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