Chitinase production by *Trichoderma viride* in submerged state fermentation

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**Abstract.** We have identified the optimal conditions for the production of chitinolytic enzymes of *T. viride* in submerged state fermentation. The production of chitinase by a new strain of fungus was carried out on the basal liquid medium, containing (%) colloidal chitin 0.5, NaNO₃ 0.2, KH₂PO₄ 0.1; MgSO₄·7H₂O 0.05 and KCl, 0.05. The activity of enzymes of the chitinase complex of the strain was evaluated using the method using dinitrosalicylic acid (DNS reagent). A quantitative determination of the activity of chitinases in a producer microorganism was established by their ability to hydrolyze 0.2% colloidal chitin (in phosphate buffer 0.05 M, pH 5.2), by the content of reducing sugars formed in this process, which were evaluated using a DNS reagent. The results of studies of the influence of various cultivation parameters showed that highest chitinolotic enzymes production by *T. viride* was obtained at pH 4.0, (301.15-303.15) K and after 144 h growth. The studied soil isolate can be further used in biotechnological research, as well as for biological control of pests and pathogens of agricultural crops.

1 Introduction

Nowadays plant diseases have become a serious problem limiting agricultural productivity worldwide. According to the Food and Agriculture Organization of the United Nations (FAO) losses in world agriculture caused by pests and phytopathogenic fungi annually represents approximately 25 percent of the potential crop [1-4]. The potential damage to food crops in the Russian Federation from phytopathogenic fungi for individual crops is estimated approximately from 5 to 15 percent. The wide prevalence, adaptability and genetic variability of pathogenic fungi, as well as their ability to form mycotoxins, representing a real danger to the health of the population and animals, constantly returns researchers in this field to this problem [5].

At the present stage, researchers in the field of phytopathology spend significant time and efforts on studying the characteristics of the morphology, physiology, biochemistry and genetics of phytopathogens, finding ways to reduce their intensity of damage and prevalence [6-12]. The use of chemical fungicides is the most commonly used method for protecting
plants in agricultural practice. However, despite all the significant benefits derived from the use of fungicides, the use of these chemicals can lead to negative consequences such as a reduction of soil fertility, disturbance of soil-ecological balance, the disappearance of insect species, the agricultural product deterioration, and many others [13-20]. In order to reduce their use is currently necessary to develop alternative and effective methods of plant protection and related drugs.

A promising direction that can be widely used in agriculture against phytopathogenic forms of fungi is the use of agents based on microorganisms that have antagonistic and other activities, for example, hydrolase activity. Thus, basisulrin based on the \textit{Bacillus amyloliquefaciens VKPM B-11008} strain simultaneously combines antagonistic activity to a wide range of plant fungal diseases, plant growth-promoting effect, as well as chitinase, phosphatase and nitrogenase activity [21].

Chitinases (EC.3.2.1.14) are a group of enzymes that are responsible for the hydrolysis of chitin, a nitrogen-containing linear polymer consisting of interconnected $\beta$-$(1\rightarrow4)$ - glycoside bonds of N-acetylglucosamine residues [3, 12, 21]. They play an important role in many biological processes, and are found in plants, microorganisms, including bacteria, fungi and actinomycetes [22-33].

In plants, chitinolytic enzymes are involved in the formation of protective reactions to pathogens [27-31]. Crustaceans, insects and other arthropods, as well as fungi, form enzymes of this group due to the need for morphogenesis of their exoskeleton (carapace) or the cell wall of the mycelium. The ability to form chitinases has been shown for many microorganisms that use chitin as a source of carbon and energy [3, 5, 12, 21, 23-26, 34]. Some bacteria of the genera \textit{Bacillus}, \textit{Streptomyces}, \textit{Serratia} and \textit{Pseudomonas} are able to hydrolyze chitin and use it as the sole carbon source. Among the fungi, the most widespread species are \textit{Alternaria alternata}, \textit{Aspergillus fumigatus}, \textit{Aspergillus terreus}, \textit{Penicillium aculeatum}, \textit{Fusarium oxysporum}, \textit{Trichoderma harzianum}, \textit{Trichoderma reesei}, \textit{Trichoderma hamatum} and \textit{Trichoderma viride} [5, 14, 32-37]. The strains of \textit{Serratia marcescens}, \textit{Streptomyces griseus} and \textit{Trichoderma harzianum} are used for the industrial production of microbial chitinase enzymes used for biocontrol of plant pathogens [5, 35, 38].

Currently, chitinases are used to control not only fungi, pathogens of agricultural crops, but also with other plant pests, such as insects and nematodes. The main advantage of chitinases with fungicidal and insecticidal activities is the high selectivity of their action [5]. In addition, it should be noted the study of enzymes of the chitinase complex of microorganisms is also of fundamental importance since they can participate in the processes of autolysis, morphogenesis and nutrition, in the relationship between plants and insects.

In connection with the foregoing, the study of new effective strains of producers of chitinolytic enzymes is a very actual task and has undoubted practical and fundamental significance.

The present work was conducted optimization of conditions for chitinase production by fungal strain of \textit{T.viride} in submerged cultivation.

2 Methods

A strain of fungus of \textit{T.viride} isolated from samples of sod-podzolic soil of the Republic of Tatarstan was used as an object of study.

For the deep growth of the fungal strain, a liquid nutrient medium of the following composition was used (%): colloidal chitin - 0.5; NaNO$_3$ 0.2; KH$_2$PO$_4$ 0.1; MgSO$_4$.7H$_2$O - 0.05; and KCl - 0.05 [35, 36]. The pH of the medium was adjusted to 5.0. The producers were cultured in 250 ml flasks with 50 ml of medium for 7 days at 120 rpm and a temperature of 301.15 K. The biomass was separated from the culture liquid by centrifugation at 8000 rpm for 10 minutes, the obtained supernatant was used for further studies.
The influence of certain physical parameters, such as incubation temperature (293.15, 297.15, 299.15, 301.15, 303.15, 305.15, 307.15, 313.15 and 323.15 K), medium pH (3, 3.5, 4, 4.5, 5, 6, 7 and 8) and incubation period (24, 48, 72, 96, 120, 144, 168 h) on chitinase production was investigated.

A quantitative determination of the activity of chitinolytic enzymes of the fungus was established using the method using dinitrosaliclyc acid [5, 12, 35]. The substrate for determining hydrolase activity was 0.2% colloidal chitin in 0.05 M solution of phosphate buffer with a pH 5.2. The test samples (0.5 ml) were kept for 5 min in a water bath (323.15 K), 0.5 ml of substrate, 1 ml of distilled water were added and mixed; after 30 minutes of incubation, the reaction was stopped by adding 3 ml of dinitrosaliclyc acid solution. The optical density was measured on a spectrophotometer 101 at 575 nm. A unit of chitinase activity was taken as a certain amount of an enzyme that catalyzes the process of chitin hydrolysis with the formation of 1 μmol of reducing sugars per min under standard conditions.

The experiments were carried out in 3 biological and 3 analytical replicates [38]. Statistical processing of the results was carried out by finding the arithmetic mean values and their standard errors using the standard software package Microsoft Office Excel 2013. The significance of differences was evaluated using the Student t-test, the differences were considered significant at p<0.05.

3 Results and Discussion

In connection with the active interest associated with the use of chitinases in various industries, the most effective methods for increasing the formation of this enzyme are being developed by enzymes. One of the main features of fungi is their ability to change metabolic processes in accordance with environmental conditions, which makes it possible to control the growth of the microorganism and increase the yield of the final product, including increasing the biosynthesis of the required protein [14, 35, 39, 40]. Various factors can influence the production of chitinolytic enzymes into the medium, including temperature, pH of the medium, and the time of cultivation of the microorganism [16, 35]. In studying the optimal growth conditions of the producer microorganism, it is necessary to take into account the final cost of the product obtained, which depends on the costs of material and fuel and energy resources.

At the first stage of the study, the most optimal temperature was determined for the production of chitinolytic enzymes in the conditions of submerged cultivation of fungus. Their formation was greatest at a temperature of (301.15-303.15) K, compared with other temperatures of cultivation (p <0.05), 3.2 ± 0.08 U/ml (Fig. 1). At a temperature of 308.15 K and higher, the level of production of extracellular enzymes decreased and a complete absence of hydrolase activity was observed at 323.15 K (results not shown).
Fig. 1. The effect of temperature on chitinase production by fungal strain of *T. viride* under conditions of submerged cultivation

The level of activity of extracellular enzymes significantly depends on the pH of the culture medium of the producer. When studying the effect of different pHs on the formation of chitinolytic enzymes, the maximum experimental result was observed when using a nutrient medium with a pH 4.0 (Fig. 2). When cultivating the strain of *T. viride* on a medium with a pH 4.0, it contributed to a 1.2-fold increase in chitinase activity compared to the initial medium (p<0.05). The activity level was also quite effective at pH 3.5 and 4.5 (decrease by 16.5 and 7.3%, respectively). While in a more alkaline environment (pH 8.0), production decreased significantly (activity decrease by 72.8%) (results not shown).

Fig. 2. The effect of pH on chitinase production by fungal strain of *T. viride* under conditions of submerged cultivation

A higher level of biosynthesis of chitinolytic enzymes in an acidic environment indicates the possibility of using hydrolases of *T. viride* in agriculture against phytopathogenic forms of fungi [35, 41]. Nevertheless, any deviation from the optimal pH was accompanied by a corresponding decrease in the yield of fungal chitinases. The results obtained was in complete accordance with the authors Fenice et al. (1998) for chitinase of *Penicillium janthinellum*
It has been shown that environmental pH affects the availability of certain metal ions, membrane permeability, internal mycelium pH, and enzymatic activity.

The data obtained are consistent with the results of foreign authors who found that the production of chitinases by the fungus of *Trichoderma viride* is significantly induced at a temperature of (301.15-303.15) K and pH 4.0 [35]. The optimal cultivation temperatures for the formation of chitinolytic enzymes of *Penicillium janthinellum*, *Verticillium lecanii viegas*, *Isaria fumosorosea*, *Penicillium sp.* LYG 0704, *Aspergillus terreus* are 295.15, 297.15, 298.15, 313.15 and 323.15 K, respectively [42-44]. The optimal pH of the nutrient medium for the production of chitinases of *Penicillium sp.* LYG 0704, *Isaria fumosorosea*, *Stachybotrys elegans*, *Verticillium lecanii viegas* are 5.0, 5.7, 7.0 and 7.5, respectively [43-45].

At the next stage, we studied the dynamics of changes in the activity of chitinolytic enzymes in the culture liquid during the growth of *T.viride* (Fig. 3).

![Fig. 3. The dynamics of chitinase production of *T.viride* under conditions of submerged cultivation](image)

As seen in figure, on day 2 a sharp increase in enzyme activity was observed in the culture liquid of fungal strain of *T.viride*. The maximum activity of chitinases was observed on the 6th day of cultivation and it was $4.42 \pm 0.11$ U/ml (p<0.05).

These results are concordant with data from other authors, who showed that the maximum enzymatic activity of chitinases of *T.viride* is illustrated on the 6th day of cultivation [35]. Similarly, highest production of chitinolytic enzymes of *Penicillium chrysogenum* was detected on days 6 and 7 [45]. On the other hand, Ghanem et al. (2010) and Lee et al. (2009) found that the maximum value of chitinase activity of *A. terreus* and *Penicillium sp.* LYG 0704 was established on the 3rd and 4th day of cultivation, respectively [45, 48].

### 4 Conclusions

The results of studies of the influence of various cultivation parameters showed that the optimal conditions for growing the strain of *T.viride* for the production of chitinolytic enzymes in submerged conditions is the use of a medium with a pH of 4.0 and a growth temperature of (301.15-303.15) K. The studied soil isolate can be further used in biotechnological research, as well as for biological control of pests and pathogens of agricultural crops. The most important advantage of this producer strain is the convenience from the point of view of cultivation, rapid growth and high activity of the microorganism,
and the possible funds obtained on its basis will be environmentally friendly and have a high specificity of action.

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