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Pilot submerged cultivation of *Trichoderma asperellum* RNCIM F-1323 micromycete

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Abstract: The study is aimed at developing a pilot industrial method for producing a biofungicide based on the fungus of *Trichoderma* genus. The cultivation was carried out by submerging in a pilot industrial bioreactor line with an inoculation bioreactor having a total volume of 10 litres and a working bioreactor with a total volume of 100 litres. Czapek's modified nutrient medium was used with yeast extract and molasses replacing sucrose, g/L: molasses – 20; yeast extract – 7; NaNO₃ – 2; K₂HPO₄ – 1; MgSO₄ – 0.5; KCl – 0.5; FeSO₄ – 0.01. Cultivation was carried out maintaining the following parameters: temperature – 27±0.5 °C; culture medium mixing speed – 600 rpm; aeration intensity – 1 L of air per 1 L of culture medium per min. The inoculum was grown on a nutrient medium of a similar composition in rocking flasks with a total and a working volume of 750 and 100 mL, respectively. Inoculated flasks were incubated using an Innova 40R shaker incubator (New Brunswick, USA) at 200 rpm for 22–24 h until a dry biomass index of 6.5–7.5 g/L was achieved. Conidia concentration was calculated using a Goryaev camera. Liquid cultivation was selected due to its relatively short process time. In addition, unlike solid-phase, the submerged cultivation provides for the accumulation of numerous secondary metabolites having antagonistic activity against phytopathogens in the finished form of the preparation. The object of the study was the *Trichoderma asperellum* RNCIM F-1323 strain with antagonistic activity against many phytopathogens. From a technical and economic point of view, the culture transfer to an inoculation bioreactor in an amount of 5 % vol. was established to be justified as a result of the experiments. The effect of various time values for cultivation in inoculation bioreactor on the growth rate of micromycetes in a working bioreactor was studied. The optimal time for transfer to a working bioreactor was determined to be within 20–24 h. At the end of the cultivation process, the conidia concentration was equal to 1.9·10⁸ conidia/mL.

Keywords: submerged cultivation, *Trichoderma*, biofungicide, pilot cultivation

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Глубинное культивирование микромицета *Trichoderma asperellum* ВКПМ F-1323 в опытно-промышленных условиях

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Резюме: Цель данного исследования – разработка опытно-промышленного метода получения био-фунгицида на основе гриба рода *Trichoderma*. Культивирование проводилось глубинным способом в опытно-промышленной линии ферментеров с ферментером-инокулятором общим объемом 10 л и рабочим ферментером общим объемом 100 л. В качестве питательной среды была использована

модифицированная среда Чапека с добавлением дрожжевого экстракта и заменой сахарозы на мелассу, г/л: меласса – 20; дрожжевой экстракт – 7; NaNO_3 – 2; K_2HPO_4 – 1; MgSO_4 – 0,5; KCl – 0,5; FeSO_4 – 0,01. Культивирование проводилось с поддержанием следующих параметров: температура – $27 \pm 0,5$ °C; скорость перемешивания культуральной среды – 600 об./мин; интенсивность аэрации – 1 л воздуха на 1 л питательной среды в мин. Инокулят выращивался на питательной среде аналогичного состава в качалочных колбах объемом 750 мл с рабочим объемом 100 мл. Инокулированные колбы инкубировались на шейкере-инкубаторе Innova 40R (New Brunswick, США) при 200 об./мин в течение 22–24 ч до достижения показателя сухой биомассы в пределах 6,5–7,5 г/л. Подсчет концентрации конидий осуществляли с использованием камеры Горяева. В качестве метода культивирования было выбрано жидкостное в связи с его относительно быстрым протеканием. Кроме того, в отличие от твердофазного культивирования глубинное позволяет аккумулировать в готовой товарной форме препарата многочисленные вторичные метаболиты, обладающие антагонистической активностью по отношению к фитопатогенам. В качестве объекта исследования выступал штамм *Trichoderma asperellum* ВКПМ F-1323, обладающий антагонистической активностью по отношению ко многим фитопатогенам. В результате проведенных экспериментов было установлено, что с технико-экономической точки зрения обосновано внесение культуры для засева ферментера-инокулятора в количестве 5 % об. Изучено влияние различной продолжительности культивирования в ферментере-инокуляторе на скорость роста микромицета в рабочем ферментере. Определено, что оптимальное время для пересева в рабочий ферментер находится в пределах 20–24 ч. По окончании процесса культивирования концентрация конидий составила $1,9 \cdot 10^8$ конидий/мл.

Ключевые слова: глубинное культивирование, *Trichoderma*, биофунгицид, опытно-промышленное культивирование

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INTRODUCTION

In all developed countries today, a tendency is observed towards the limitation of chemical pesticide use in agriculture. As a consequence, research effort is actively aimed at the development of biological products forming an alternative to chemical pesticides. As a general rule, these biological products are formed on the basis of natural strains of microorganisms having useful properties for application in agriculture. Families of biological products used as biopesticides currently presented on the market are described by researchers [1, 2].

One of the most promising directions in the development of biological protective agents consists in the creation of biofungicides based on microorganisms characterised by antagonistic activity against plant pathogens. Thus, many fungal cultures of the *Trichoderma* genus are distinguished by their effectiveness against diseases of legumes, grains and fruit crops caused by other fungi and bacteria, resulting in the expediency of developing biological products based on *Trichoderma* genus micromycetes [3–6].

Biological products created on the basis of *Trichoderma* genus fungi typically have a dry commodity form, for example: Sternifag SP and Gliokladin SP (Agrobiotechnology CJSC), RootShield (Green Methods, Cuba), GMax Tricon (Green Max AgroTech, India) and others. However, during the

preparation of a dry fungus product, a native solution is formed as a waste product with many secondary metabolites having antagonistic, cellulolytic and growth-promoting activity [7–10]. In this regard, a liquid commodity form of *Trichoderma* genus fungi is advisable in creation of biological products on their basis. At the same time, in order to create a stable biological product with a sufficient shelf life in this form, maximum conidium formation with simultaneous hydrolysis of the mycelium is necessary to be ensured during the cultivation [11]. Therefore, the present study was aimed at the development of the technology of submerged cultivation of the *Trichoderma* genus fungus for obtaining a biofungicide.

EXPERIMENTAL PART

The *Trichoderma asperellum* RNCIM F-1323 strain (Organic Park LLC, Republic of Tatarstan, Russia) with antagonistic activity against many phytopathogens represented the object of the study [12].

The cultivation was carried out in a pilot industrial bioreactor line with an inoculation bioreactor having a total volume of 10 L and a working bioreactor having a total volume of 100 L (BI Bio, China).

During the cultivation, the following parameters were controlled: temperature – $27 \pm 0,5$ °C; cul-

ture medium mixing speed – 600 rpm; aeration intensity – 1 L of air per 1 L of culture medium per min. Continuous measurement was carried out using Mettler Toledo 405-dpas-sc-k8s/120 potentiometric sensors and InPro 6820/12/120 polarographic sensors (Switzerland) without adjusting the pH of the medium or the concentration of dissolved oxygen (pO_2).

A modified Czapek nutrient medium was used with yeast extract and molasses replacing sucrose, g/L: molasses – 20; yeast extract – 7; $NaNO_3$ – 2; K_2HPO_4 – 1; $MgSO_4$ – 0.5; KCl – 0.5; $FeSO_4$ – 0.01 [13].

The inoculum was grown on a nutrient medium of a similar composition in rocking flasks with a total and working volume of 750 and 100 mL respectively. Inoculated flasks were incubated by an Innova 40R shaker incubator (New Brunswick, USA) at 200 rpm for 22-24 h until a dry biomass index of 6.5-7.5 g/L was reached.

Determination of absolutely dry biomass was carried out according to a known method [14] using an Ohaus Advanturer Pro AV 264 C analytical balances (China) and an Eppendorf 5804 R centrifuge (Germany).

Micromycete conidia were calculated according to the standard method presented in [15] using a Goryaev camera with 50-fold sample dilution.

RESULTS AND DISCUSSION

The amount of culture required for inoculation was determined at the initial stage of the work. Culture volume values of 1, 5 and 10 % of the nutrient medium volume in the bioreactor were selected. Moreover, cultivation was carried out only in inoculation bioreactors without subculturing to working bioreactors.

Taking into account that, throughout the entire cultivation process, the bioreactors maintain a constant temperature, the intensity of mixing and aeration of the medium, as well as the concentration of dissolved oxygen, can be considered as one of the con-

rol parameters of cultivation, since the value of this parameter decreases with increasing concentration of viable cells actively consuming oxygen and increases following the start of conidia formation and hydrolysis of mycelium due to a decrease in concentration of aerobic cell culture. Thus, it is possible to estimate the growth phase of the microorganism according to the obtained concentration plots for dissolved oxygen. In this case, a weak or absent change dynamics in the oxygen concentration immediately following inoculation is a possible means of indicating the culture being in a lag phase associated with growth retardation following subculturing in fresh nutrient medium [16]. A short lag phase duration is evidence of the activity of the culture and the presence of young cells in it.

The dynamics of changes in the acidity of the culture fluid appear to be no less important for the analysis of the cultivation process. In the lag phase of the cultivation process, no change in pH value is observed or else it changes very slightly due to the low activity of microorganisms; this is also true of the concentration of dissolved oxygen. Subsequently, a sharp acidification of the medium occurs due to the active consumption of, first of all, glucose metabolised in aerobic respiration to acetyl-CoA entering the Krebs cycle with the formation of organic acids [17]. Excess acid is removed from the cell into the culture fluid, resulting in the acidification of the latter. A further increase in pH may indicate the use of synthesised acids in terms of a carbon source due to the exhaustion of glucose in the nutrient medium, as well as the synthesis of secondary metabolites, including antibiotic substances.

Figures 1 and 2 depict the changes in the medium acidity and the concentration of dissolved oxygen during cultivation. It should be noted that a decrease in the inoculum volume from 10 to 5 % slightly increases the lag phase of the cultivation process. A further decrease in the volume of the inoculum to 1% of the nutrient medium volume leads to an increase in the duration of the lag phase by 6 hours as compared to 10 % of the inoculum.

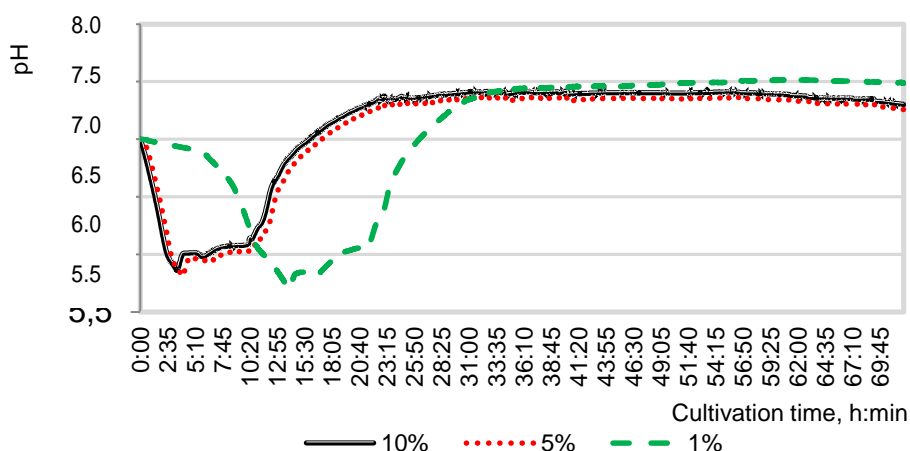


Fig. 1. pH change in the medium during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 1. Изменение pH среды в процессе культивирования *T. asperellum* ВКПМ F-1323

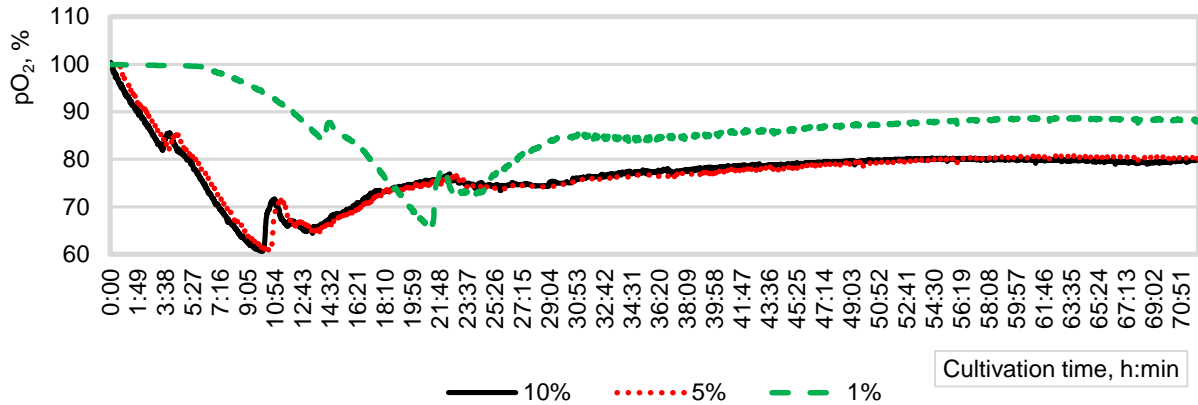


Fig. 2. Change in the concentration of dissolved oxygen pO_2 during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 2. Изменение концентрации растворенного кислорода pO_2 при культивировании *T. asperellum* ВКПМ F-1323

In Figure 3, the concentration of biomass during cultivation is shown. According to the data obtained, the difference in the values of absolutely dry biomass between the 10 and 5 % variants of the inoculum volume was within the statistical error throughout the entire measurement time. When using a 1 % inoculum volume, biomass accumulation occurred more slowly. Thus,

after 24 h of cultivation, the ADB value for 1 % of inoculum was on average by 27 % lower as compared to 10 % of inoculum. It is important to note that the total fermentation time directly depends on the rate of biomass accumulation, since a larger amount of biomass utilises the existing substrate faster; accordingly, the cultivation process is completed more quickly.

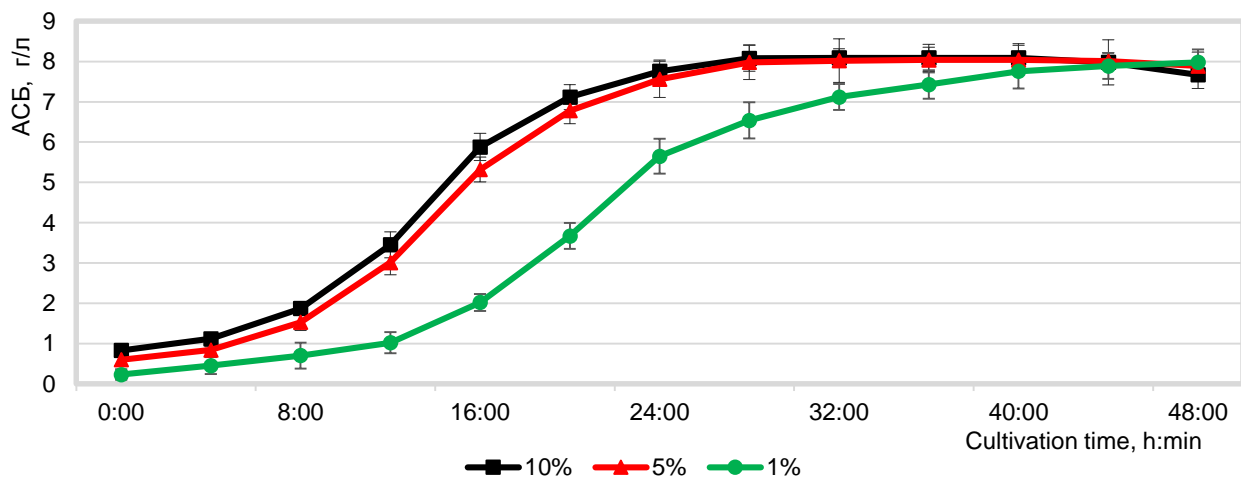


Fig. 3. Dependence of the absolutely dry biomass content on the inoculum volume during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 3. Зависимость содержания абсолютно сухой биомассы от объема инокулята в процессе культивирования *T. asperellum* ВКПМ F-1323

Thus, the accumulation of biomass by the *T. asperellum* micromycete in the inoculation bioreactor was established to occur faster in the case of an inoculum volume of 5–10 %, with no significant difference being observed between the amounts of 5 and 10 %. A decrease in the volume of the inoculum to 1 % negatively affects the rate of biomass accumulation. Therefore, from an economic point of view, a 5 % inoculum value is optimal.

At the next stage, the time optimal for the transfer of the culture from the inoculation to the working bioreactor was determined. The volume of the inoculum was set to 5 % of the nutrient medium volume in the working bioreactor. For the purposes of the study, cultivation times of 16, 20, 24 and 28 h were selected for the inoculation bioreactor.

Figures 4 and 5 depict changes in the medium acidity and the concentration of dissolved oxygen during the cultivation process.

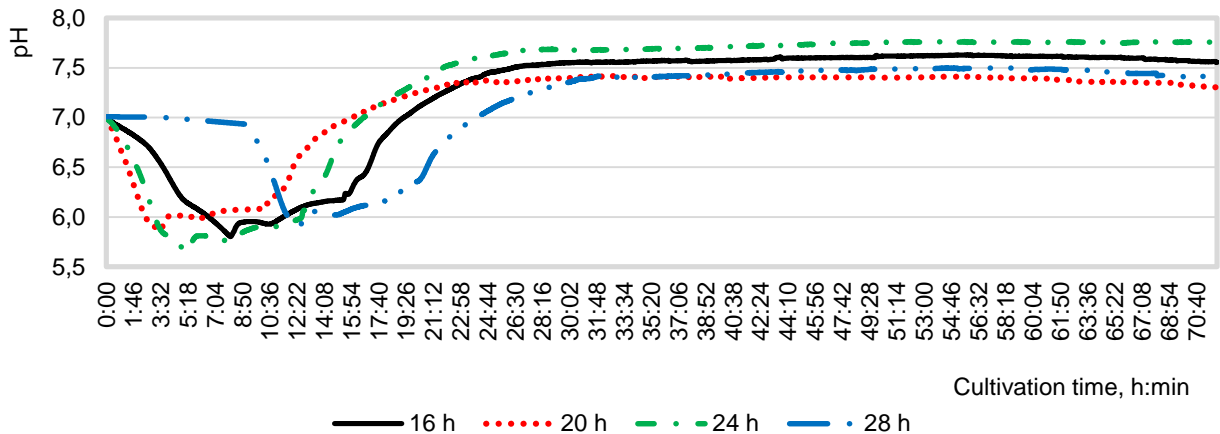


Fig. 4. pH change in the medium during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 4. Изменение pH среды при культивировании *T. asperellum* ВКПМ F-1323

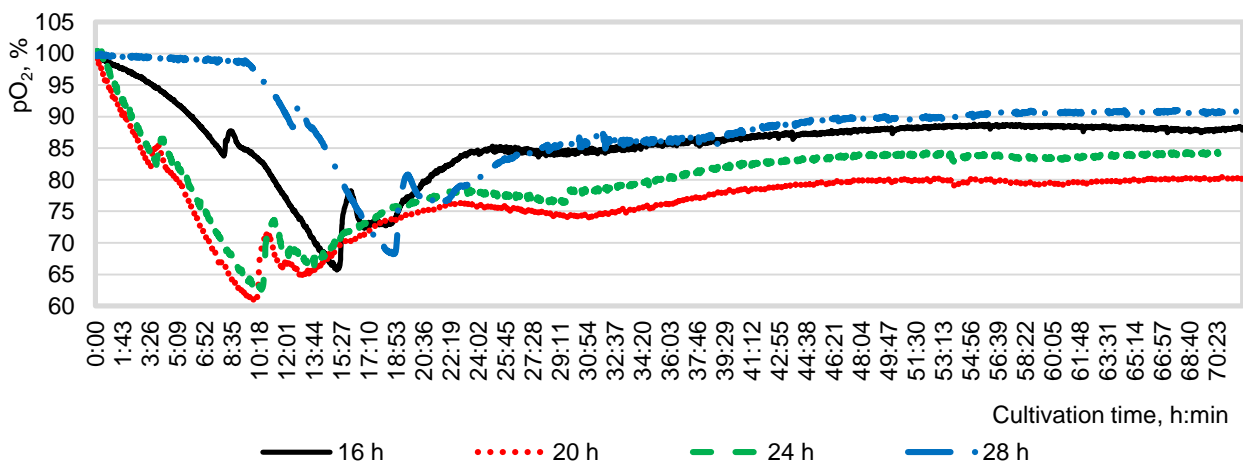


Fig. 5. Change in the concentration of dissolved oxygen during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 5. Изменение концентрации растворенного кислорода при культивировании *T. asperellum* ВКПМ F-1323

As can be seen from the data presented in Fig. 4 and 5, the optimal time range for subculturing is 20–24 h, while an increase in cultivation time in the inoculation bioreactor has a greater negative effect on the duration of the lag phase than its decrease in the working bioreactor. In the case of a short cultivation time (16 h) in the inoculation bioreactor, the lag phase duration in the working bioreactor can be assumed to increase due to insufficient biomass. At the same time, with an increase in cultivation time in the inoculation bioreactor to 28 h, the conidiation process starts to result in additional time required for the culture transition to the active growth phase following transfer to the working bioreactor.

In Figure 6, the change in the concentration of absolutely dry biomass during cultivation in the working bioreactor is provided.

As can be seen from the plots presented in Fig. 6,

when the inoculum is incubated before subculturing for 20–24 h, the most rapid biomass accumulation proceeds in the working bioreactor. Moreover, under these inoculum subculturing conditions, a steady decrease in the biomass concentration is observed after 48 h of cultivation; this is probably due to hydrolysis of the mycelium. In the case of inoculum incubation for 16 h, biomass accumulation was slower compared to the incubation time of 20–24 h. The slowest accumulation of biomass to its maximum values, starting only from the 48th day, was observed when the inoculum was incubated for 28 hours prior to subculturing.

The obtained data are comparable with changes in the acidity of the medium and the concentration of dissolved oxygen (see Fig. 4 and 5), as well as with the nature of the change in the concentration of conidia during cultivation (Fig. 7). The number of conidia was counted using a Goryaev camera.

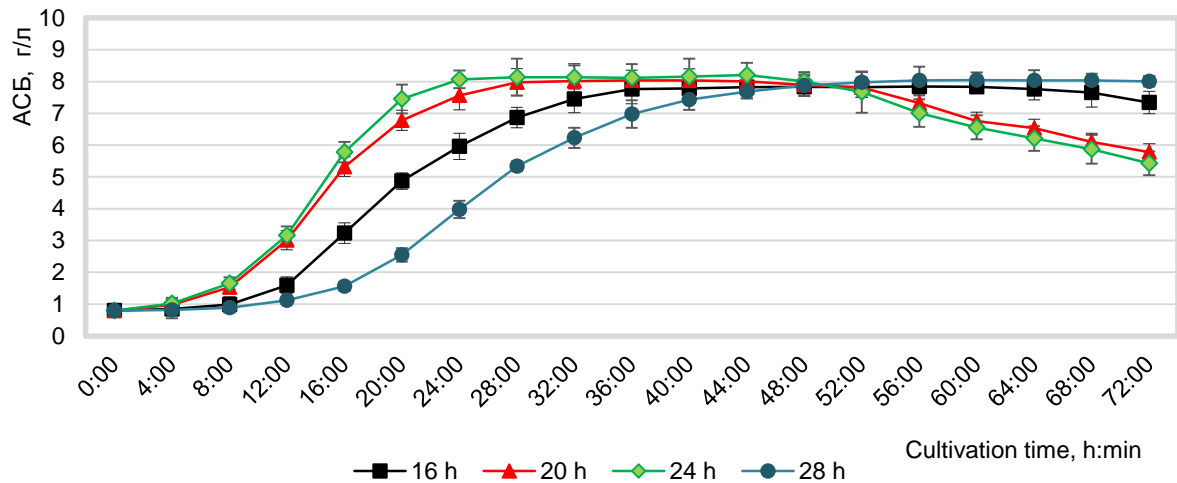


Fig. 6. Absolutely dry biomass concentration during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 6. Концентрация абсолютно сухой биомассы в процессе культивирования *T. asperellum* ВКПМ F-1323

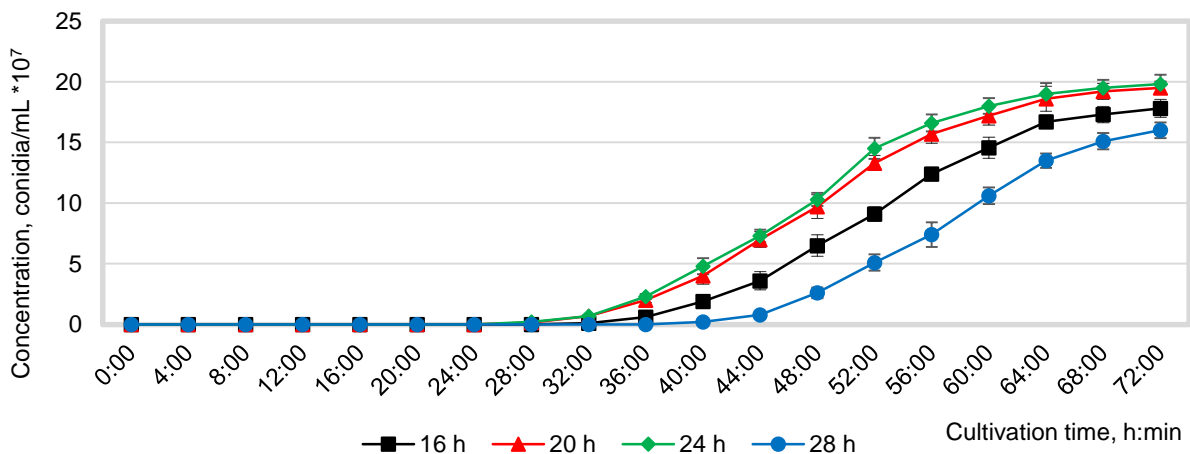


Fig. 7. Conidia concentration during the cultivation of *T. asperellum* RNCIM F-1323

Рис. 7. Изменение концентрации конидий в процессе культивирования *T. asperellum* ВКПМ F-1323

From the moment of inoculating the working bioreactor to the start of the conidium formation process, the shortest time of 32 hours passed for cultivation times in the inoculation bioreactor of 20 and 24 hours with the difference between these two options was within the statistical error during the entire cultivation process. When the cultivation time in the inoculator is reduced to 16 hours, the conidiation process begins 4 hours later. Following 72 hours of cultivation in the working bioreactor, the concentration of conidia is lower by an average of 10 % compared with the 24-h cultivation of the inoculum. In the case of inoculum cultivation for 28 hours, the conidiation process begins later. In addition, at the end of the experiment, the concentration of conidia was 19.2 % lower compared to the best option.

CONCLUSION

According to the results of the experimental study of submerged cultivation of *Trichoderma asperellum* RNCIM F-1323 under experimental industrial conditions, the following recommendations can be proposed:

- the amount of inoculum should be 5 % of the nutrient medium volume in the inoculation bioreactor;
- the cultivation time for *Trichoderma asperellum* RNCIM F-1323 in the inoculation bioreactor should be between 20 and 24 hours;
- the optimal cultivation time for *Trichoderma asperellum* RNCIM F-1323 in the working bioreactor is 72 hours with a $1.9 \cdot 10^8$ conidia/mL conidia concentration or 1.9 times higher than the declared titer of the preparation.

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Contribution

Danis D. Ziganshin, Anna A. Egorshina, Mikhail A. Luk'yantsev, Alexander S. Sirotkin carried out the experimental work, analyzed the experimental results and prepared the text of the manuscript. Danis D. Ziganshin, Anna A. Egorshina, Mikhail A. Luk'yantsev, Alexander S. Sirotkin have equal author's rights and bear equal responsibility for plagiarism.

Conflict interests

The authors declare no conflict of interests regarding the publication of this article.

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