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## Phytate hydrolysing activity of the *Aspergillus niger* L-4 micromycete strain

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**Abstract:** The aim of the study was to study the phytase synthesis capability of *Aspergillus niger* L-4 strain. The method for determining phytase activity is based on establishing the content of inorganic phosphates as a result of the action of phytase on the substrate under certain standard conditions by binding them with a vanadium-molybdenum reagent to form a coloured complex. The use of phytases for the hydrolysis of phytates in animal feed is important from the point of view of preserving the environment: when phytate complexes are destroyed, phosphorus is released, which performs an important structural and regulatory function, ensuring the normal development of bone and dental tissues and supporting their safety and integrity. Phosphoric acid is involved in the synthesis of kinases responsible for the normal course of chemical reactions in cells, in fat metabolism, as well as in the synthesis and breakdown of starch and glycogen. This reduces the release of undigested phosphorus into the environment. The object of the study consisted of native solutions obtained by culturing an industrial strain of acid-forming *A. niger* L-4 on various carbohydrate-containing media. The *A. niger* L-4 strain, previously selected at the All-Russian Scientific Research Institute of Food Additives for fermentation of molasses, has the ability to synthesise extracellular phytase. This paper presents the results of studies of phytase activity during the cultivation of *A. niger* L-4 on carbohydrate-containing media. It was found that in order components of the sucrose-mineral medium provide an elevated level of low-molecular-weight sugars necessary for increasing the productivity of phytase biosynthesis. Phytase activity in the native solution was shown to increase over 72 hours of fermentation to reach a value of  $25.8 \pm 0.1$  units/cm<sup>3</sup>. The phytase activity was 1.5 times higher than the fermentation process of a corn starch hydrolysate with a dextrose equivalent DE =  $21 \pm 1$  %, ensuring the productive biosynthesis of citric acid.

**Keywords:** phytase, *A. niger* L-4, corn starch, sucrose

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## Фитатгидролизирующая активность штамма *Aspergillus niger* Л-4

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**Резюме:** Целью исследования являлось изучение способности штамма *Aspergillus niger* Л-4 синтезировать фитазу. Метод определения фитазной активности основан на определении содержания неорганических фосфатов в результате воздействия фитазы на субстрат при определенных

стандартных условиях путем связывания их ванадий-молибденовым реагентом с образованием окрашенного комплекса. Использование фитаз для гидролиза фитатов в кормах для животных важно с точки зрения сохранения окружающей среды: при разрушении фитатных комплексов высвобождается важный элемент – фосфор, который выполняет структурную и регуляторную функцию, обеспечивает нормальное развитие костных и зубных тканей, поддерживает их прочность и целостность. Фосфорная кислота участвует в синтезе киназ, ответственных за нормальное течение химических реакций в клетках, метаболизм жиров, а также синтез и расщепление крахмала и гликогена. Это уменьшает выброс неусвоенного фосфора в окружающую среду. Объектом исследования являлись нативные растворы, полученные при культивировании промышленного штамма-кислотообразователя *A. niger* Л-4 на различных углеводсодержащих средах. Штамм *A. niger* Л-4, ранее селекционированный во Всероссийском научно-исследовательском институте пищевых добавок для ферментации мелассы, обладает способностью синтезировать внеклеточную фитазу. В данной работе представлены результаты исследований фитазной активности при культивировании штамма *A. niger* Л-4 на углеводсодержащих средах. Установлено, что для повышения продуктивности биосинтеза фитазы необходимо более высокое содержание низкомолекулярных сахаров. Установлено, что компоненты сахарозоминаральной среды обеспечивают повышенный уровень фитазной активности ферментов синтезируемых микромицетом *A. niger* Л-4. Выявлено, что фитазная активность в нативном растворе увеличивается в течение 72 ч ферментации и достигает значения  $25,8 \pm 0,1$  ед/см<sup>3</sup>. По сравнению с процессом ферментации гидролизата кукурузного крахмала с декстрозным эквивалентом  $DE = 20,9 \pm 0,5$  %, который обеспечивал продуктивный биосинтез основного метаболита – лимонной кислоты, фитазная активность была в 1,5 раза выше.

**Ключевые слова:** фитаза, *Aspergillus niger* Л-4, гидролизат кукурузного крахмала, сахароза

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## INTRODUCTION

Micromycete *A. niger* synthesises enzymes (amylases, phytases, invertases, cellulases, etc.) and organic acids (citric, gluconic, itaconic, and kojic acids). By isolating acids and enzymes in the environment, aspergillus breaks down polymers into simple molecules that are absorbed back into micromycete cells [1–3].

A significant quantity (more than 80 %) of phytic acid salts (phytates), which bind divalent metal cations, were found in the soil, along with peptides and low molecular weight metabolites associated with the formation of inaccessible and difficult to assimilate compounds [4–5].

Phytates are mainly localised in the seeds and bran of crops. When phytate complexes are broken down, phosphorus is released. This element performs a vital structural and regulatory function, ensuring the normal development of bone and dental tissues, as well as helping maintain their safety and integrity throughout life [6–8]. Phosphoric acid is involved in the synthesis of kinases responsible for the normal course of chemical reactions in cells, fat metabolism, as well as the synthesis and breakdown of starch and glycogen [9–11]. In the absence of phosphoric acid, fermentation and respiration – both of which are vital for all living things – are inhibited [12, 13].

For the effective hydrolysis of phytates, phytases are used to catalyse the cleavage of

phytate complexes to residues of myo-inositol and phosphoric acid, the latter representing a convenient source of phosphorus. The use of phytases is important for increasing the availability of phosphorus from phytates in compound feeds for farm animals [14, 15]. This is also important from the point of view of preserving the environment, since it allows the release of undigested phosphorus into the environment to be reduced.

The importance of phosphorus and other elements associated with phytates poses a challenge for biotechnologists to find new sources of phytate-hydrolysing enzymes to increase the biological value of plant materials.

The aim of the study was to investigate the ability of *Aspergillus niger* L-4 to hydrolyse extracellular phytase, as well as to inform the selection of optimal cultivation conditions for *Aspergillus niger* micromycete for highly productive phytase biosynthesis.

## EXPERIMENTAL PART

The object of the study are native solutions obtained by culturing *A. niger* L-4 micromycetic acid-forming agent on various carbohydrate-containing media. The strain of micromycete, a producer of citric acid, was previously selected at the All-Russian Scientific Research Institute of Food Additives for the fermentation of molasses. For the study, crystalline sugar (STATE STAND-

ARD 33222-2015) and a corn starch hydrolysate with DE = 21 ± 1% (STATE STANDARD 32159-2013) were used as a carbohydrate source. The source of nitrogen was ammonium nitrate (STATE STANDARD 22867-77), the source of phosphorus was potassium phosphate monosubstituted (STATE STANDARD 4198-75).

The composition of the fermentation medium, g/dm<sup>3</sup>: carbohydrate substrate (conversion to glucose) – 150; ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>) – 2.5; magnesium sulphate seven-water (MgSO<sub>4</sub>·7H<sub>2</sub>O) – 0.25; potassium phosphate monosubstituted (KH<sub>2</sub>PO<sub>4</sub>) – 0.16; pH 6.5 [16].

The process was carried out at a temperature of:

– at the stage of inoculum mycelium production – (36±1) °C for sugar-mineral medium, (32±1) °C – for hydrolysis of starch;

– at the fermentation stage – from (29±1) °C to (34±1) °C for the sugar-mineral medium and starch hydrolysate.

The duration of the process was 120 hours.

The ages of inoculum mycelium were 24, 36, and 48 hours. The amount of inoculum mycelium to the volume of the initial nutrient medium was 15 %.

Phytase activity (PhA) was evaluated by the

colorimetric method according to GOST (RF state standard) 31487-2012. The acid content was determined by titration. The protein content was determined according to the Lowry method. The biomass content with a residual moisture content of 10 % was determined by drying at a temperature of 105±5 °C for 24 hours.

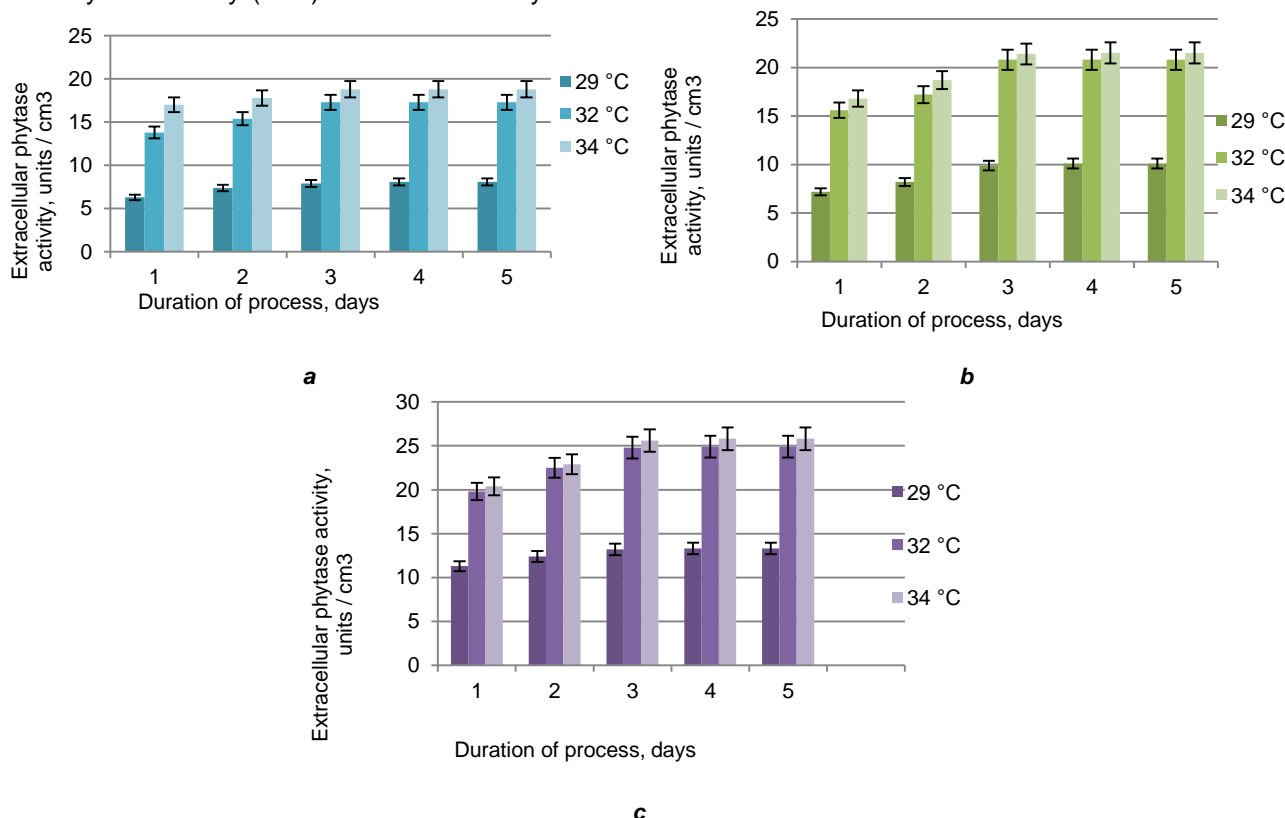
The experimental data were processed using mathematical statistics methods and Excel XP programs.

## RESULTS

Productive biosynthesis of target metabolites is affected by conditions such as age and amount of seed mycelium, fermentation temperature.

Previous studies have shown that the productive biosynthesis of amylolytic enzymes by *A. niger* micromycetic acid-forming strains is achieved using seed mycelium in an amount of 15 % of the volume of the nutrient medium having ages of 24–36 h at a fermentation temperature of 32±1 °C regardless of the carbohydrate source [17, 18].

Figure 1 show the dynamics of changes in extracellular PhA for inoculum mycelium at ages of 24 hours, 36 hours and 48 hours.



**Fig. 1. Dependence of extracellular phytase activity during the cultivation of the strain *A. niger* L-4 on sucrose-mineral medium.**

**Seed mycelium: a – 48 hours; b – 36 hours; c – 48 hours**

**Рис. 1. Зависимость экстрацеллюлярной фитазной активности от времени культивирования штамма *A. niger* Л-4 на сахарозоминаральной среде.**

**Возраст посевного мицелия, ч: а – 24; б – 36; с – 48**

Increased extracellular phytase activity detected at temperatures 32 °C and 34 °C. Regardless of the age of the seed mycelium at a temperature of 29 °C, the extracellular phytase activity was low. The level of PhA for seed mycelium at the age of 24 and 36 hours at a given temperature differed from the level of PhA at temperatures of 32 °C and 34 °C by more than 1.5 times. Phytase activity was observed to increase within 3 days, followed by a slowdown in activity growth. This

phenomenon was noticeable for both 24-hour and 36- and 48-hour seed mycelium at all studied fermentation temperatures of the sugar-mineral medium. It is possible that phytase biosynthesis is inhibited due to an increase in the accumulation of acid in the culture fluid by the end of the process.

Table 1 shows the experimental data obtained at the final stage of the fermentation process (120 h).

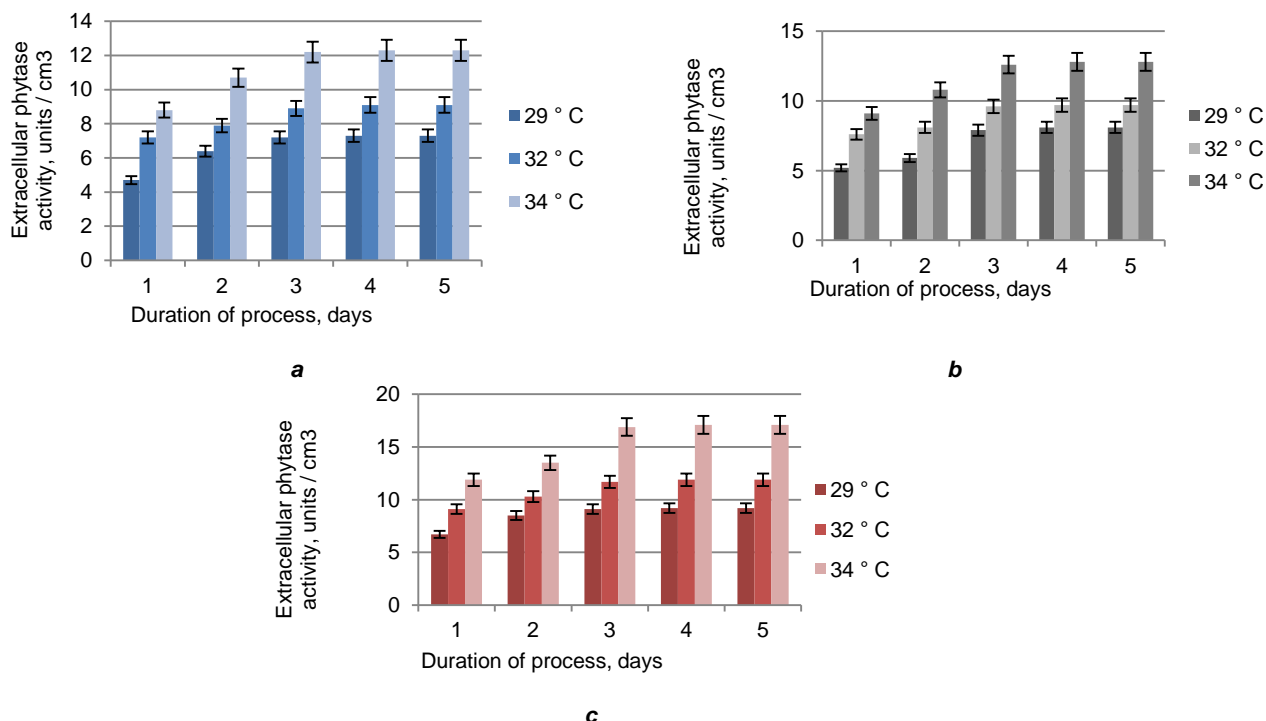
**Table 1**

**Results of fermentation of the sucrose-mineral medium  
(native solution, end of the process 120 h)**

**Таблица 1**

**Результаты по ферментации сахарозо-минеральной среды  
(нативный раствор, конец процесса, 120 ч)**

Cultivation conditions		Main characteristics			
The age of seed mycelium, h	Temperature, °C	Protein content, mg/cm <sup>3</sup>	The amount of synthesised acid, g	Phytase activity	
				u/cm <sup>3</sup>	u/mg protein
24	29	3.8±0.1	5.8±0.1	8.1±0.1	2.1±0.1
	32	3.5±0.1	6.1±0.1	17.3±0.1	4.9±0.1
	34	3.3±0.2	5.3±0.1	18.8±0.1	5.7±0.2
36	29	2.1±0.1	5.9±0.1	10.1±0.1	4.8±0.1
	32	2.2±0.1	6.3±0.1	20.8±0.1	9.5±0.1
	34	2.1±0.1	5.4±0.1	21.5±0.1	10.2±0.2
48	29	2.0±0.1	6.1±0.1	13.3±0.1	6.7±0.2
	32	1.8±0.2	6.5±0.1	24.9±0.1	13.8±0.2
	34	1.1±0.1	5.6±0.1	25.8±0.1	23.4±0.1



**Fig. 2. Dynamics of changes in extracellular phytase activity during cultivation of the *A. niger* L-4 strain on a starch hydrolysate with DE=21±1%. The age of the seed mycelium: a – 24 hours; b – 36 hours; c – 48 hours**

**Рис. 2. Динамика изменения внеклеточной фитазной активности при культивировании продуцента лимонной кислоты – штамма *A. niger* Л-4 на гидролизате крахмала с DE = 21±1%. Возраст посевного мицелия, ч: а – 24; б – 36; с – 48**

Table 2

Fermentation results of corn starch hydrolysate with DE = 21±1%  
(native solution, end of process, 120 h)

Таблица 2

Результаты по ферментации гидролизата кукурузного крахмала с ДЕ = 21±1 %  
(нативный раствор, конец процесса, 120 ч)

Name of substrate	Enzymatic activity, u/cm <sup>3</sup>	pH	Amount of synthesised acid, g	Protein content, mg / cm <sup>3</sup>
Starch Hydrolysate	17.3±0.2	1.6	6.8±0.1	0.8±0.1
Crystalline sugar (sucrose mineral medium)	25.8±0.1	1.9	5.6±0.1	1.1±0.1

The research results showed that the maximum level of extracellular PhA is achieved using a 48-hour seed mycelium at a process temperature of 34 °C. The specific PhA (23.4±0.1 u/mg protein) exceeded that for the process proceeding at a temperature of 32 °C by 1.5–1.7 times.

Figure 2 show the dynamics of changes in PhA during cultivation of *A. niger* L-4 micromycete on a corn starch hydrolysate with DE=20.9±0.5 %.

The results of the studies showed that the phytase activity taking place during the fermentation of starch hydrolysate with DE=20.9±0.5 %, as well as during fermentation of the sugar-mineral medium, increased throughout the process, becoming most prominent at a process temperature of 34 °C with the use of seed mycelium having an age of 48 hours

The protein concentration at the end of the fermentation process of both the sucrose mineral medium and the starch hydrolysate decreased, which may be due to the synthesis of proteinases, as was revealed in other works [19]. The protein content during the fermentation of starch hydrolysate and of the sucrose-mineral medium was at the same level (Table 2).

A comparative analysis of the data showed that, in order to increase the productivity of phytase biosynthesis, a higher content of low molecular weight sugars is necessary. The studied starch hydrolysate contains glucose in an amount of 3±1 %, maltose 20±1 % and dextrins 77±1 % in

the total amount of carbohydrates. When cultivating an *Aspergillus* strain, maltose and dextrans are hydrolysed by their own amylolytic enzymes into glucose, which is involved in the process of acid formation. Sucrose-mineral medium contains only sucrose, which in the process of fermentation, under the influence of its own enzymes, hydrolyses to glucose and fructose. It is of interest to study the process of phytase biosynthesis during the fermentation of a corn starch hydrolysate with a deeper degree of hydrolysis.

Taking a comparative approach, the authors of [20] established the following parameters for productive phytase biosynthesis by means of *A. niger* micromycete-acid former: carbon source – sucrose (1.0 %); nitrogen source – ammonium nitrate (0.5 %); temperature – 30 °C; pH = 5.5.

## CONCLUSION

The *Aspergillus niger* L-4 micromycete strain shows a high extracellular phytate hydrolysis activity. The above results allow us to conclude that the sucrose-mineral medium is an advantageous medium for productive phytase biosynthesis using *A. niger* L-4. To increase phytase activity, it is necessary to conduct studies to optimise cultivation parameters: concentration of carbohydrate and nitrogen-containing sources, macro- and microelements, pH and temperature, oxygen concentration, etc.

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### **Contribution**

Nargul M. Musta Ogly, Natalya Yu. Sharova carried out the experimental work. The authors on the basis of the results summarized the material and wrote the manuscript. All authors 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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