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Impact of cultivation conditions on xylanase production and growth in *Paenibacillus mucilaginosus*

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Abstract: *Xylanase is an enzyme that hydrolyses β-1,4 bonds in plant xylan. This enzyme is applied in the bioconversion of agro-industrial waste for xylooligosaccharide hydrolysate production to improve digestibility and nutrition value of animal feed, food processing, the utilisation and faster decomposition of crop debris in soil, as well as in cellulose bleaching and other industries. The current trend focuses on using renewable resources, such as agricultural waste, as substitutes for expensive purified xylan in producer screening and xylanase synthesis. This work aimed to determine the impact of Paenibacillus mucilaginosus cultivation conditions on the xylanase production yield. Rice bran ferment lysate along with birch and beech timber xylans were used as a carbon source. Temperature, medium pH, pH correction factors, inoculant incubation time, carbon and nitrogen sources and concentrations were the studied criteria of xylanase biosynthesis and growth in bacteria P. ucilaginosus strain 560. We show that the xylanase biosynthesis and cultivation in P. mucilaginosus strain 560 are more practical and cost-effective with the use of a rice bran ferment lysate-based nutrient medium. Inductors contained in the rice bran ferment lysate improve the xylanase biosynthesis. Calcium ions also facilitate biosynthesis in the studied strain. Cultivation recommendations are: carbon source concentration in medium 0.5% of total reducing substances content; 0.2% carbamide as optimal nitrogen source; calcium hydroxide as an agent for medium pH correction to 6.0±0.2; cultivation temperature 30±1 °C. Under the specified conditions, cultivation of P. mucilaginosus does not require inoculate preprocessing, and a maximal xylanase activity in stationary culture reaches 20 U/mL.*

Keywords*: rice bran, birch, beech, xylan, Paenibacillus mucilaginosus, culturing, xylanase*

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Влияние условий культивирования на продуцирование ксиланазы и рост бактерий *Paenibacillus mucilaginosus*

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Резюме: *Ксиланаза – фермент, гидролизующий β-1,4-связи в ксиланах. Этот фермент используется для получения из отходов агропромышленного комплекса гидролизата ксилоолигосахаридов с целью улучшения энергетической ценности и повышения усвояемости кормов для животных, обработки пищевых продуктов, утилизации и ускорения разложения поживных остатков в почве, а также в технологии отбелки целлюлозы и других областях. В настоящее время представляет интерес использование возобновляемых ресурсов, в частности, отходов сельскохозяйственного производства, в качестве субстрата вместо дорогого очищенного ксилана для скрининга продуцентов и выделения ксиланаз. Целью настоящей работы являлось определение влияния условий*

культивирования бактерий Paenibacillus mucilaginosus на продуцирование ксиланаз. В качестве источника углерода использовали ферментолизат рисовой шелухи, ксилан, выделенный из березы и бука. Изучено влияние температуры, pH среды, факторов корректировки pH среды, продолжительности инкубации инокулята, источ-ников углерода и азота, и также их концентраций на биосинтез ксиланаз и рост штамма 560 P. mucilaginosus. Установлено, что для биосинтеза кисланазы культивирование штамма 560 P. mucilaginosus перспективно и экономически целесообразно проводить на питательной среде, приготовленной на основе ферментолизата рисовой шелухи. Присутствующие в составе ферментолизата рисовой шелухи индукторы улучшают биосинтез ксиланаз. Показано положительное влияние ионов кальция на биосинтез ксиланаз у рассматриваемого штамма. Рекомендуемые условия культивирования: концентрация источника углерода в питательной среде по общему количеству РВ – 0,5%; в качестве источника азота целесообразно использовать 0,2% карбамид; при корректировке pH среды до 6,0±0,2 необходим гидроксид кальция; температура культивирования бактерий – 30±1 °С. В указанных условиях культивирования P. mucilaginosus не требуется предварительного приготовления посевного материала, а максимальная активность синтезируемой ксиланазы в стационарной фазе роста бактерий достигает значения 20 ед./мл.

Ключевые слова: *рисовая шелуха, береза, бук, ксилан, Paenibacillus mucilaginosus, культивирование, ксиланаза*

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INTRODUCTION

The second most abundant renewable natural polysaccharide after cellulose, xylan is a major hemicellulose of grain and wood [1]. This complex polysaccharide consists of β-1,4-xylopyranosyl residues chained through β-1,4-glycosidic bonds [2]. Side chains are linked to the backbone with β-(1→3)-glycosidic bonds. Some hydroxyl groups in xylose residues can be acetylated and attached with 4-O-methyl-D-glucuronic acid and L-arabinofuranose [2, 3]. The xylan structure varies depending on the plant taxon and extraction method [4].

Xylan is abundant in hardwood (15–30%) and conifer timber (7–10%). A high xylan content (about 30%) is found in straw, stems and other parts of annual plants and grasses (cereals, including sorghum, sugar cane cake, stems, cobs and husk of corn) [5]. Hardwood xylan is O-acetyl-4-O-methylglucuronoxylan. Conifer timber contains arabino-4-O-methylglucuronoxylan, which is distinguished from hardwood xylan by the absence of acetyl groups and presence of arabinofuranose branches. Grasses and annual plants usually possess arabinoxylans [6, 7]. Linear unsubstituted xylan was also found in esparto grass [8], tobacco [9] and some sea algae [10] and contains xylopyranosyl residues linked by 1,3-β- and 1,4-β-bonds [10, 11].

Xylans are the main antinutrient components of plant material that hamper nutrient absorption in the gastrointestinal tract of monogastric animals. Xylan is hydrolysed by xylanases (1,4-β-D-xylanases, EC 3.2.1.8) used for xylooligosaccharide (XOS) hydrolysate production from agricultural waste to improve digestibility and nutritive value of animal feed, in food processing, in the utilisation and effective decomposition of crop debris in soil, as well as in cellulose bleaching and other industries [12]. Xylanases are the main endoenzymes hydrolysing β-1,4-bonds in xylan, the major hemicellulose polymer [13]. The current trend focuses on the use of renewable resources, such as agricultural waste, as substitutes for expensive purified xylan in producer screening and xylanase synthesis [14]. Fungi are known promising producers of xylanases. However, today's biotechnology opts for bacteria as primary xylanase producers, which are distinguished from mycelial fungi by a higher growth rate and effective production and absorption of carbon from various types of plant matter. Bacteria can also produce xylanase in large volumes and at moderate enzyme purification costs[15].

Paenibacillus bacteria are able to hydrolyse various carbohydrates and produce numerous extracellular enzymes, including xylanase. This xylan-degrading genus includes the species of *P. favisporus*, *P. phyllosphaerae*, *P. barcinonensis* and *P. panacisoli* [16–19]. Besides the benefits of their enzymatic action, the biomass and other products of *Paenibacillus* metabolism can supplement feed additives to replenish the diet of farm animals and birds with biologically active substances [20]. Sustainable growth in animal and poultry farming demands for the development of high-quality compound feeds containing enzyme additives. The Russian market is dominated by import fodder enzyme preparations, whereas their domestic production is very limited. The search for new efficient xylanase producer strains in the animal feed industry and the development of scalable fermentation technologies

for the substitution of imported fermented fodder preparations is a pressing issue.

The present work aimed to determine the impact of *Paenibacillus mucilaginosus* cultivation conditions on the xylanase production yield.

EXPERIMENTAL

Xylanase producer. Strain 560 of bacterium *P. mucilaginosus* was provided by the Russian Collection of Agricultural Microorganisms (RCAM, All-Russian Research Institute for Agricultural Microbiology, St. Petersburg).

Nutrient growth media. Submerged cultivation of *P. mucilaginosus* was carried out on the Alexandrov's nutrient medium modified as follows, %: NaCl – 0.02, K_2HPO_4 – 0.2, $MgSO_4.7H_2O$ – 0.05, $CaCO_3$ – 0.01, $(NH_4)_2SO_4$ – 0.1, yeast extract – 0.1 [21].

Rice bran fibre ferment hydrolysate containing 0.5% of the total reducing substances (RS) content was used as a carbon source. The bran was pretreated with 2.5% sodium hydroxide, with a solid-surface to sodium hydroxide saline ratio of 1:8, treatment temperature was 120±1 °C and treatment time 20 min. The fibre was then rinsed with tap water and exposed for 24 h to the Accellerase 1500 enzyme complex containing exoglucanase, endoglucanase, hemicellulase and beta-glucosidase at 55±1 °C, pH 5.5±0.3. This ferment lysate contained RS available for bacterial growth. Silicon-containing lye segregated from fibre was used as a medium pH correction factor.

Birchwood xylan was also used as a carbon source [22]. Xylan was extracted from birchwood chips (*Betula pendula*) through oxygen-free steaming at 150–155 °C and 0.60–0.65 MPa excess pressure. Xylan was precipitated from the resulting water extract by intensive vortexing in an ethanol/water solution (85:15) with overnight exposure for complete coagulation. After decantation, xylan precipitate was vacuum sieved with a filter paper (Black ribbon). Eluted xylan was vacuum dried at 40 °C for 48 h.

Beechwood xylan (Cath Roth) was used as a carbon source in comparison assays.

Nutrient medium was autoclaved at 120 °C and 1 atm. Sterile medium was corrected to neutral pH with calcium hydroxide. Cultivation was carried out with 250 mL Erlenmeyer flasks in 100 mL medium stirred continuously at 200 rpm on an ES-20 incubator shaker for 3 days at 30 °C. The flasks were inoculated at 5% relative to medium volume.

Experiments were designed as OFAT (One-Factor-At-a-Time). The OFAT approach was used to study the influence of cultivation conditions (substrate concentration, medium pH, temperature, inoculant incubation time, nitrogen source, nitrogen source concentration, carbon source) on the growth and metabolic yield in *P. mucilaginosus* bacteria. The method varies one tested factor per trial, while leaving the others constant [23]. The OFAT experimental design is detailed in Table 1.

Table 1. Experimental design
Таблица 1. Планирование эксперимента

Experiment number	Substrate concentration, %	Temperature, °C	pH corrector	pH	Carbon source	Inoculate Incubation time, h	Nitrogen source and concentration
1	0.25; 0.5; 0.75; 1	30	Calcium hydroxide	7	Rice bran ferment lysate	24	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
2	Opt. value as in Trial 1	25, 30, 35	Calcium hydroxide	7	Rice bran ferment lysate	24	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
3	Opt. value as in Trial 1	Opt. value as in Trial 2	Calcium hydroxide, sodium hydroxide, Si-containing lye	7	Rice bran ferment lysate	24	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
4	Opt. value as in Trial 1	Opt. value as in Trial 2	Opt. value as in Trial 3	6, 7, 8, 9	Rice bran ferment lysate	24	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
5	Opt. value as in Trial 1	Opt. value as in Trial 2	Opt. value as in Trial 3	Opt. value as in Trial 4	Rice bran ferment lysate, birch xylan, beech xylan	24	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
6	Opt. value as in Trial 1	Opt. value as in Trial 2	Opt. value as in Trial 3	Opt. value as in Trial 4	Opt. value as in Trial 5	0, 12, 24, 36, 48	0.1% $(NH_4)_2SO_4$ + 0.1% yeast extract
7	Opt. value as in Trial 1	Opt. value as in Trial 2	Opt. value as in Trial 3	Opt. value as in Trial 4	Opt. value as in Trial 5	Opt. value as in Trial 6	Total concentration 0.2%
8	Opt. value as in Trial 1	Opt. value as in Trial 2	Opt. value as in Trial 3	Opt. value as in Trial 4	Opt. value as in Trial 5	Opt. value as in Trial 6	0; 0.02; 0.1; 0.2; 0.3; 0.4% of opt. nitrogen source in Trial 7

Trial 1 assessed the xylanase activity and growth parameters of *P. mucilaginosus* strain 560 under rice bran ferment lysate concentrations varying from 0.25 to 1% total RS. Temperature, pH, inoculate incubation time, nitrogen sources and consumption rate were set constant. The RS concentration in ferment lysate corresponding to the maximal xylanase activity and optimal growth parameters in Trial 1 was fixed downstream in Trial 2, which tested the effect of temperature on bacterial growth and xylanase yield under other fixed parameters (pH, incubation time, nitrogen source and consumption rate). Trials 3–8 are designed likewise by varying one cultivation parameter at a time.

Assessment of growth parameters. Specific growth rate, bacterial biomass generation time and total yield were estimated as recommended in [24]. Biomass was pelleted by centrifugation with a 5418 R Eppendorf microcentrifuge at 12,000 rpm for 10 min. Biomass yield was determined thermogravimetrically with an MX-50 moisture analyser.

Assessment of xylanase activity. The xylanase activity and residual RS content of undegraded carbohydrates in supernatant after a 12, 24, 48 and 72-h submerged cultivation of *P. mucilaginosus* were measured by adding concentrated sulfuric acid in the ratio 1:1. Enzymatic activity was measured as in [25], with certain modifications. Xylanase activity was assessed with a 1% beechwood xylan substrate (1 g of xylan per 100 mL of acetate buffer, pH 6). Enzymatic activity was measured relative to the RS value [26]. The measurement procedure was as follows: 0.12 mL of supernatant with 1.2 mL of substrate were incubated for 1 h at 50 °C followed by the addition of 0.6 mL of 3,5-dinitrosalicylic acid (DNS reagent). In the control, 1.2 mL of substrate was mixed with 0.6 mL of DNS reagent and 0.12 mL of supernatant. Tubes with the substrate, culture medium and DNS reagent were boiled in a water bath for 10 min, cooled down and 6 mL of distilled water was added before optical density measurement at 540 nm. One xylanase activity unit was defined as the amount of enzyme needed to hydrolyse 1 g of substrate (30% of reaction total) to monosugars in 1 h under assumed pH and temperature.

Trials were in the form of biological and analytical assays performed in triplicate, and statistical analyses were performed using the MS Excel 2010 and Prism 7 software.

DISCUSSION

Effect of substrate concentration on growth and xylanase biosynthesis. Cost-efficient and therefore practical sources of carbon are substrates derived from recycled plant matter, such as crop debris and timber stands. Grain husk, straw, bran and wood shavings are typically rich in xylans.

Our trials demonstrate an impact of the substrate concentration, such as rice bran ferment lysate, on the growth and secreted xylanase activity in *P. mucilaginosus* strain 560. Higher substrate concentrations corresponded to a higher enzyme activity (Fig. 1, *a*), lower generation time and increased specific growth rate in bacteria (Table 2). Maximal xylanase activity reached 7.66 U/mL after 24 h of cultivation, with the total rice bran ferment lysate RS value of 0.5%, effective growth conditions and maximal biomass yield of 40%.

Effect of cultivation temperature. In 0.5% ferment lysate medium trials, a cultivation temperature of 30±1 °C facilitated both effective xylanase production and optimal bacterial growth (Fig. 1, *b*). A higher temperature of 35 °C was associated with a higher specific growth rate and 2-fold reduced generation time compared to culturing at 25 °C, and 1.5-fold reduction compared with 30 °C. However, the biomass yield at 25 or 35 °C diminished compared with culturing at 30 °C (see Table 2).

The effect of medium pH. Xylanase activity and growth in *P. mucilaginosus* was significantly influenced by the nutrient medium pH (Fig. 2) and pH correction factors. Table 2 shows that noncrystalline silicon-containing lye as a pH corrector facilitates bacterial growth and increases the specific growth rate. However, the use of silicon-containing lye or sodium hydroxide correctors was associated with a lower xylanase activity and reduced biomass yield (Fig. 2, *a*; see Table 2).

A maximal yield was observed with calcium hydroxide (see Table 2), which is likely explained by the regulatory role calcium ions play in many cellular processes. Calcium is a known stabilising factor in the outer lipopolysaccharide membrane and cellular wall in Gram-negative bacteria [27] and a stimulator of bacterial protein biosynthesis resulting in higher biomass yield and enzyme activity [28]. A maximal xylanase activity of 11 U/mL was observed after a 48 h cultivation of *P. mucilaginosus* on a calcium hydroxide-corrected medium (see Fig. 2, *a*), which may be related to the calcium-mediated stabilisation and regulation of the enzyme activity [29–31].

The calcium hydroxide adjustment of the medium's pH from 6 to 9 resulted in the arrest of bacterial growth at pH 9. Adding calcium hydroxide for pH adjustment of 6 to 8 accelerated the specific growth rate and had a 2-fold reduction on generation time (6 h at pH 6 *vs.* 3 h at pH 8, see Table 2).

This result conforms with another study [32], where the growth of rhizobacteria is shown to be co-affected by pH and calcium ion concentrations. In our trials, a maximal biomass yield of 38% is observed at a neutral medium pH of 7.0 (see Table 2). The maximal xylanase activity reaches 15 U/mL under pH 6.0 following a 48h cultivation on a rice bran ferment lysate medium (Fig. 2, *b*).

Effect of carbon source. Cultivation trials with various carbon sources demonstrated a peak biomass accumulation (about 60%) with beechwood xylan (see Table 2).

Rice bran ferment lysate was identified as the most effective source of carbon for xylanase produc-

Table 2. Effect of cultivation conditions on growth kinetics in P. mucilaginosus strain 560
Таблица 2. Влияние условий культивирования на кинетические параметры роста штамма 560 бактерий P. mucilaginosus

Parameter	Range	Specific growth rate, h^{-1}	Generation time, h	Biomass yield, %
Substrate concentration, %	0.25	0.17±0.05	4.10±0.59	37.68±2.62
	0.5	0.22±0.03	3.09±0.41	40.11±2.83
	0.75	0.26±0.05	2.71±0.25	32.09±2.65
	1	0.23±0.05	2.96±0.30	33.13±2.63
Temperature, °C	25	0.11±0.04	6.45±0.66	18.26±1.52
	30	0.15±0.03	4.54±0.48	38.48±2.72
	35	0.24±0.03	2.86±0.14	26.55±3.02
pH correction factor	Calcium hydroxide	0.21±0.02	3.29±0.27	29.23±2.69
	Sodium hydroxide	0.31±0.04	2.25±0.27	18.92±1.45
	Si-containing lye	0.45±0.04	1.54±0.12	17.20±1.65
pH	6	0.11±0.01	6.89±0.57	19.24±2.02
	7	0.15±0.03	4.54±0.18	38.48±2.67
	8	0.20±0.05	3.52±0.10	19.91±1.82
Carbon source	Birchwood xylan	0.16±0.02	4.24±0.55	44.57±2.85
	Beechwood xylan	0.20±0.03	3.48±0.25	59.43±3.02
	Rice bran ferment lysate	0.21±0.02	3.29±0.27	39.23±2.12
Inoculate incubation time, h	0	0.16±0.03	4.36±0.12	43.30±2.63
	12	0.15±0.03	4.57±0.15	34.43±2.70
	24	0.09±0.01	7.47±0.81	21.38±2.22
	36	0.10±0.01	6.77±0.70	33.75±2.68
	48	0.13±0.02	5.55±0.71	56.16±3.08
Nitrogen source	No nitrogen	0.12±0.01	5.84±0.61	16.68±1.82
	NH_4NO_3	0.19±0.02	3.72±0.22	22.40±2.12
	$(NH_4)_2SO_4$	0.19±0.02	3.63±0.20	25.57±2.07
	Yeast extract	0.11±0.01	6.68±0.25	33.16±2.82
	$(NH_4)_2SO_4$ + yeast extract	0.15±0.01	4.67±0.53	37.10±2.68
	Corn extract	0.06±0.01	12.17±0.74	49.31±2.64
	Pepton	0.15±0.01	4.68±0.46	29.87±2.12
	Carbamide	0.17±0.03	4.08±0.46	15.29±1.92
	Betafin	0.23±0.05	3.00±0.47	11.39±1.22
Carbamide content, %	0.02	0.21±0.04	3.25±0.36	29.18±2.27
	0.1	0.16±0.01	4.33±0.45	18.88±1.62
	0.2	0.15±0.01	4.51±0.35	15.21±1.65
	0.3	0.08±0.01	8.84±0.70	10.44±1.60

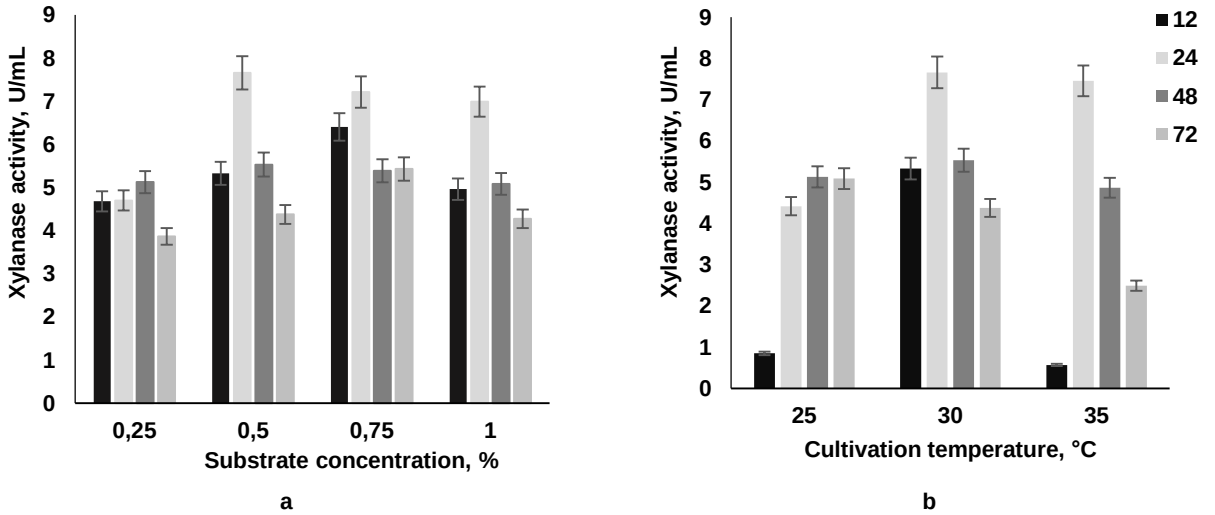
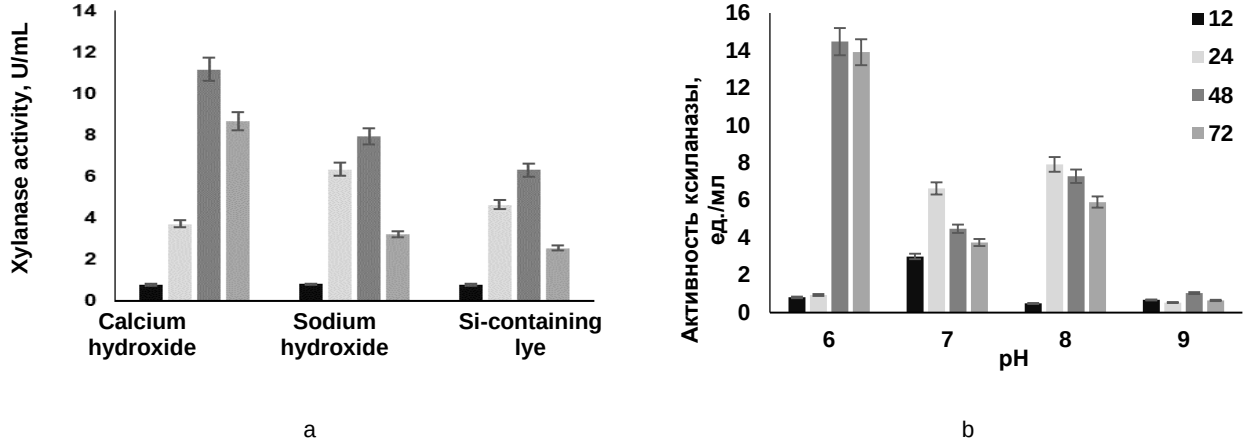


Fig. 1. Effect of rice bran ferment lysate carbohydrate concentration (a) and cultivation temperature (b) on xylanase activity (U/mL) in *P. mucilaginosus* strain 560

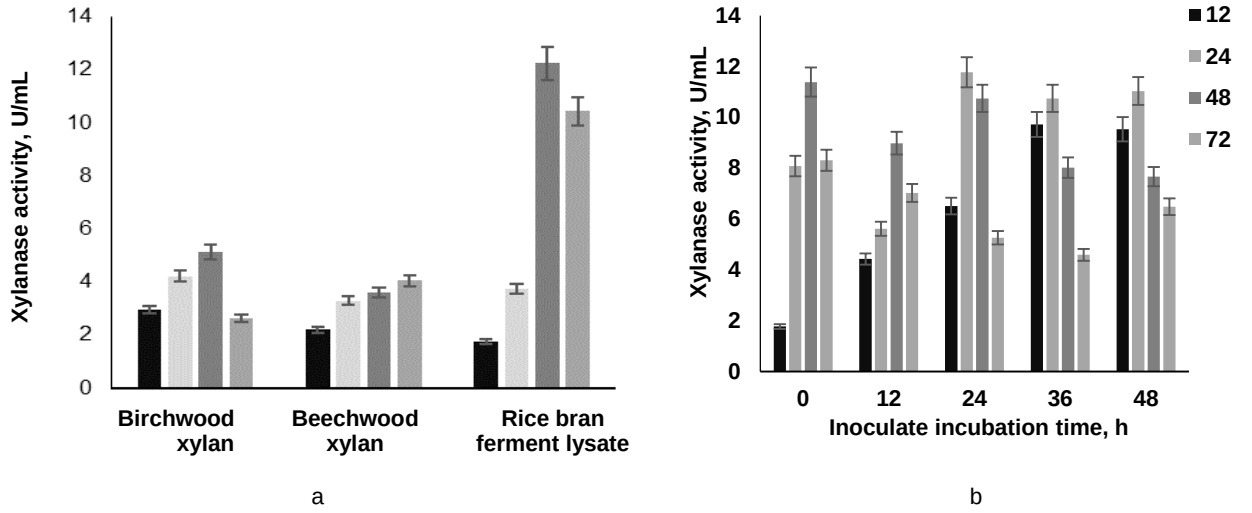
Рис. 1. Влияние концентрации углеводов в ферментолизате рисовой шелухи (a) и температуры культивирования (b) на ксиланазную активность (ед./мл) штамма 560 *P. mucilaginosus*



a b

Fig. 2. Effect of pH correction factors (a) and acidity (b) on xylanase activity (U/mL) in *P. mucilaginosus* strain 560

Рис. 2. Влияние факторов корректировки pH среды (a) и кислотности среды (b) на ксиланазную активность (ед./мл) штамма 560 бактерий *P. mucilaginosus*



a b

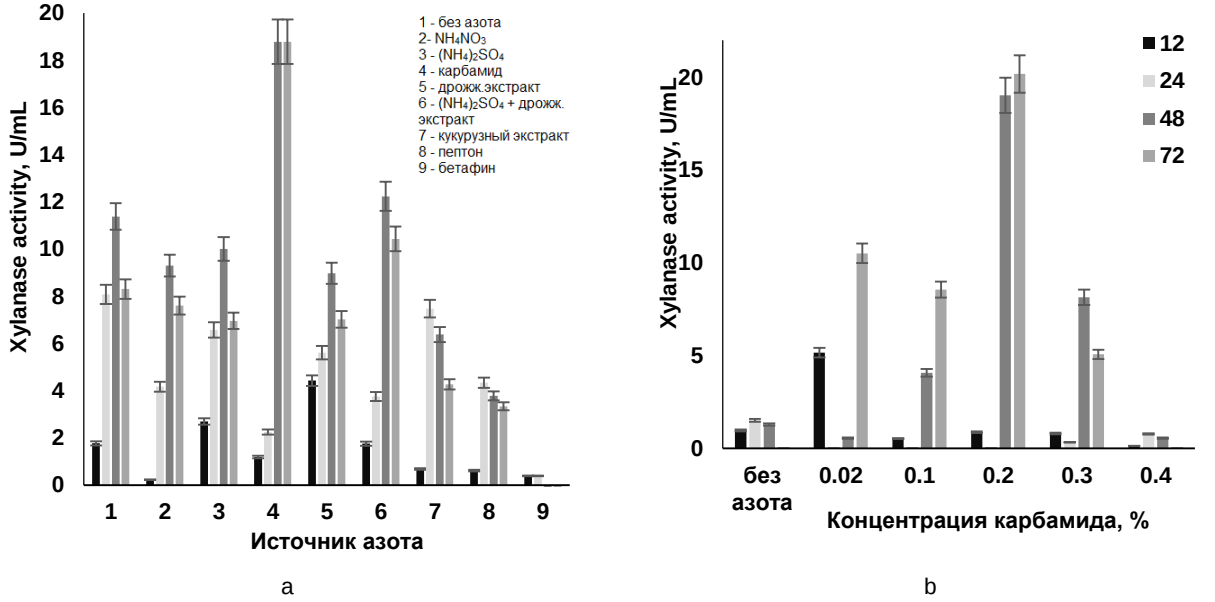
Fig. 3. Effect of xylan source (a) and incubation time (b) on xylanase activity (U/mL) in *P. mucilaginosus* strain 560

Рис. 3. Влияние источника ксилана (a) и продолжительности инкубации инокулята (b) на ксиланазную активность (ед./мл) штамма 560 бактерий *P. mucilaginosus*

tionin bacteria *P. mucilaginosus* strain 560 (Fig. 3, *a*). This observation may relate to the presence of specific inductors produced in the partial hydrolysis of ferment lysate, which boost the xylanase biosynthesis as reported by [33] in experiments examining fungal xylanase production in *Aspergillus* and *Trichoderma*. As shown in [34], specific inductor substances play a key role in xylanase biosynthesis regulation. Among those are xylose, xylobiose, xylooligosaccharides, xylose heterodisaccharides and their positional isomers formed during the enzymatic hydrolysis of rice bran fibre. Inoculate incubation time under the trial conditions was found to have an insignificant effect on bacterial growth and xylanase production (Fig. 3, *b*; see Table 2). Accordingly, the inoculum pretreatment is not required for experimental assays.

Effect of nitrogen source and concentration. Trials with various nitrogen sources demonstrated a maximal xylanase activity after a 48 h cultivation with carbamide supplemented medium (Fig. 4, *a*). A carbamide content of 0.2% increased the xylanase activity by 2–4 times compared to the control (no nitrogen) (Fig. 4, *b*). Under optimal cultivation conditions, the maximum xylanase activity in the stationary phase reached 20 U/mL, giving a 2-fold increase compared to *P. campinasensis BL11* cultured under optimal conditions on a lye-treated rice bran-straw lysate medium (xylanase 10.5 U/mL) [35].

A combined application of ammonium sulphate and yeast extract in the medium increased the *P. mucilaginosus* biomass yield (see Table 2).



a b

Fig. 4. Effect of nitrogen source (a) and concentration (b) in medium on xylanase activity (U/mL) in *P. mucilaginosus* strain 560

Рис. 4. Влияние источника азота на ксиланазную активность (ед./мл) штамма 560 бактерий *P. mucilaginosus*: a – источник азота; b – концентрация источника азота в питательной среде

CONCLUSIONS

We identify the rice bran ferment lysate-based nutrient medium as optimal for the xylanase production in *P. mucilaginosus* strain 560. Calcium supplementation positively affects bacterial growth and xylanase biosynthesis. Recommended cultivation conditions are: carbon source concentration in the medium of 0.5% of total RS content; 0.2% carbamide as the optimal nitrogen source; calcium hydroxide as medium pH corrector to 6.0±0.2; cultivation temperature 30±1 °C. These conditions do not require inoculate pre-treatment of *P. mucilaginosus* strain 560, and a maximal xylanase activity reaches 20 U/mL in stationary culture.

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Contribution

Dung T. Ha, Albert V. Kanarsky, Zosia A. Kanarskaya, Andrei V. Shcherbakov, Elena N. Shcherbakova, Andrey V. Pranovich carried out the experimental work, 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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Ха Т.З., Канарский А.В., Канарская З.А., Щербаков А.В., Щербакова Е.Н., Пранович А.В. выполнили экспериментальную работу, на основании полученных результатов провели обобщение и написали рукопись. Авторы имеют на статью равные авторские права и несут равную ответственность за плагиат.

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