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Regulatory Activity of Heterologous Gene-Activator *xlnR* of *Aspergillus niger* in *Penicillium canescens*

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Abstract—The gene encoding the *xlnR* xylanolytic activator of the heterologous fungus *Aspergillus niger* was incorporated into the *Penicillium canescens* genome. Integration of the *xlnR* gene resulted in the increase in a number of activities, i.e. endoxylanase, β-xylosidase, α-*L*-arabinofuranosidase, α-galactosidase, and feruloyl esterase, compared to the host *P. canescen*s PCA 10 strain, while β-galactosidase, β-glucosidase, endoglucanase, and CMCase activities remained constant. Two different expression constructs were developed. The first consisted of the nucleotide sequence containing the mature *P. canescens* phytase gene under control of the *axhA* promoter region gene encoding *A. niger* (1,4)-β-*D*-arabinoxylan-arabinofuranohydrolase. The second construct combined the *P. canescens* phytase gene and the *bgaS* promoter region encoding homologous β-galactosidase. Both expression cassettes were transformed into *P. canescens* host strain containing *xlnR*. Phytase synthesis was observed only for strains with the *bgaS* promoter on arabinose-containing culture media. In conclusion, the *bgaS* and *axhA* promoters were regulated by different inducers and activators in the *P. canescens* strain containing a structural tandem of the *axhA* promoter and the gene of the *xlnR* xylanolytic activator.

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Arabinose, which is formed by cleavage of hemicellulose and pectins, induces the synthesis of major secreted β-galactosidase and xylanase enzymes of the fungus *Penicillium canescens* [1, 2]. One of the strains of this fungus (*P. canescens* F178 VKPM) has defect at the arabitol-dehydrogenase step of the enzymatic catabolism chain of arabitol. Due to this fact, cells of the fungus cultivated on arabinose-containing substrates accumulate substantial amount of arabitol and, correspondingly, the synthesis of enzymes induced by arabitol increases [3]. Compared with the wild type strain *P. canescens* F178, the synthesis of these enzymes in *P. canescens* strains with multiple copies of the β-galactosidase and xylanase genes increased by 7-10-fold [4, 5]. The phenomenon of superproduction is supposed to be associated with the absence of inducer limit in the regulatory mechanism of the synthesis of arabinose-induced excreted proteins [3].

The target of the activator in the fungal cell is a regulatory protein, transcriptional activator, which promotes initiation of transcription by interaction with specific nucleotide sequences in promoter regions of regulated genes near the starting point of transcription. A number of fungal activator-encoding genes including xylanase activators *xlnR* of *Aspergillus niger* and *xlnR* of *P. canescens* have been cloned [6, 7]. Comparative examination of the enzyme spectra in *P. canescens* strains, including single and multiple numbers of the *xlnR* gene as well as in deletions revealed a group of arabinose-induced xylanolytic genes that are under control of xylanase acti-

Abbreviations: BP medium, medium containing beet pulp and peptone; *p*-NP, *p*-nitrophenyl; RS, reducing sugars.

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vator. The enzymes hydrolyzing bonds between the major chain of xylan and modified arabinose residues including (1,4)-β-*D*-arabinoxylan-arabinofuranohydrolase AbfA and α-*L*-arabinofuranosidase AbfB of *P. canescens* are significant. β-Galactosidase and AbfA were found to be not under control of the *P. canescens xlnR* gene [7]. The similar group of *A. niger* xylanolytic genes, which are under control of *xlnR*, is induced by xylose and arabinose with the exception of the *axhA* gene encoding (1,4)-β-*D*-arabinoxylan-arabinofuranohydrolase AxhA. The molecular nature of the inducer of this gene is unknown, but it is suggested to take part in the molecular structure of xylan [8].

Penicillium canescens is of interest for biotechnological industry as a novel enzyme producer. The scheme of using features of genetic regulation includes genetic design of strains in which structural genes of target proteins are under control of arabinose-induced strong promoters of β-galactosidase (*bgaS*) and xylanase (*xylA*) genes. Also, in multiple-gene-containing producer strains, competition between promoter regions of multiple genes for regulatory proteins is known to occur. Specifically, the *bgaS* and *xylA* genes were indicated to show this effect [2]. Due to this, supplementing the regulatory scheme of *P. canescens* with a structural analog from the heterologous fungus *A. niger*, especially by *axhA* promoter and xylanase activator gene *xlnR*, looks quite attractive because their functional interaction different from arabinose inducer is required.

The phytase enzyme (myo-inositol-hexakisphosphate-3(6)phosphohydrolase; EC 3.1.3.8 and 3.1.3.26) from different species is commercially produced as widely used feed additive. *Penicillium canescens* is known to secrete only a small amount of this enzyme [9]. In the present investigation, *P. canescens* was used as basic organism for construction using plasmid transformation of multiple-gene strains of the producer of phytase proper, which was used as reporter protein in the present study. For the goal of enzymatic synthesis of phytase, in one group the structural gene of *P. canescens* phytase was combined with β-galactosidase (*bgaS*) promoter, and in another group it was combined with *axhA* promoter that was under regulatory control of the heterologous xylanase activator gene *xlnR* of the fungus *A. niger* inserted into *P. canescens*.

MATERIALS AND METHODS

Plasmid transformation and selection of transformants. Strain *P. canescens* PCA 10 (*niaD*$^-$) was used as a recipient for plasmid transformation [10]. The transformation method and composition of minimal media and a medium containing beet pulp and peptone (BP) for cultivating *P. canescens* were described earlier [4]. In transformation experiments, the quantitative ratio between selective marker (*niaD*$^+$) containing plasmid pSTA and plasmid with cloned target gene was 1 : 10.

Fungal mycelium was transferred from growth medium to inducer-containing medium as described earlier [2]. Phytase-producing transformants were selected on immunological plates. Transformants that grew on selective minimal medium were individually replated into wells containing 100 μl BP medium minus phosphates. After 144 h growth, 10 μl of culture fluid was taken from each well, 10-fold diluted with sodium acetate buffer, and then 10 μl of the resulting solution was transferred into wells of a second plate with 60 μl of 1.4 mM sodium phytate solution. After incubation at 38°C for 60 min, the reaction was stopped by adding 70 μl of 10% trichloroacetic acid. Then 130 μl of freshly prepared ammonium-molybdenum reagent (13 mM $FeSO_4 \cdot 7H_2O$/8.1 mM $(NH_4)_6Mo_2O_{24} \cdot 4H_2O$/0.533 M H_2SO_4) was added to the wells, and after incubation at room temperature for 30 min the optical density of the solutions was measured at 750 nm.

Cloned genes and plasmid constructions. For phytase gene cloning, a phage library of *P. canescens* F178 genes and a phage clone selection method described earlier were used [11]. For clone screening, radioactively labeled 1 kb PCR-fragment was synthesized on *P. canescens* DNA template using the following oligonucleotide primers: PPHYD1, 5′ CTG TTG ATG GCG GTT ATC AAT GC 3′; PPHYR1, 5′ GGC GAC ATT GCA TCA TCT CGA C 3′.

Nucleotide sequences in primers were determined based on conserved nucleotide sequences of fungal phytases from the GenBank database. Among clones with positive signal, one clone with the phytase gene in a 12 kb DNA fragment was selected. A DNA fragment containing the full gene of *P. canescens* phytase was subcloned in bacterial vector pUC57, and the pPHYA plasmid was thus obtained. Then the nucleotide sequence encoding the structural part of the phytase gene was combined with the nucleotide sequence encoding the promoter region and signal peptide of the *bgaS* gene of *P. canescens* β-galactosidase. The resulting fragment was integrated into bacterial vector pUC19 and the pPrPHY expression plasmid was obtained (Fig. 1).

A DNA fragment with the structural part of the *P. canescens* phytase gene and the sequence encoding the *bgaS* signal peptide that is under control of the promoter region of the *A. niger axhA* gene was obtained by PCR synthesis using the *axhA* gene sequence published in GenBank (AN: Z78011) [8].

The promoter region of *axhA* was amplified by PCR using the following primers: AXHPR-LIC5, 5′TACTTCCAATCCATGCTGATTGGGATTCTGCAGGAATTC3′; AXH/PHY-REV, 5′GAAGACAAAAGCTTCATTTTCGTATGACTTTATCCGCT3′. The genome DNA of *A. niger* isolated according to a standard protocol [12] was used as a template.

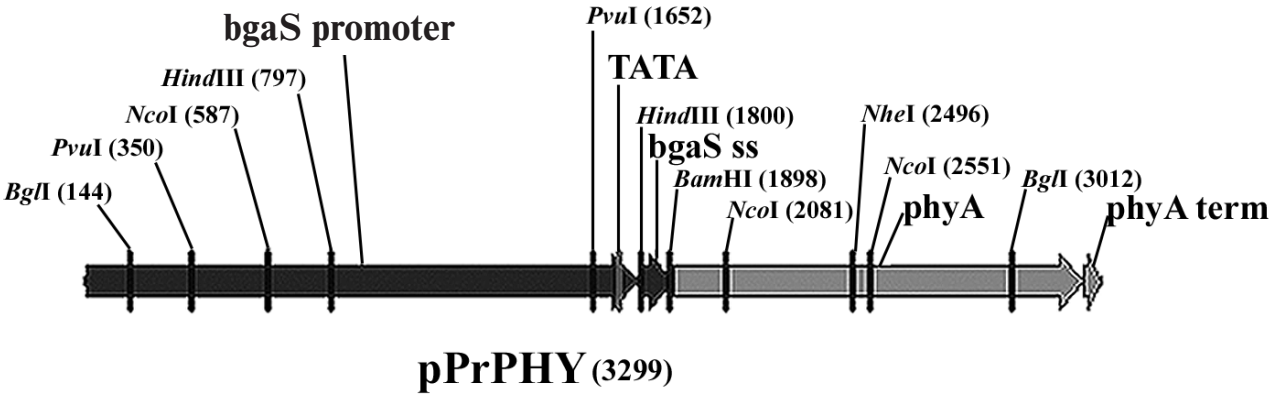

Fig. 1. Scheme of plasmid pPrPHY.

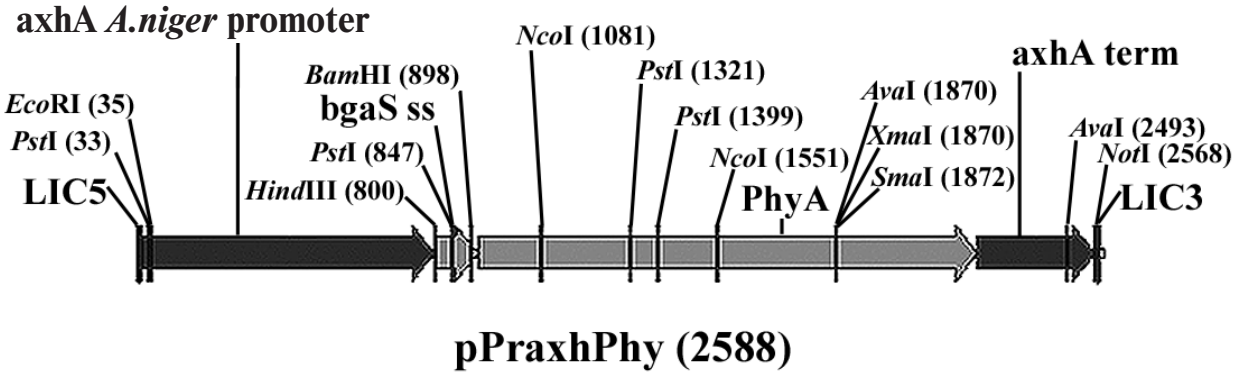

Fig. 2. Scheme of plasmid pPraxhPhy.

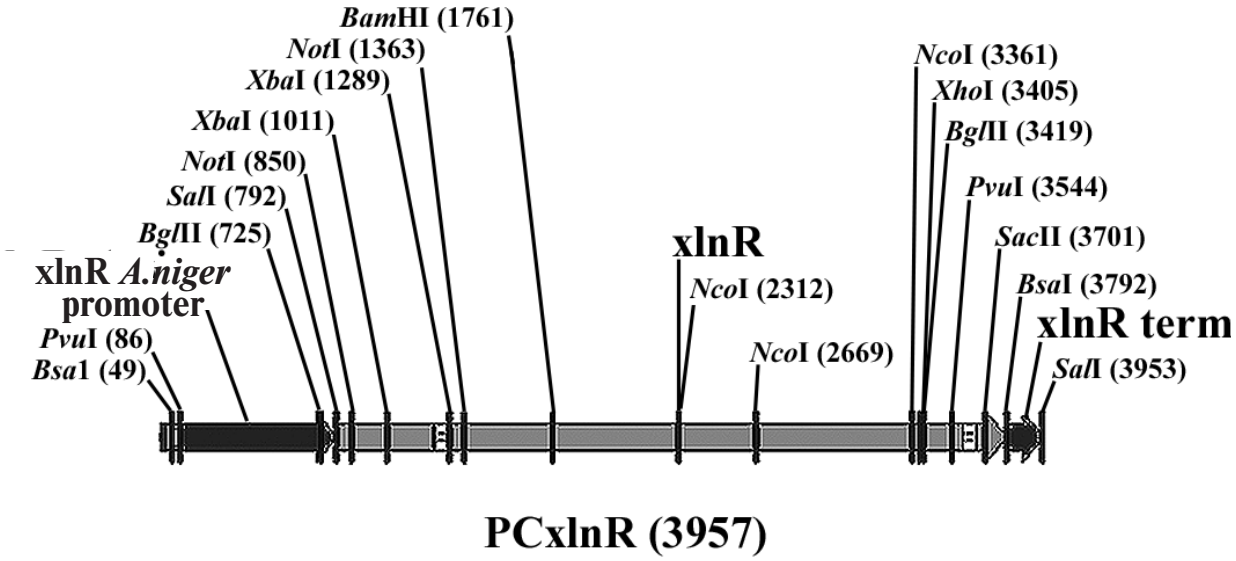

Fig. 3. Scheme of plasmid PCxlnR.

A DNA fragment with the structural part of the *P. canescens* phytase gene and the sequence encoding the *bgaS* signal peptide was also obtained by PCR using the following primers (with earlier obtained pPrPhy plasmid as a template): AXHTR-LIC3, 5′TATCCACCTTTACTGCGGCCGCAACTGGAAACAAAG3′; AXH/PHY-FWD, 5′AGCGGATAAAGTCATACGAAAATGAAGCTTTTGTCTTC3′. The two DNA nucleotide sequences

were combined by PCR using AXHPR-LIC5 and AXHTR-LIC3 primers. Then the DNA fragment obtained from the amplification was subcloned into bacterial vector pUC-LIC containing special DNA regions at the 5′- and 3′-ends that allowed cloning by the method of independent ligation [13]. As a result, expression plasmid pPraxhPhy was obtained (Fig. 2).

For cloning the xylanase activator gene *xlnR*, the phage library of *A. niger* genes was used. Screening was carried out using earlier the resulting gene-specific 550 bp DNA fragment *xlnR* [7]. The DNA fragment carrying the full *xlnR* gene of *A. niger* was inserted into bacterial vector pUC57, and expression plasmid PCxlnR was thus obtained (Fig. 3).

Determinations of biochemical characteristics of the enzymes. Electrophoresis under denaturing conditions (in the presence of SDS) was carried out in 12% polyacrylamide gel using a Mini Protean cell (Bio-Rad, USA). Protein bands in the gel were stained with Coomassie Brilliant Blue R-250 (Ferak, Germany). As a standard, protein mixture MW-SDS-200 (30-200 kDa; Sigma, USA) was used.

Enzymatic activity determinations. Enzymatic activities on the polysaccharide substrates—CMC (sodium salt of carboxymethyl cellulose of medium viscosity), β-glucan from barley, glucuronoxylan from birch wood, and arabinoxylan from oat spelts (all from Sigma, USA)—were determined from initial rates of the formation of reducing sugars (RS) at pH 5.0 and 50°C by the modified Somogyi–Nelson method [14] as well as by the bicinchoninate method [15].

The activity on cellobiose (Merck, Germany) was determined from the initial rate of glucose formation at pH 5.0 and 40°C by the oxidase–peroxidase method [12].

Enzymatic activities with synthetic *p*-nitrophenyl substrates—*p*-NP-α-*L*-arabinofuranoside, *p*-NP-α-*D*-glucopyranoside, *p*-NP-β-*D*-galactopyranoside, *p*-NP-β-*D*-glucopyranoside, and *p*-NP-β-*D*-xylopyranoside (all from Sigma)—were measured from initial rates of formation of colored reaction product (phenolate ion) at pH 5.0 and 40°C [16].

Feruloyl esterase activity was determined from the initial rate of hydrolysis of feruloyl oligosaccharides of wheat arabinoxylan (Megazyme, Australia) at pH 5.0 and 40°C estimated by the increase in free ferulic acid concentration (spectrophotometric detection at 335 nm).

Phytase activity was estimated from the initial rate of phosphate ion formation during hydrolysis of sodium phytate (from rice, Sigma) at pH 5.0 and 38°C. Free phosphate concentration was measured using ammonium-molybdenum reagent [17].

All the activities mentioned above were expressed in international units per milliliter of culture liquid (U/ml). One activity unit corresponds to the amount of an enzyme hydrolyzing 1 μmol of substrate bonds per minute.

RESULTS AND DISCUSSION

Transformation of *P. canescens* with plasmid containing DNA fragment with full *xlnR* gene of *A. niger*. Protoplasts of *P. canescens* PCA 10 (*niaD*$^-$) were cotransformed with plasmids pSTA (*niaD*$^+$) and PCxlnR, and obtained transformants were cultured in flasks with BP medium. Total xylanase activity was determined in culture liquid aliquots, and transformants with enhanced activity (25-35%) were selected. Electrophoretic and enzymatic features of the secreted proteins were determined in the selected transformants. Results of electrophoresis of culture liquid after growth of the RN-3 transformant (selected as the best) are presented in Fig. 4. Compared with the enzyme complex of the recipient strain *P. canescens* PCA 10, the enzyme complex secreted by *P. canescens* RN-3 strain showed appreciable broadening of a protein band at 29-31 kDa (corresponding to xylanase XylA) as well as protein bands at 45-66 kDa. The bands corresponding to α-*L*-arabinofuranosidase AbfB, feruloyl esterase, and α-galactosidase α-Gal A of *P. canescens* are located in this region. Results of determination of enzymatic activities in culture liquids obtained after growth of *P. canescens* RN-3 and PCA 10 strains are presented in Table 1. Compared with PCA 10, the expected increase in endoxylanase, β-xylosidase, α-*L*-arabinofuranosidase, α-galactosidase, and feruloyl esterase activities was observed for RN-3 strain. Thus, the activity of the *P. canescens* RN-3 enzyme complex measured with arabinoxylan as substrate (feruloyl esterase activity) was

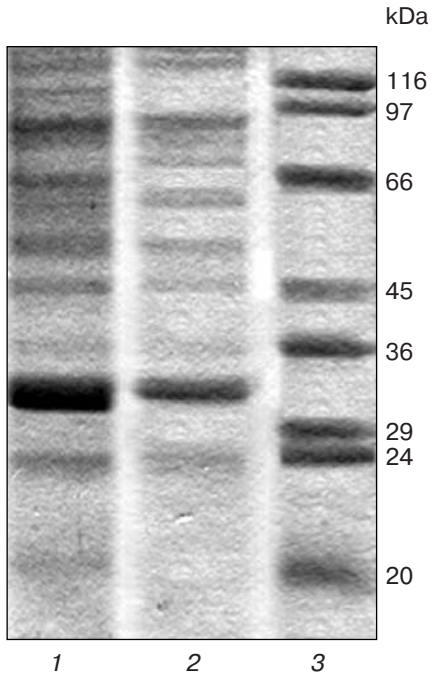

Fig. 4. Electrophoresis of proteins secreted by *P. canescens* strains: *1*) RN-3; *2*) PCA 10; *3*) marker proteins.

Table 1. Activity of enzyme complexes secreted by *P. canescens* PCA 10 and RN-3 strains

Enzyme	Activity, U/ml	
	PCA 10	RN-3
Xylanase (on the birch wood glucuronoxylan)	41.1	277
Xylanase (on the oat spelts arabinoxylan)	26.3	167
β-Xylosidase (with *p*-NP-β-*D*-xylopyranoside)	0.2	2.3
α-*L*-Arabinofuranosidase (on *p*-NP-α-*L*-arabinofuranoside)	2.3	5.6
Feruloyl esterase (on hydrolysate of wheat arabinoxylan)	0.11	2.8
α-Galactosidase (on *p*-NP-α-*D*-galactopyranoside)	2.4	24.4
β-Galactosidase (on *p*-NP-β-*D*-galactopyranoside)	10.6	14.6
β-Glucosidase (on *p*-NP-β-*D*-glucopyranoside)	1.6	0.8
β-Glucosidase (on cellobiose)	1.2	1.1
Cellulase (on CMC)	4.1	3.0
Endoglucanase (on barley β-glucan)	6.1	6.3

three orders of magnitude higher than that of *P. canescens* PCA 10, with *p*-NP-α-*D*-galactopyranoside and *p*-NP-β-*D*-xylopyranoside one order of magnitude higher, with glucurono- and arabinoxylan more than 5 times higher, and with *p*-NP-α-*L*-arabinofuranoside 2 times higher. In contrast, β-galactosidase, β-glucosidase, endoglucanase, and cellulase (CMCase) activities of enzyme complexes produced by the primary and novel strains of *P. canescens* did not differ at all or differed insignificantly. On the whole, it can be concluded that the heterologous transactivator of *A. niger* encoded by the *xlnR* gene is effectively expressed in *P. canescens* growing in multi-component BP medium and shows the same selectivity toward the group of xylanolytic genes as in *A. niger* [6].

Transformation of *P. canescens* PCA 10 and *P. canescens* RN-3-7 strains with pPrPHY and pPraxhPhy plasmids. *Penicillium canescens* RN-3 strain was obtained by transformation of auxotrophic strain (*niaD*$^-$) using incorporation of marker plasmid pSTA(*niaD*$^+$) and, correspondingly, had phenotype *niaD*$^+$. For transformation experiments with this strain on phytase gene incorporation under control of *axhA* gene promoter, the procedure of conversion of this strain into the recipient *niaD*$^-$ was carried out. Mutagenesis and selection of the strain with the required phenotype (*niaD*$^-$) were carried out by the method of Aleksenko et al. [10]. The selected variant was designated as *P. canescens* RN-3-7. The phytase gene under the control of the *A. niger bgaS* and *axhA* gene promoters was incorporated using cotransformation with pSTA(*niaD*$^+$) marker plasmid and pPrPHY plasmid of *P. canescens* PCA 10 strain (regulation by *bgaS* gene promoter) as well as with the same marker plasmid and plasmid pPraxhPhy of *P. canescens* RN-3-7 strain (regulation by *xlnR* activator of *A. niger axhA* gene promoter). In addition, cotransformation of *P. canescens* PCA 10 strain by pPraxhPhy plasmid was carried out (regulation by *axhA* gene promoter but without *A. niger xlnR* activator).

As a result of the screening of the transformants for the ability to produce phytase, two strains were selected. In Phy-19 strain, the phytase structural gene was under the control of promoter of the β-galactosidase *bgaS* gene, and in strain *axhA*PhyF8 it was under control of the promoter of the *A. niger axhA* gene. In experiment on transformation of *P. canescens* PCA 10 strain by pPraxhPhy plasmid, more than 100 transformants with *niaD*$^+$ genotype were examined, but none produced phytase. It seems that the regulatory proteins including *P. canescens* XlnR activator are not functional with the *A. niger axhA* gene promoter.

To investigate the ability of arabinose to induce phytase synthesis, strains were cultivated in minimal medium with 1% fructose (growth time 24 h), and then collected and washed mycelium was transferred to inducing medium containing arabinose at different concentrations (1, 5, and 10 mM). In addition, Phy-19 and *axhA*PhyF8 strains were grown (for 120 h) in flasks containing BP medium [4]. Results are given in Table 2. It is seen that both strains produce phytase effectively (near 200 U/ml) in BP medium. However, in experiments in which fungal mycelium

Table 2. Induction of β-galactosidase and phytase synthesis by Phy-19 and *axhA*PhyF8 strains

Enzyme	Arabinose concentration in inducing medium, mM	Activity, U/ml	
		Phy-19	*axhA*PhyF8
Phytase (in BP medium)		200	196
β-Galactosidase	1	0.23	0.87
	5	0.16	0.44
	10	0.01	0.02
Phytase	1	5.74	0.09
	5	2.09	0.07
	10	1.66	0.04

was transferred to the inducing medium with arabinose, synthesis of phytase was observed only in Phy-19 strain, but not in *axhA*PhyF8 strain. At the same time, the *axhA*PhyF8 strain synthesized β-galactosidase, i.e. the mechanism of induction by arabinose of the *bgaS* gene under the experimental conditions was not impaired in this strain. It can be concluded that the *bgaS* and *axhA* gene promoters of strain RN-3-7 are regulated by different inducers and activators, i.e. in contrast to *bgaS* and *xylA* promoters of *P. canescens* PCA 10 primary strain, the *bgaS* and *axhA* gene promoters of strain RN-3-7 are regulated independently.

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