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A NEW PRODUCER OF A RECOMBINANT AFLATOXIN-DEGRADING ENZYME OBTAINED VIA HETEROLOGOUS EXPRESSION IN *Pichia pastoris*

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Abstract

Contamination of food and feed with mycotoxins causes significant economic losses in the food and feed industry and poses a serious threat to the human health and animal life because of mutagenic, carcinogenic and other disruptive properties of these secondary metabolites of fungi. Enzymatic degradation of mycotoxins represents an efficient and environmentally safe alternative to the chemical decontamination of agricultural and food products. In this study, a synthetic *adtz* gene encoding ADTZ, an aflatoxin-degrading oxidase from *Armillaria tabescens*, was integrated into the genome of a *Pichia pastoris* GS115 strain under the control of a glyceraldehyde-3-phosphate dehydrogenase promoter. To amplify the *adtz* gene, oligonucleotide sequences were constructed with specific restriction sites HindIII and NotI added to the 5' end. The *adtz* gene-containing pPIG-ADTZ plasmid obtained with the use of the pPIG-1 vector was linearized by digestion with restriction endonuclease ApaI, followed by transforming the cells of *P. pastoris* recipient strain GS115 by electroporation. The transformed yeast cell were selected on YPD medium with an antibiotic. PCR amplification, restriction analysis and Sanger sequencing confirmed insertion of the target gene. As a result, 54 transformed clones containing the target gene were obtained, and the most productive clone secreting the recombinant ADTZ-14 (2.1 mg/ml of the total extracellular protein) was selected. Recombinant ADTZ represented a monomeric protein (78±3 kDa) possessing a high affinity to aflatoxin B_1 (AFB_1). Saving the functional properties of the recombinant protein was shown using experiments on assessment of its ability to degrade AFB_1 during short-time and prolonged incubation. The obtained protein was able to degrade AFB_1 by 14 % after a 2-h incubation at 40 °C; after 72 and 120 h of incubation at 30 °C, the content of AFB_1 in ADTZ-14 culture liquid (CL) reduced by 50 and 80 %, respectively, compared to content in CL of non-transformed control GS115. These data suggest a quite high biotechnological potential of a new recombinant ADTZ preparation in relation to the decontamination of agricultural products contaminated with AFB_1. Thus, the earlier developed expression system intended to increase the copy number of heterologous genes in *Pichia pastoris* was first used to obtain a recombinant protein able to degrade AFB_1. Using this approach, we transformed yeast cells with the pPIG-ADTZ plasmid and obtained 154 recombinant clones of *P. pastoris*, 77 % of which contained the target sequence of the *adtz* gene. Productivity of the best transformant (clone ADTZ-14) was 2.1 mg of protein per 1 ml of culture liquid, and about half of the pool of the extracellular proteins fell to the share of recombinant ADTZ able to degrade 80 % of AFB_1 incubated in cell-free culture broth at 30 °C and pH 7.0.

Keywords: aflatoxin B_1, mycotoxins, enzymatic degradation, ADTZ from *Armillaria tabescens*, synthetic *adtz* gene, recombinant proteins, heterologous expression, *Pichia pastoris*

Aflatoxins, a group of structurally similar secondary metabolites of the

fungal genus *Aspergillius* widely distributed in nature, are known as dangerous mycotoxins that contaminate feed and other agricultural products [1-4]. Currently, more than 20 aflatoxins (AF), their derivatives and closely related compounds have been identified [5]. Contamination of feeds for livestock and poultry with B- and G-type AF raises the most serious concern [6, 7]. These mycotoxins are derivatives of difuranocoumarin which have a bifuran group linked to the coumarin core and a cyclopentane (in B-type AF) or lactone ring (in G-type AF) [8, 9]. Due to the toxicity, carcinogenicity and mutagenicity of these compounds, and their resistance to heat treatments [10, 11], feed and other crop products contaminated with AF above the concentrations allowed by hygienic regulations are not suitable for direct use or further processing into food products. Globally, contamination with these mycotoxins, especially AFB_1 which surpasses all other AFs in hepatotoxicity and danger to warm-blooded animals [6, 7], causes serious economic damage to both agriculture and the food industry, and also create risks for human health [1, 3].

For decontamination, physical, chemical, and microbiological methods are used, which, however, have a number of well-known limitations [1, 12]. Therefore, there is a constant search for other effective, environmentally friendly means and methods of AF degradation and detoxification that do not affect the quality of agricultural products. From this point of view, an approach based on the ability of a number of fungi [13-15] and bacteria [16-19] to synthesize enzymes that transform AF to non-toxic or less toxic compounds seems to be very promising [20, 22]. The use of cell-free preparations containing such enzymes makes it possible to avoid problems that may arise when using the producers themselves (for example, deterioration of the organoleptic properties of processed products, a decrease in their nutritional value). In addition, enzyme preparations are technologically more convenient for feed processing [23] and, unlike those for the food industry, do not require expensive multi-stage purification of the target product.

It is known that some xylotrophic basidiomycetes of the genera *Pleurotus* [24, 25], *Phanerochaete*, and *Armillaria* [26-28] can be sources of enzymes for AF degrading and detoxifying. An enzyme with oxidase activity [28] called by the authors aflatoxin-detoxifizyme (ADTZ) was isolated from the mycelium of *Armillaria tabescens* using hydrophobic and metal chelate chromatography. It turned out that ADTZ can catalyze the opening and subsequent hydrolysis of the difuran ring [29], a structure associated with B-type AF toxicity. Further studies have shown that ADTZ is a 76 kDa monomeric protein with high affinity for AFB_1 [29]. Upon contact with ADTZ, the toxicity and mutagenicity of AFB_1 were significantly reduced [28, 30].

These data indicate the prospects for the development of detoxifying drugs containing ADTZ. However, their creation is primarily hampered by the lack of available technology for obtaining intracellular ADTZ from *A. tabescens* mycelium and, in part, by the fact that deep cultures of *A. tabescens* requires liquid media of complex composition, including very specific and expensive components [31], or a multi-stage fermentation procedure [28]. These obstacles could be overcome by using a heterologous expression system and creating an accessible producer of the recombinant ADTZ protein. However, there is still no suitable system for obtaining extracellular heterologous ADTZ in an amount sufficient for its use in decontamination of crop products. Nevertheless, a number of modern works [32, 33] report on the successful use of *Pichia pastoris* yeast cells as recipients for heterologous expression.

Previously, we adapted the expression system in *P. pastoris* by modifying the integration vector to increase the copy number of heterologous genes in the yeast chromosome (the integration vector and its preparation are patented) [34]. In the present study, this approach was used for the first time to create a new producer of the aflatoxin-degrading enzyme.

Our goal was to optimize and use this system for the heterologous expression of ADTZ in *Pichia pastoris* GS115 and to evaluate the ability of a cell-free culture liquid (CL) preparation of the resulting *P. pastoris* ADTZ-14 strain containing the extracellular recombinant ADTZ enzyme to degrade AFB_1.

Materials and methods. For the expression of the *adtz* gene encoding aflatoxin-detoxifizim, the yeast strain *Pichia pastoris* GS115 (syn. *Komagataella phaffii*) (Thermo Fisher Scientific, USA) was used. Yeast cells were cultured for 3 days at 30 °C in liquid YPD medium (glucose 20.0 g/l; yeast extract 10.0 g/l; meat peptone 20.0 g/l). For plasmid DNA, *Escherichia coli* XL1-Blue (Agilent, USA) was grown at 37 °C in Luria-Bertrani medium (tryptone 10 g/l; yeast extract 5 g/l; NaCl 5 g/l; pH 7.2-7.5). The pPIG-1 plasmid was used to express ADTZ [34]. The *adtz* gene encoding the aflatoxin degradation enzyme in *A. tabescens* (GenBank AY941095.1) was synthesized at ZAO Evrogen (Russia) according to the codon compositions in *P. pastoris.*

PCR mix for the *adtz* gene amplification (50 μl) contained 1× buffer with 3 mM $MgCl_2$ and 5 U Taq polymerase (NEB, UK), 0.2 μM ADTZ-fwd (5′-gaagcttctATGGCTACTACAACTG-3′) and ADTZ-rev (5′-cgcggccgcTTACAATCTTCTCTC-3′) oligonucleotides, and 0.1 ng DNA as a matrix. The reaction was carried out under the following conditions: 95 °C for 15 s, 62 °C for 15 s, and 72 °C for 120 s (25 cycles) (a T-100 amplifier, Bio-RAD, USA). The PCR products were evaluated electrophoretically (a 1% agarose gel, a Sub-Cell GT Cell, Bio-RAD, USA).

The amplification product, vector pPIG-1, was digested with HindIII and NotI restriction endonucleases according to the manufacturer's recommendations (NEB, UK).

The processed fragments were ligated with T4 DNA ligase (ZAO Evrogen, Russia), and *Escherichia coli* XL1-blue cells (Agilent, USA) were transformed with the 2 μl mixture by the heat shock method. Transformants were selected on Luria-Bertrani agar medium containing ampicillin (100 μg/ml). The pPIG-ADTZ plasmid was isolated from ampicillin-resistant transformants using the Plasmid Mini-prep kit (ZAO Evrogen, Russia). The presence of the target gene insert in the pPIG-ADTZ plasmid was confirmed by PCR amplification, restriction analysis as described above, and Sanger sequencing. Sequencing was performed in both directions from primers used for the gene amplification. Gene sequencing and synthesis of primers used for amplification were performed at OOO Sintol (Russia).

The pPIG-ADTZ plasmid was linearized by digestion with restriction endonuclease ApaI (NEB, UK) according to the manufacturer's protocol and transferred into *P. pastoris* GS115 by electroporation [35]. Transformants were selected on a YPD agar medium supplemented with 200 μg/ml antibiotic zeocin (Thermo Fisher Scientific, USA). DNA was isolated from antibiotic-resistant colonies [36] and the ADTZ insert was checked by PCR.

The recombinant ADTZ protein was produced by culturing the *P. pastoris* ADTZ-14 producer strain in 24-well plates (3 ml YNB liquid medium, 30 °C, aeration 200 rpm, 3 days). Every 24 hours, 40% glucose solution in 20 mM

potassium phosphate buffer (pH 6.0) was added to the wells to a final concentration of 2%.

The recombinant ADTZ in cell-free CL was detected by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (DNS-PAGE, Mini-PROTEAN® Tetra, Bio-RAD, USA). Total protein concentration was measured by the Lowry method [37].

The degradation kinetics of AFB_1 and AFG_1 was studied in experiments with short-term incubation of *P. pastoris* ADTZ-14 cell-free CL. Commercial preparations of AFB_1 and AFG_1 (VNIIVSGE, Russia) were dissolved in 20 mM Na-phosphate buffer (pH 6.7) to a final concentration of 2.5 μg/ml each. The concentration was controlled using the molar extinction coefficients $\varepsilon = 21800$ and $\varepsilon = 17700$ (at $\lambda = 362$ nm) for AFB_1 and AFG_1, respectively. The CL of the *P. pastoris* ADTZ-14 transformant was incubated with toxins in the wells of a thermostated autosampler plate (30 or 40 °C). A 5 μl aliquots were taken from the reaction mixture every 30 min for 2.5 h and the AF content was determined by reverse-phase chromatography on a thermostated (30 °C) Kromasil Ethernity 5-C18 column (4.6×250 mm) (Akzo Nobel, Sweden) equipped with an appropriate guard column. An Agilent 1200 chromatographic system (Agilent Technologies, USA) with diode array detection was used. Chromatographic separation was performed in a water/acetonitrile gradient (from 40% to 68% acetonitrile in 20 min, detection at 360, 235, and 225 nm, slit width 8 nm). The degree of AFB_1 and AFG_1 degradation was assessed by the change in the area of the corresponding chromatographic peak. The CL of the untransformed strain *P. pastoris* GS115 was a control.

To assess the ability of the recombinant ADTZ enzyme to degrade AFB_1 during prolonged incubation, 1 ml of *P. pastoris* ADTZ-14 CL samples (2.1 mg total protein/ml) after pre-sterilization by filtration (membranes with a pore size of 0.22 μm, Millipore, USA) were added with 1.0 μg of AFB_1 (Sigma-Aldrich, USA) dissolved in a minimum volume of methanol. Samples (1 ml) of CL of non-transformed strain *P. pastoris* GS115 addedd with the same amount of AFB_1 were used as a control. Samples were incubated for 3 and 5 days at pH 7.0 and 30 °C. The post-incubztion AFB_1 concentration was measured using high-performance liquid chromatography (HPLC) on a thermostated (27 °C) Symmetry C18 column (5 μm, 150×4.6 mm) in isocratic elution mode (mobile phase methanol:water 60:40, 10 μl sample injected, $\lambda = 362$ nm) using a Waters 1525 Breeze system with a Waters UV 2487 detector (Waters Corp., USA) [12, 38]. Prior to HPLC analysis, CL samples were diluted 100-fold with the mobile phase. Toxin concentrations were measured in the linear detection range in the test and control samples, The concentrations were calculated from the calibration curve for the AFB_1 standard (Sigma-Aldrich, USA). The percentage of degradation was determined relative to the amount of toxin detected in the corresponding control sample.

Statistical processing of AFB_1 quantification data was performed using the STATISTICA 6.1 program (StatSoft, Inc., USA). Significance of differences at $p \leq 0.05$ was confirmed using Student's *t*-test for independent variables. The table and figures indicate the mean values (*M*) of two measurements for each of the three biological repetitions with standard deviations (±SD).

Results. The gene for the aflatoxin-degrading enzyme from *A. tabescens* was cloned by PCR using the developed oligonucleotides.

The size of the amplification product corresponding to the synthesized *adtz* gene was 2088 bp. Sequencing of the obtained product confirmed its identity with the sequence of *A. tabescens* (GenBank AY941095.1) (Fig. 1).

```
ADTZ_sint           1     ATGGCTACTACAACTGTTCACAGAGAGAGATTCTTGGCTGACAAGTCTGCTCCATTGTGT  60
ADTZ_A.tabescens    92    ATGGCCACCACAACTGTCCACCGGGAGCGATTCCTGGCAGATAAGTCTGCTCCTTTGTGT  151
ADTZ_sint           61    GGTATGGACATCAGAAAGTCTTTCGATCAACTGTCTTCCAAAGAGAAGCTGTACACTCAC  120
ADTZ_A.tabescens    152   GGTATGGATATTAGAAAGTCATTTGATCAGCTCAGCTCTAAGGAAAAGCTCTACACGCAT  211
ADTZ_sint           121   TATGTCACAGAAGCCTCATGGGCTGGTGCTCGTATCATTCAAGCTCAATGGACTCCACAA  180
ADTZ_A.tabescens    212   TACGTGACCGAAGCTTCTTGGGCGGGCGCAAGAATCATCCAGGCTCAGTGGACCCCGCAG  271
ADTZ_sint           181   GCTACTGACTTGTATGATCTGTTGATTCTGACCTTCTCTGTCAATGGTAAGCTGGCAGAT  240
ADTZ_A.tabescens    272   GCGACAGATCTATATGATCTGTTGATCCTTACGTTCAGCGTAAATGGAAAGCTCGCCGAC  331
ADTZ_sint           241   TTGAATGCTCTCAAGACTTCTTCTGG--TTTGAGTGAAGATGACTGGGAAGCCTTGATTC  298
ADTZ_A.tabescens    332   CTGAATGCCCTTAAGACGTCGTCAGGCCTTTCAGAG--GACGATTGGGAGGCCTTGATAC  389
ADTZ_sint           299   AGTACACTGTTCAAGTTTTGTCCAACTTGGTGAACTACAAGACCTTCGGATTCACTAAGA  358
ADTZ_A.tabescens    390   AGTACACGGTCCAGGTATTGAGCAATCTTGTCAACTACAAGACGTTCGGATTTACGAAGA  449
ADTZ_sint           359   TCATTCCTAGAGTTGATGCTGAGAAATTCGAATCTGTTGTCAAAGCATCTTCCAATGCTG  418
ADTZ_A.tabescens    450   TCATTCCCCGCGTCGACGCAGAAAAGTTTGAGTCAGTGGTCAAAGCCTCTAGCAACGCAG  509
ADTZ_sint           419   ATCAAGGTTCTGCCTTGTTCACCAAATTGAAACAACACATCTATGCCTTGTCTCCAGAGT  478
ADTZ_A.tabescens    510   ACCAGGGCTCGGCACTATTCACCAAGTTGAAACAACACATATATGCGCTTTCTCCTGAGT  569
ADTZ_sint           479   CTGCCTTGTTCATTGGTAAGCGTAAAGATGGTCATGTTTCCAACTACTACTTGGGTGAAC  538
ADTZ_A.tabescens    570   CAGCGCTATTCATTGGCAAAAGGAAGGACGGTCACGTATCAAATTACTATCTTGGTGAAC  629
ADTZ_sint           539   CAGTTGGTGATGCAGAAGTTGATGCCATTCAGAATGTTGCTGAGAAATTGGGTGTTGACA  598
ADTZ_A.tabescens    630   CTGTTGGAGATGCTGAGGTCGATGCTATCCAGAATGTCGCTGAGAAGTTAGGCGTTGATA  689
ADTZ_sint           599   TTTTGAACACTAGAGTTAAGAAGAATGGTGCTGGAGATTACACTCTGTTGGTTGCTTCTG  658
ADTZ_A.tabescens    690   TCCTCAATACTCGCGTGAAGAAGAATGGAGCGGGTGATTACACGCTCTTAGTTGCCTCTG  749
ADTZ_sint           659   CCAAAACATCTCCTCCATCTGTTCATGACTTCCAGATTGATTCTACTCCTGCCAAGTTGA  718
ADTZ_A.tabescens    750   CTAAAACCAGTCCACCCTCCGTGCATGACTTCCAAATCGACTCAACTCCGGCTAAATTGA  809
ADTZ_sint           719   CCATTGAGTATGGTGACTATGCTTCTTCCTTGACTAAAGTTGTTGCAGCTTTGCAAGAAG  778
ADTZ_A.tabescens    810   CGATTGAGTATGGCGACTACGCGTCATCTCTAACGAAGGTTGTCGCCGCCCTTCAGGAGG  869
ADTZ_sint           779   CTAAACAGTATACTGCTAATGATCATCAATCTGCTATGATTGAAGGCTATGTCAAGTCCT  838
ADTZ_A.tabescens    870   CCAAACAGTATACCGCGAACGATCATCAATCAGCGATGATCGAAGGCTATGTCAAGTCGT  929
ADTZ_sint           839   TCAACTCTGGTTCCATTCCAGAACACAAAGCTGCCTCAACTGAGTGGGTTAAGGACATTG  898
ADTZ_A.tabescens    930   TCAACTCAGGATCAATTCCGGAACACAAAGCTGCGTCAACAGAATGGGTGAAAGATATTG  989
ADTZ_sint           899   GTCCAGTTGTTGAGTCCTACATTGGTTTCGTTGAGACCTATGTTGATCCATATGGTGGAA  958
ADTZ_A.tabescens    990   GACCGGTTGTAGAGTCCTACATCGGGTTCGTCGAAACCTATGTCGACCCATATGGCGGAC  1049
ADTZ_sint           959   GAGCTGAGTGGGAAGGTTTCACAGCCATTGTTGACAAACAACTGTCTGCCAAGTATGAAG  1018
ADTZ_A.tabescens    1050  GCGCGGAATGGGAGGGTTTCACTGCCATCGTCGACAAGCAGCTGAGTGCGAAGTACGAAG  1109
ADTZ_sint           1019  CTCTGGTGAATGGAGCACCAAAGTTGATCAAGTCATTGCCATGGGGTACTGACTTCGAAG  1078
ADTZ_A.tabescens    1110  CATTGGTTAACGGTGCTCCTAAGTTGATCAAGAGTCTTCCGTGGGGAACGGACTTCGAGG  1169
ADTZ_sint           1079  TTGATGTGTTCAGAAAACCAGACTTCACTGCTTTGGAAGTTGTTTCCTTCGCTACTGGTG  1138
ADTZ_A.tabescens    1170  TTGACGTCTTCAGGAAGCCGGACTTTACTGCGTTGGAAGTCGTATCATTTGCAACAGGAG  1229
ADTZ_sint           1139  GTATTCCTGCTGGTATTAACATTCCAAACTATTATGAAGTCAGAGAAAGTACTGGTTTCA  1198
ADTZ_A.tabescens    1230  GTATTCCTGCCGGAATCAATATACCAAACTATTATGAAGTCCGGGAAAGCACAGGGTTTA  1289
ADTZ_sint           1199  AAAATGTTTCATTGGCTAACATTCTGGCTGCTAAAGTTCCAAACGAAGAGTTGACTTTCA  1258
ADTZ_A.tabescens    1290  AGAATGTTTCGCTAGCGAATATTTTGGCGGCCAAGGTACCAAACGAGGAGTTAACTTTCA  1349
ADTZ_sint           1259  TTCATCCAGACGATGTTGAGTTGTACAATGCTTGGGATTCTAGAGCTTTCGAGCTGCAAG  1318
ADTZ_A.tabescens    1350  TCCATCCTGATGACGTAGAACTATATAACGCTTGGGATAGTCGCGCGTTTGAACTTCAGG  1409
ADTZ_sint           1319  TTGCTAATCATGAGTTGTTGGGACATGGTAGTGGCAAGTTGTTCCAAGAAGGTGCAGATG  1378
ADTZ_A.tabescens    1410  TGGCCAACCACGAACTTTTGGGTCATGGCTCCGGCAAGCTTTTCCAAGAAGGTGCTGATG  1469
ADTZ_sint           1379  GTAAACTGAACTTCGATCCTGAGAAAGTCATCAATCCATTGACTGGCAAACCTATCACTT  1438
ADTZ_A.tabescens    1470  GGAAACTGAACTTCGATCCCGAAAAGGTCATAAACCCTCTGACTGGAAAGCCGATAACTT  1529
ADTZ_sint           1439  CTTGGTACAAACCAGGTCAAACACCAGATTCTGTGTTGGGTGAAGTTAGTTCTTCCATGG  1498
ADTZ_A.tabescens    1530  CATGGTATAAGCCAGGGCAAACGCCGGATTCTGTTTTAGGCGAAGTGTCGTCGTCAATGG  1589
ADTZ_sint           1499  AAGAGTGCAGAGCTGAGACTGTTGCCTTGTACTTGGTTTCCAATTTGGACATCTTGAAAA  1558
ADTZ_A.tabescens    1590  AAGAATGTCGGGCGGAGACCGTAGCGCTCTACTTGGTTAGCAACCTCGATATTCTTAAAA  1649
ADTZ_sint           1559  TCTTCAACTACGTTGACAAACAAGATATTGAAGACATTCAGTACATCACTTTCTTGTTGA  1618
ADTZ_A.tabescens    1650  TTTTCAATTACGTCGACAAGCAAGACATTGAAGATATCCAGTACATCACGTTCTTGCTTA  1709
ADTZ_sint           1619  TGGCTAGAGCTGGTTTGAGAGCTTTGGAATTCTATGATCCTGCTACCAAGAAACATGGTC  1678
ADTZ_A.tabescens    1710  TGGCCCGCGCTGGTCTGCGGGCACTAGAGTTTTATGATCCAGCCACCAAGAAGCACGGAC  1769
ADTZ_sint           1679  AAGCTCACATGCAAGCTCGTATGGGTATCACTCAGTACTTGATTCAAGCTGGTATTGCTA  1738
ADTZ_A.tabescens    1770  AGGCACATATGCAGGCCAGAATGGGCATAACCCAGTACCTGATTCAAGCTGGGATTGCGA  1829
ADTZ_sint           1739  GATTGGAATTGATTCAAGATGCTAATGGTGAATTGGAGAACTTGTATGTTAGAGTTGATA  1798
ADTZ_A.tabescens    1830  GACTTGAATTGATCCAGGATGCCAACGGCGAACTCGAAAACTTATACGTTCGGGTTGACC  1889
ADTZ_sint           1799  GAGAGAAAGTCTTGTCCAAAGGCAAAGAAGTTGTTGGTCAACTGTTGATTGAATTGCAAG  1858
ADTZ_A.tabescens    1890  GGGAGAAAGTGTTGTCCAAAGGAAAGGAGGTTGTTGGTCAATTGCTGATCGAACTCCAAG  1949
ADTZ_sint           1859  TTAG--AAAGTCAACTGCTGATGGTACAGGATCTAGAGACTTCTACACTACCTTGACTGA  1916
ADTZ_A.tabescens    1950  TCCGGAAAAGT--ACCGCAGACGGCACCGGCTCCCGAGATTTCTACACAACGCTGACCGA  2007
ADTZ_sint           1917  ACCAATCTCTGGTTGGGAAGGTAAGATCAGAGACATTGTGCTGAAGAAGAAGTTGCCTCG  1976
ADTZ_A.tabescens    2008  ACCAATCTCTGGATGGGAGGGCAAGATCCGAGACATCGTTTTGAAGAAGAAGCTTCCTCG  2067
ADTZ_sint           1977  TAAGATCTTCGTTCAACCTAACACCTTCGTTGTCAATGGTGAAGTTCAGTTGAAAGAGTA  2036
ADTZ_A.tabescens    2068  AAAAATCTTTGTCCAACCCAATACATTTGTCGTCAACGGCGAAGTCCAGCTCAAAGAGTA  2127
ADTZ_sint           2037  TCCATTGACTGCAGCTGGAGTGATTGAATCCTTCATTGAGAGAAGATTGT            2086
ADTZ_A.tabescens    2128  TCCTTTGACGGCTGCCGGGGTAATTGAAAGTTTCATTGAGAGACGATTGT            2177
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Fig. 1. Sequence alignment visualization of the codon-optimized *adtz* gene and the natural *adtz* gene

of *Armillaria tabescens* (GenBank AY941095.1, https://www.ncbi.nlm.nih.gov/genbank/). Optimization of the codon composition was carried out by ZAO Evrogen (Russia) using the codon frequency table for *Pichia pastoris* (https://www.kazusa.or.jp).

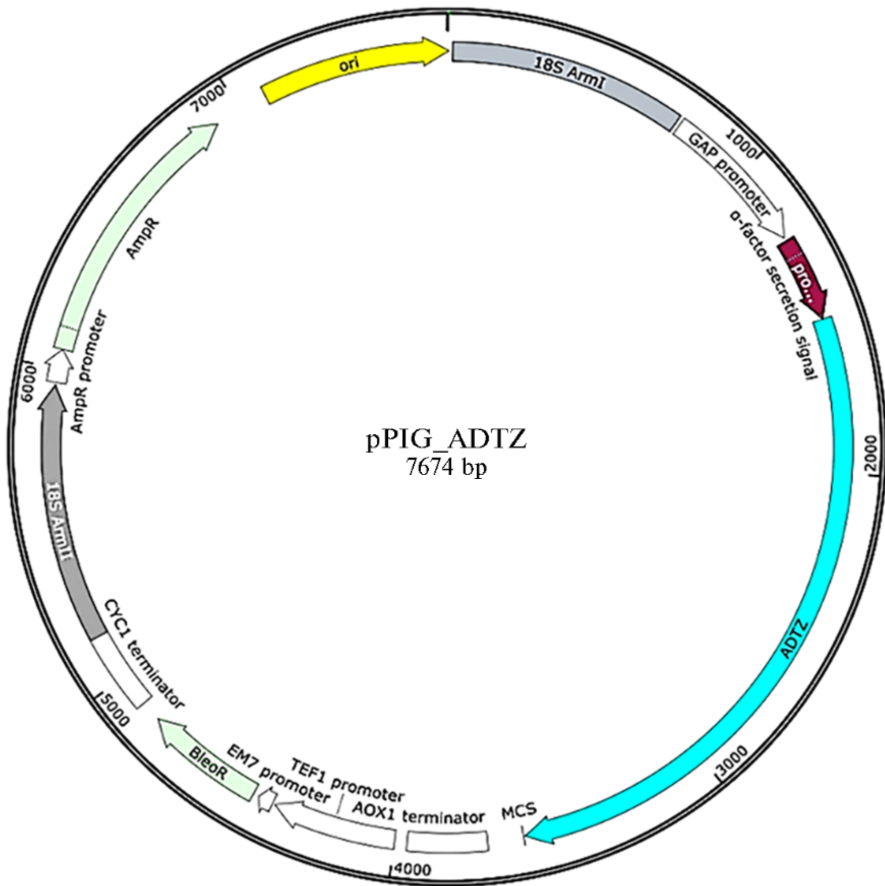
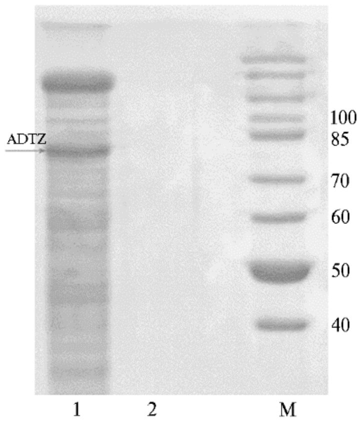


Fig. 2. A map of the pPIG-ADTZ plasmid obtained by cloning the sequence of the synthesized aflatoxin-detoxphyzyme (ADTZ) ***adtz* (*synthetic_ADTZ*) gene into the pPIG-1 vector.**

The resulting recombinant gene was integrated into the pPIG-1 vector by the restriction ligation method. Restriction analysis of the resulting new plasmid, after double digestion with HindIII and NotI, resulted in 5500 and 2100 bp products, confirming the correct integration of the target sequence into the pPIG-1 vector. The resulting recombinant plasmid (Fig. 2) was called pPIG-ADTZ.

Plasmid pPIG-ADTZ, linearized with restriction endonuclease ApaI, was electroporated into competent *P. pastoris* GS115 cells, and transformants were selected on YPD medium added with zeocin. Cloning resulted in 154 clonal colonies. Genomic DNA isolated from 70 randomly selected transformed clones was analyzed by PCR to identify the *adtz* gene insert. PCR analysis of the DNA of these transformants grown on the selective media showed that at least 54 clones contained the target *adtz* insert. Among them, the ADTZ-14 clone turned out to be the most productive and was used for further work. Already after 72 h of culture, expression of ADTZ in this clone led to the accumulation in the CL of the extracellular recombinant protein. The size of this protein according to the SDS-PAGE analysis was 78±3 kDa (Fig. 3), while in the CL of the nontransformed recipient *P. pastoris* GS115 we did not find proteins of a comparable size. In the CL of the transformed ADTZ-14 clone, the total protein concentration was 2.1 mg/ml.



Fid. 3. Electrophoregram of the culture liquid proteins of the strain *Pichia pastoris* ADTZ-14 (1) **transformed with the recombinant pPIG-ADTZ vector containing the synthesized aflatoxin-detoxifizyme (ADTZ) *adtz* gene, and the recipient *P. pastoris* GS115** (2). M — molecular weight marker PageRuler™ 26614 (Thermo Fisher Scientific, USA).

Comparison of the degradation kinetics of AFB_1 and AFG_1 in CL of *P. pastoris* ADTZ-14 showed that the recombinant enzyme is capable of destroying both toxins; however, its efficiency against AFB_1 turned out to be significantly higher than for AFG_1. Thus, under the action of CL of the producer of recombinant extracellular ADTZ, already after 2 h of incubation, the concentration of AFB_1 toxin decreased by approximately 14% compared to the initial level, while for AFG1 the decrease was only 4% (Fig. 4).

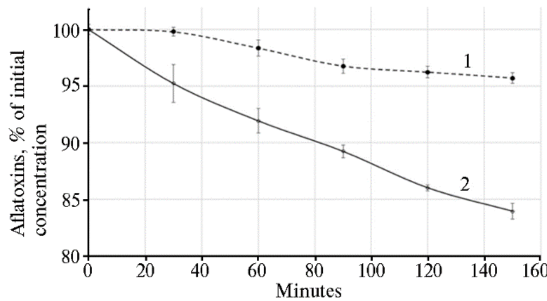


Fig. 4. Degradation kinetics of aflatoxins G_1 (1) **and B_1** (2) **in the cell-free culture fluid of the *Pichia pastoris* ADTZ-14 strain transformed with the pPIG-ADTZ recombinant vector conyaining the synthesized aflatoxin-detoxphyzyme** (ADTZ) ***adtz* gene at 40 °C and pH 6.7** (n = 3, M±SD).

The obtained results were consistent with the data of other authors who noted the high specificity of intracellular ADTZ from *A. tabescens* to AFV_1 [29]. In this regard, we studied the degradation activity of recombinant ADTZ with respect to the indicated toxin during its longer incubation with cell-free CL of the *P. pastoris* ADTZ-14 strain.

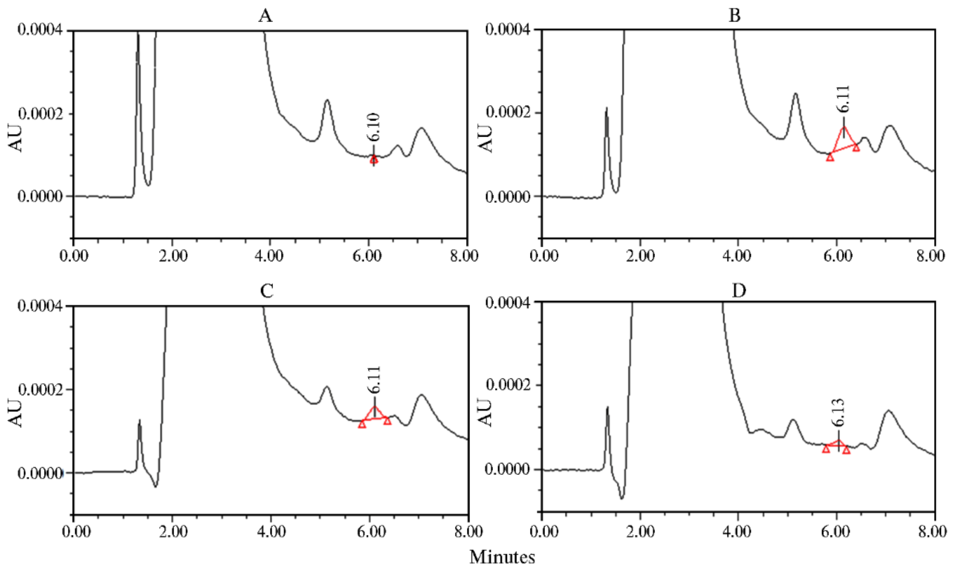


Fig. 5. Chromatograms of culture liquid (CL) **samples of Pichia pastoris** (incubation at 30 °C and pH 7.0).

A: CL of the non-transformed recipient strain GS115 (control).

B: CL GS115 + aflatoxin B_1 (APB[1], 1 μg/ml) after incubation (control sample). The peak on the chromatogram corresponds to 10 ng of AFB_1 in the injected sample.

C and D: CL of the *P. pastoris* ADTZ-14 strain transformed with the pPIG-ADTZ recom-

binant vector containing the synthesized aflatoxin-detoxiphyzyme (ADTZ) *adtz* gene, + AFV_1 (1 μg/ml) after 3 and 5 days of incubation, respectively.

In these experiments, it was found that after 3 days the concentration of the toxin added to the CL decreased by almost 2 times, and after 5 days the efficiency of its degradation reached 80% (Fig. 5, Table).

Dynamics of enzymatic degradation of aflatoxin B_1 (AFB_1) **in the cell-free culture liquid** (CL) **of the *Pichia pastoris* ADTZ-14 transformant straindecreting the recombinant aflatoxin-detoxphyzyme (ADTZ)** (n = 6, M±SD)

Strain	Incubation time				
	0 h	72 h		120 h	
	AFB_1, μg/ml	AFB_1, μg/ml	degradation, %	AFB_1, μg/ml	degradation, %
ADTZ-14	0.97±0.01	0.57±0.06	41.2[a]	0.19±0.04	80.4[b]
GS115 (control)	0.98±0.01	1.01±0.01	0.0[c]	0.90±0.05	0.1[c]

N o t e. The ADTZ-14 strain was obtained by transformation of the *P. pastoris* GS115 recipient with the pPIG-ADTZ recombinant vector with the synthesized aflatoxin-detoxphyzyme (ADTZ) *adtz* gene. Before incubation, AFB_1 was added to the culture liquid (CL) to a concentration of 1 μg/ml; for 0 h, the concentrations detected in the CL samples before incubation are indicated (opening from 96 to 99%).
[abc] Differences between percent degradation marked with different letters are statistically significant at $p \leq 0.05$.

The data we presented here indicate a rather high biotechnological potential of the new producer of recombinant ADTZ and expand the so far limited spectrum of recombinant enzymes of other xylothorophic fungi degrading AFB_1 obtained using the system of heterologous expression in *P. pastoris* [39].

It should also be noted that the ADTZ-14 producer was characterized by a rather high level of expression of extracellular proteins for *P. pastoris*. Probably, the use of a synthetic gene with optimized codons contributed to an increase in the productivity of yeast cells. A similar approach has been successfully used previously for the expression of bacterial α-amylase in *P. pastoris* [40]. However, according to recent data, the use of synthetic genes can lead to protein misfolding, degradation, and a decrease in its activity and stability. This may be the cause of partial degradation of secreted recombinant proteins [41], which, as noted above, we also observed in our experiments with electrophoretic analysis of the CL of the transformed clone ADTZ-14. Therefore, it is necessary to continue research on increasing the efficiency of AFB_1 degradation by recombinant ADTZ, additional testing of the effect of this recombinant enzyme on other aflatoxins, as well as experiments on the treatment of crop products contaminated with AFB_1 with the enzyme preparation. It is also possible that heterologous expression using other eukaryotes, e.g., filamentous fungi which are used for bioprocessing of feed to increase its nutritional value, will allow for obtaining new producers of highly active extracellular ADTZ. Such producers could be promising for the simultaneous decontamination of plant raw material contaminated with aflatoxin and increasing the availability of their nutritional components.

Thus, this article is the first report on the production of a recombinant protein capable of cleaving AFB_1 using an expression system developed by us earlier to increase the copy number of heterologous genes in *Pichia pastoris*. Yeast cells were transformed with the pPIG-ADTZ plasmid and 154 recombinant clones of *P. pastoris* were generated, 77% of which contained the target sequence of the ADTZ synthetic aflatoxin-detoxiphyzyme *adtz* gene. The protein yield of the most productive ADTZ-14 transformant was 2.1 mg/ml cell-free culture liquid, and in this case, about half of all extracellular protein pool was recombinant ADTZ. Incubation of AFB_1 with this recombinant ADTZ led to 80% degradation of the added toxin. The transformant strain *P. pastoris* ADTZ-14, secreting functional ADTZ, can be a producer of an accessible and sufficiently active recombinant enzyme for AFB_1 degradation. Based on *P. pastoris* ADTZ-14, preparations for the enzymatic degradation of AFB_1 can be developed in the future. Confirmation

of the decontamination potential of the recombinant enzyme will indicate the feasibility of optimizing biotechnology to increase the yield of the target product and develop its formulation.

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