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Gene from *Aureobasidium pullulans* from Egypt

Abstract

Sequancing of pullulanase from the fungus *Aureobasidium pullulans* isolated from Egypt soil; Genomic DNA of pullulanase was determined for the first time using PCR, according to Baser program, Pullulanase nucleotide collection from *Aureobasidium pullulans* was blasted which showed similarity using NCBI significant alignment with *Aureobasidium namibiae* CBS 147.97 hypothetical protein partial mRNA and 46 % with *Aureobasidium pullulans* JQ624241 and AF470619; Identified sequenced fragment was 2051 bp. and G+C content is 50.5% with molecular mass 63 KDa.

Keywords: pullulanase, *Aureobasidium pullulans*, genomic, Sequence, amino acid

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Introduction

Aureobasidium pullulans (De Bary) Arnaud, distributed in large numbers on dead plants in 1918, isolated from air, food, soil, textiles and wood.[1] *Aureobasidium pullulans* was isolated from the soil in Fayoum Governorate soil and identified by AUMC 2997.[2] Pullulanase as pullulan 6- glucanohydrolase (EC 3.2.1.41) cleaves α-1,6-glycosidic linkages in pullulan, a polysaccharide produced by *Aureobasidium pullulans* that consists of α-1,6 linked maltotriose units. Together with glucoamylases, β-amylases, and α-amylases, pullulanases utilized in the saccharification of starch.[3] The utmost production of pullulanase from Klebsilla aerogenes NCIM 2239 (78.62U/ml) was spotted at 48 h. Temperature was 37°C and optimized pH was 7.0. The *Klebsilla* Sp. was found to display maximum pullulanase production using peptone and starch.[4] Redefinition the four varieties of *A. pullulans* as separate species, the 25.43, 29.62 Mb genomes of those four varieties of *A. pullulans* encode between 10266 and 11866 predicted proteins.[5] The specific pullulanase (DBEs) and isoamylase found and purified in the developing maize endosperm. Based on the similarity with the cDNA of *Oryza sativa*, which encodes pullulanase (DBE), the cDNA clone Zpu1 was isolated. The comparative of ZPU1, protein product with 18 other DBEs recognized motifs to both pullulanase type enzymes and isoamylase as specific class sequence blocks. Zpu1 hybridized of genomic DNA defined as single copy gene, zpu1 located on chromosome no. 2. mRNA Zpu1was considerable in endosperm during biosynthes of starch, but wasn't determine within the root or leaf. Anti-ZPU1 antiserum specifically known ZPU1 protein in developing endosperm approximately 100 kD, but n't found in leaves. Isoamylase (DBEs) and pullulanase were purified from maize kernels extracts. Zpu1 cDNA cloned from hybrid maize endosperm cDNA library by rice cDNA fragment was used as hybridization probe. The entire three overlapping bacteriophage l clones (designated l3C, l14-1, and l17C) (accession no. AF080567) created Zpu1 cDNA nucleotide sequence of 3261 bp. ZPU1 of about 106kD contains the initial ATG, a continuous ORF of 2886 bp, and can predict a polypeptide of 962 amino acids. Protein is high similar in sequence to rice, shown 78% identity between 880 aligned residues with three gaps within the alignment.[6] Cloned and sequenced the pullulanase from Spinach by peptide sequences of purified enzyme. Its ORF codes for a protein of 964 aa which act as pullulanase precursor. The N-terminal transit peptide is composed of mature protein and 65 amino acids, calculated molecular mass is 99 kDa for 899 amino acids.[7] A gene encoding the dextrinase limit of the starch debranching enzyme, LD of *Hordeum vulgare*. The gene encodes a 904-aa residue protein with a measured molecular mass of 98.6 KDa. This is also in accordance with the SDS-PAGE calculation of a value of 105 KDa. The coding sequence is interrupted by 26 introns, which are between 93 and 825 bp in length. Vary 27 exons in length from 53 - 197 bp. Sequenced peptide fragments occupy 70% of the entire protein sequence, showing 62% and 77% identity with spinach and rice starch debranching enzymes, 37% homogeneity with *Klebsiella* pullulanase. Sequence alignment supports the multi-domain architecture and and recognises secondary components of the catalytic barrel substrate structure, catalytic residues and specificity-related motifs that are distinctive to members of the glycoside hydrolase family 13 that cleave alpha-1,6-glucosidic bonds.[8] Type I pullulanase a completely unique from *Fervidobacterium nodosum* Rt17-B1. Sequence review showed that the type I pullulanase standard motif of the enzyme was (YNWGYDP). Purified FN-pululanase recombinant on SDS-PAGE as a band with a molecular mass of about 95 kDa. At pH 5.0 and 80°C, the enzyme showed the best activity, with a basic activity of 25.93 U/mg.[9]

Materials and methods

Isolation of total RNA

RNA extraction was performed using *Aureobasidium pullulans* (AUMC 2997), which was isolated from Fayoum Governorate soil: SV Total RNA isolation system (Promega Kits), determination of RNA concentration by site http://www.pubquizhelp.com/other/dnacalculator.html was Absorbance value OD_{260}=5.5nm. Using spectrophotometer (Jenway 6305), the concentration of RNA was 22µg/µl. Then preparation of running buffer (5X TBE buffer pH 8), and agarose gel 1%, loading dyes 6X MBI Fermentas and preparation of RNA sample.

Isolation of total DNA

Wizard® Genomic DNA Purification Kit (Promega Kits no. Cat. # A1120), determination of DNA concentration, preparation of agarose gel 1% and preparation of DNA sample. Absorbance value OD_{260}=5 nm, the Concentration of DNA was 25µg/µl.

Protocol of RT-PCR and PCR

Protocol RT, (Access Quik™ RT-PCR system (promega kits). Transeliminator (ms major science, model muv 2020.312) was used to check on agarose gel 1 % and gel is running at 60 mA. (2µl loading dye 6X is mixed with 10 µl from the sample) then, Sweep up the product of PCR (Wizard SV gel and PCR cleaning system, promega kits) check again by transeliminator, sequencing for sample by (Gatac

Home Company 'Germany' with Sanger sequencing protocol), gave result of sequence, database was searched for sequence producing significant alignments using NCBI http://www.ncbi.nlm.nih.gov and sequence alignment with NCBI.

I. Design the primers as shown in the table 1 in the result.

II. We had 48 samples of PCR- product (Promega Kits) & RT- PCR (Promega Kits) after design primers No. 1, 2 and 3 with different concentration and different programs and we had 2 good samples no. 1& 2 Then, 5 samples of PCR- product & RT- PCR after design primer No. 4 with different concentration and different programs and we had 3 good samples no. 3, 4 & 5 After that, we had 25 samples of PCR- product after design primers No. 5, 6 and 7 with different concentration and different programs and we had one good sample no. 6 and also, 43 samples of PCR- product after design primers No. 8, 9 and 10 with different concentration and different programs and we had 7 good samples no. 7, 8, 9, 10, 11, 12 and 13 last, we had 66 samples of PCR- product after design primers No. 11, 12 and 13 with different concentration and different programs and we had 10 good samples no. 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23 as Figure 1.

III. Transeliminator was used to check the bands of agarose gel.

IV. Digram of sequence as Figure 1.

V. Alignments with NCBI for all sequence.

VI. DNA Baser program v2.80 for overlapping of sequences was used, samples from no. (1- 22) were loaded to the program according to identity percentage, overlapping and assembly scores.

VII. Partial sequence of pullulanase: Molecular mass (http://www.bioinformatics.org/sms2/dna_mw.html).

1. Sequences significant alignments with NCBI: www.ncbi.nlm.nih.gov.

2. Translation of nucleotides to protein sequence: https://web.expasy.org/translate/ and http://www.bioinformatics.org/sms2/protein_mw.html

VIII. Pairwise Sequence Alignment between *Aureobasidium pullulans* JQ624241 and AF470619 as Figure 2. (https://www.ebi.ac.uk/Tools/psa/emboss_stretcher/nucleotide.html).

IX. Open reading frame (https://www.ncbi.nlm.nih.gov/orffinder/).

Sequence on NCBI

Many changes were make to publish the sequence in http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html, convert DNA sequence into its reverse complement by http://www.bioinformatics.org , and then Change the translation to reading frame 5'3' Frame 1. The final sequence recorded under GenBank accession number BankIt1512066 *Aureobasidium* (JQ624241).

Research involving human participants and/or animals:

I am not using any animal studies, my study on fungi (*Aureobasidium pullulanse*), the gen recorded on NCBI under no. (GenBank accession number BankIt1512066 *Aureobasidium* (JQ624241)).

Results and discussion

Design the primers; shown at Table 1.

Table 1 Primers design

No. of Primer	Forward (F)	Reverse (R)	Reasons
1St.	5' -YTNTGGGCNCYNACNGCN -3'	5'-GGNCTRATRCCNACCTTRATR -3'	Designed as amino acids according to conserved regions among all pullulanase I as this: In Spinach *(Spinacia oleracea L.), Zea mays, Thermus sp.* and *Bacillus stearothermophilus*: YNWGYNP, In *Fervidobacterium sp., Thermotoga maritima, Bacteroides thetaiotaomicron, Bacillus acidopullulyticus, Caldicellulosiruptor saccharolyticus, Klebsiella aerogenes and Klebsiella pneumoniae*: YNWGYDP, Also, in *Spinacia oleracea, Zea mays and Klebsiella*: LWAPTAQ
2nd	5' AACATAGATGGTGTAGAGGGT 3'	5'-GATAGTTTCTATCGGCTGCAT- 3'	Similar to primer used for sequencing of Spinach gene.
3rd	5' TGCTTTCACTTCACAGGACTCAGC 3'	5' TCCATCTTCATCTCGGATGGCAGT -3'	Primer design according to Spinach sequence with help of: www.idtdna.com/Scitools/Applications/Primerquest/
4th	5'-TTACCTATGTCGGCTCCT-3'	5'-TAACAAATTTGGTTTCAGTA-3'	According to defined parts of sequence from samples no. 10, 45 in comparison to *Spinacia oleracea* and *Zea mays*.
5th	5'- GTTGAGCTGCAACCAGAAAATG -3'	5'- CCCTGTGTTGTGAAAGTG -3'	According to sequence of *Zea mays* and definition parts from our sequence.

Table continued...

No. of Primer	Forward (F)	Reverse (R)	Reasons
6th	5'- CCAAGGCTTGATTCCTTCTCTG -3'	5'- GAGGTCCATCGGAGTCTTTAGAC- 3'	According to sequence of *Zea mays* and defined parts from our sequence.
7th	5'- ACCTGTCTCACAACAACTTTC -3'	5'- CAGCCAATAGAAACTATC -3'	According to sequence of *Zea mays* and definition parts from our sequence.
8th	5'- CGCACAGGGGCTCTTGTTGG -3'	5'- GGAAAATAGGGTGGTTCACC -3'	According to sequence of *Zea mays* and defined parts from our sequence.
9th	5'- CCCTGTGGTTTGGGGCGTTC -3'	5'-GATCAAGCGACTTAGATCGTAG -3'	According to sequence of *Zea mays* and defined parts from our sequence.
10th	5'- GTTGACGGGTTCAGATTTGATC -3'	5'- CGCAGACTGCCTGGCTAACG -3'	According to sequence of *Zea mays* and defined parts from our sequence.
11th	5'- CCG TCG TCA GCT TCG CCG -3'	5'- GTT CTC CAA TCT TGG CTT CAT C- 3'	According to sequence of *Zea mays* and defined parts from our sequence:
12th	5'-GTT GAT GTT GCC ACC TTG GTG-3'	5'- GCA CAA TAT CAC TTG CTG -3'	Designed of the twelfth primer according to sequence of *Zea mays* and definition parts from our sequence:
13th	5'- CAG ATG GTC CAA GTC GTA TC -3'	5'- GGA TGC AAC TGA AGC CGC AG-3'	According to *Zea mays* sequence and defined parts from our sequence:

Diagram of different sequences; shown as Figure 1.

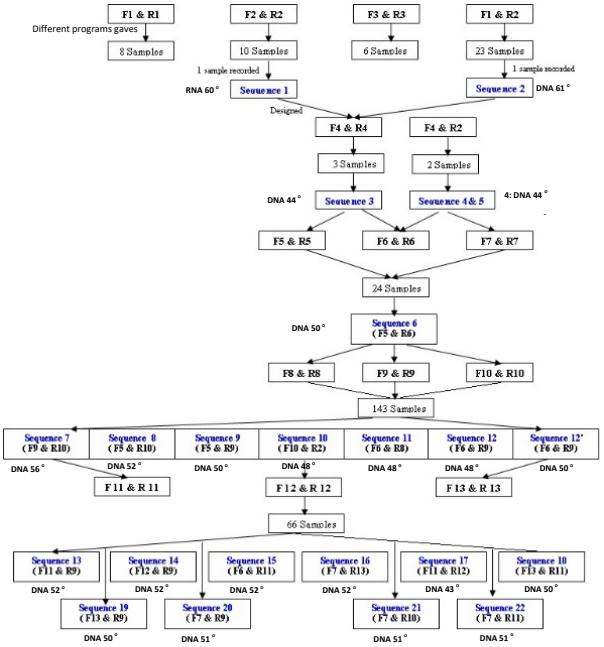

Figure 1 Diagram of different sequences.

Shown the samples of PCR- product & RT- PCR; shown as Figure 2.

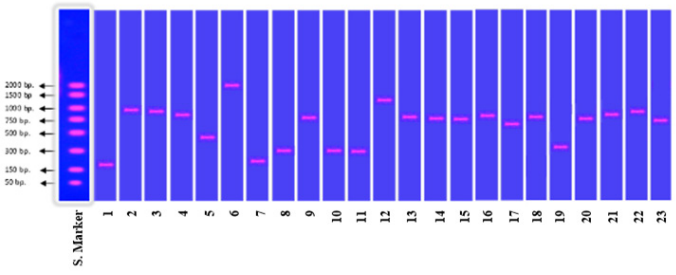

Figure 2 The samples of PCR- product & RT- PCR.

Partial sequence of pullulanase: https://www.ncbi.nlm.nih.gov/nuccore/JQ624241; Analyze sequence: Length: 2051, Molecular mass 633894.37 Da (63 KDa), G+C content: 50.5 %, GenBank accession; number BankIt1512066 *Aureobasidium* JQ624241.

Sequences significant alignments with NCBI

Somewhat similar sequences (blastn): *Aureobasidium namibiae* CBS 147.97 hypothetical protein partial mRNA 77%.

Blastn two sequences (AF470619 and JQ624241); shown as Figure 3.

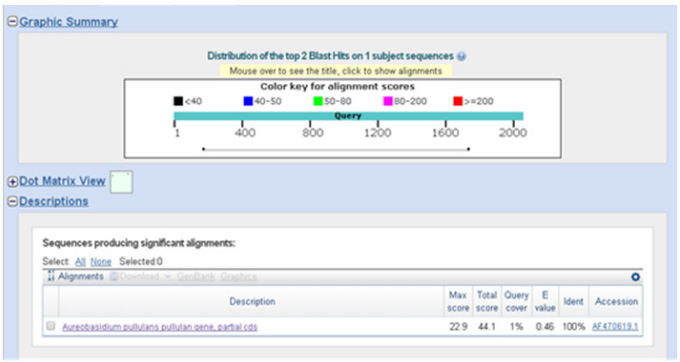

Figure 3 Alignment between AF470619 and JQ624241.

1. Translation of nucleotides to protein sequence: Translation gave number of amino acids: 683, Molecular weight: 74.23 kDa.
2. Pairwise Sequence Alignment (NUCLEOTIDE) by EMBOSS Stretcher between *Aureobasidium pullulans* JQ624241 and AF470619: Length: 2118, Identity: 976/2118 (46.1%), Similarity: 976/2118 (46.1%), Gaps: 369/2118 (17.4%).

Open reading frame*:* Determined ORFs founded was 22.

Discussion

Pullulanase as pullulan 6- glucanohydrolase (EC 3.2.1.41) cleaves α-1,6-glycosidic linkages in pullulan, a polysaccharide produced by *Aureobasidium pullulans* that consists of α-1,6 linked maltotriose

units.[3] The α-1,6 bond in pullulan is hydrolyzed by pullulanase, which belongs to the family of glycosyl hydrolases 57.[10] The pullulanase from *Aureobasidium pullulans* cleaves the α-1,6-glycosidic linkages of pullulan and starch. Pullulanase gene was not sequenced from *Aureobasidium pullulans* and this is the first record of pullulanase gene sequence from Egypt, although it was deduced from *Oryza sativa*, *Spinacia oleracea* and *Zea mays* according to.[2,11]

In this work, genomic DNA of pullulanase from *Aureobasidium pullulans*, was partially sequenced to 2604 bp (Accession no. BankIt 1512066, JQ624241), with the molecular weight is 63 KDa, which is similar to the 73 KDa value estimated by SDS-PAGE, and the G + C content is 50.5%. Shows that the 25.43-29.6 2 Mb genomes of these four varieties of *A. pullulans* encode 10266 and 11866 predicted proteins.[6] Taking the purified enzyme peptide sequence as a starting point, and using PCR technology and cDNA library screening, the spinach pullulanase gene was cloned and sequenced. The cDNA for pullulanase nucleotide sequence of 3437 bp Accession no. X83969.[7] Based on the homology with rice (*Oryza sativa*), a cDNA clone Zpu1 was isolated from *Zea mays* endosperm, which encodes a pullulanase-type starch debranching enzyme. The complete cDNA nucleotide sequence of Zpu1 (accession number NM 001111450) is 3261 bp. according to.[6] Pullulanase is purified from rice endosperm to homogeneity. A cDNA clone encoding the full length of rice endosperm debranching enzyme was isolated, and determined its nucleotide sequence to be 3322 bp. The registration number D50602 conforms to.[12] Determine the genomic DNA (EMBL / GenBank / DDBJ accession number AB012915) containing the rice (Oryza sativa, cv. Norin-8) gene encoding pullulanase-type starch debranching enzyme (EC 3.2.1.41). Along the 15,248 bp DNA, the pullulanase gene is divided into 26 exons. The four pullulanase consensus regions are located in the middle of the sequence and are separated by long introns and 1-3 exons. Comparison of rice resumes. The genome structure of Norin-8 pullulanase is the same as the "limit dextrinase" of barley pullulanase.[13] The EMBL/GenBank/ DDBJ accession number (AF022725) indicates that most pullulanase exons are highly conserved. The base alignment of the nucleotide base of rice exon 8 with the barley exon 8-intron 8-exon 9 fragment showed that the 85 bp internal sequence of rice exon 8 was originally an intron The absence of barley and spinach further indicates this possibility.[8] EMBL / GenBank / DDBJ accession number (X83969) pullulanase amino acid residues encoded by 85 bp fragments. NCBI nucleotide shows the nucleotide sequence of the Zpu1 gene of the pullulanase-type starch debranching enzyme from corn, and determined that part of the cds (accession number DQ 195078) is 3601bp. Another maize pullulanase type starch debranching enzyme Zpu1 mRNA, the complete cds (accession number AF 080567) was determined to be 3261 bp. Also, *Oryza sativa* Indica Group cultivar 9308 pullulanase (PUL) gene, complete cds (Accession no. GQ 150892) was determined of 14272 bp. and another *Oryza sativa* Indica Group cultivar Minghui 63 pullulanase (PUL) gene, complete cds (Accession no. GQ 150896) was determined of 13788 bp. The pullulanase-type debranching enzyme gene of the *sorghum bicolor* cultivar SC748-5 was determined, and part of the cds (accession number EF 089721) was determined to be 1209 bp. and another *Sorghum bicolor* cultivar Tx2911 pullulanase type debranching enzyme gene, partial cds (Accession no. EF 089724) was determined of 1219 bp. Recording with *Culex quinquefasciatus* pullulanase, mRNA (Accession no. XM 001870454) was determined of 2007 bp. *Ricinus communis* pullulanase, putative, mRNA (Accession no. XM 002532734) was determined of 2895 bp. and another Accession no. of *Ricinus communis* pullulanase, putative, mRNA (Accession no. XM 002539938) was determined of 890 bp.[7]

The ORF code (accession number BankIt 1512066) of pullulanase from *Aureobasidium pullulans* was determined for 22 open reading frames. In addition, according to records, it is determined that the open reading frame code of the spinach pullulanase gene is a 964 protein with a molecular weight of 106 KDa, which represents the precursor of pullulanase. The N-terminal transit peptide contains 65 amino acids, the mature protein contains 899 amino acids, and the calculated molecular mass is 99 KDa.[5] It shows that the complete Zpu1 (Accession No. NM 001111450) cDNA nucleotide sequence has been determined from three overlapping phage clones. Zpu1 contains a continuous open reading frame starting with 2886 bp ATG. It is estimated that the polypeptide Zpu1 of about 962 amino acids is approximately 106 KDa. The sequence of Zpu1 protein is highly similar to that of rice RE, showing 78% identity in 880 alignment residues. There are three gaps in the alignment. Zpu1 also exhibits extensive similarity with spinach leaf characteristic pullulanase DBE. (Accession No. X83969); these two proteins have no gaps between 882 aligned residues and are 59% identical. The genome and cDNA sequence from barley can predict another pullulanase type DBE (accession number AF022725); Zpu1 is identical to the deduced amino acid sequence at 79% of the 832 aligned residues, with only one amino acid interval. Zpu1 is 46% identical to the pullulanase of *Klebsiella aerogenes.*[6] Pullulanase from the developing rice endosperm (Accession No. D50602). The cDNA contains an OPR of 2958 bp. The mature debranching enzyme of rice seems to be composed of 912 amino acids, and the predicted relative molecular mass is 102069 Da.[12] Similarly, from barley (Hordeum vulgare) shown that the gene encoding starch debranching enzyme limit dextrinase LD, encodes a protein with 904 amino acid residues, and the calculated molecular mass is 98.6 KDa. This is consistent with the 105 KDa value estimated by SDS-PAGE. The sequenced peptide fragments cover 70% of the entire protein sequence, have 62% and 77% homology with spinach and rice starch debranching enzymes, and have 37% homology with *Klebsiella pullulanase*. On the other hand, NEB Cutter shown the nucleotide sequence from Zpu1 gene, partial cds (Accession no. DQ 195078) was determined for three open reading frame that predicts a 156, 119 and 115aa. *Oryza sativa* Indica Group cultivar 9308 pullulanase, complete cds (Accession no. GQ 150892) was determined five open reading frame that predicts a 186, 142, $100^{(1)}$, $100^{(2)}$, $100^{(3)}$- amino acid and *Oryza sativa* Indica Group cultivar Minghui 63 pullulanase (PUL) gene, complete cds (Accession no. GQ 150896) was determined five open reading frame that predicts a 215, 134, 133, 127, 100 aa.[8] The record indicated that the clone and sequencing of the 8.1 kb insert encoding pullulanase from F. pennavorans Ven5 and three large ORFs were identified and identified. The functions can be assigned to ORF1 and ORF2 based on the sequence homology identified by the BLAST algorithm. The G + C content of the entire code is 40.3%.ORF1 encodes pullulanase by subcloning the pullulanase into pUC18 and observing the activity of E. coli transformants on a red-stained pullulan plate. This gene is called pulA. is 2,550 bp, encodes a protein of 849 amino acids, and has a predicted molecular weight of 96.6 KDa. The G + C content of pulA is 41.9%. ORF2 is 2,694 bp, encodes a protein of 897 amino acids, and has a predicted molecular mass of 103.5 KDa. The G + C content of ORF2 is 41.8%. ORF3 is 1,272 bp, encodes a protein of 423 amino acids, and has a predicted molecular mass of 46.1 KDa.

Similarity by the somewhat similar sequences (blastn) for sequence of pullulanase from *Aureobasidium pullulans* (Accession no. BankIt 1512066) shows a high similarity of 77% with that of the mRNA of the hypothetical protein part of *Aureobasidium namibiae* CBS 147.97. *Culex quinquefasciatus* (Acession no. XM 001870454.1) (69%), *Zea mays* (Acession no. DQ195078.1) (51%), *Zea mays* (Acession no. NM 001111450.1) (51%).[14] The comparison record of spinach

sequence with other starch degrading enzymes shows that it is highly similar (58%) to the pullulanase of Klebsiella aerogenes and Bacillus stearothermophilus. At the same time, the rice pullulanase sequence is 80% identical to the spinach enzyme.[7] Pairwise Sequence Alignment (NUCLEOTIDE) by EMBOSS Stretcher between *Aureobasidium pullulans* JQ624241 and AF470619 shown Similarity: 976/2118 (46.1%).

Conclusion

Genomic DNA of pullulanase (EC 3.2.1.41), glycosyl hydrolase family 57 from *Aureobasidium pullulans*, was determined for the first time using polymerase chain reaction, according to Baser program, which was assembled from 16 sequenced using amplified 22 samples, total identified sequenced fragment was 2051 bp. and G+C content is 50.5% with molecular mass 63 KDa. NCBI were used to predict 22 ORF. Translation of nucleotide sequence resulted 683 amino acids, Molecular weight was 74.23 kDa. Pullulanase nucleotide collection from *Aureobasidium pullulans* showed similarity with *Aureobasidium namibiae* CBS 147.97 hypothetical protein partial mRNA 77%, while *Aureobasidium pullulans* JQ624241 and AF470619 which showed 46.1% similarity. This is the first record of pullulanase sequence gene from *Aureobasidium pullulans* from Egypt.

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Conflicts of interest

Not have any conflict of interest statement.

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