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Cloning and Analysis of *pyr*G Gene Encoding Orotidine 5-Monophosphate Decarboxylase of *Aspergillus oryzae* Strain S1

(Pengklonan dan Analisis Gen *pyrG* yang Mengekodkan Orotidina 5-monofosfat Dekarboksilase *Aspergillus oryzae*)

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ABSTRACT

In this study, the pyrG gene which encodes for orotidine 5-monophosphate decarboxylase (OMP decarboxylase) of Aspergillus oryzae strain S1 was cloned and analysed. This 1.8kb A. oryzae pyrG encompasses the 5'-regulatory flanking region (465 bp), open reading frame (899 bp) and 3'-regulatory region (475 bp). The pyrG contained one intron at position 623-687 bp based on the AUGUSTUS and FGENESH (SoftBerry) analysis corresponding to the intron present in the pyrG of A. oryzae (Accession Number: Y13811). In silico analysis showed that the enzyme encoded by the A. oryzae S1 pyrG gene has a theoretical molecular weight of 30.28 kDa and theoretical pI value of 5.92. This enzyme is hydrophilic, located in a region outside of the transmembrane and it functions in the cytoplasm. Five motives such as N-glycosylation site, protein kinase C (PKC) phosphorylation site, casein kinase II (CK-2) phosphorylation site, N-myristolation site and orotidine 5-monophoshate decarboxylase active site have been identified in the pyrG amino acid sequence. The three dimensional structure of this enzyme generated via protein homology modeling using the bioinformatic software, Swiss Model, shows that OMP decarboxylase is a protein with an α/ß barrel structure possessing 8 ß-strands surrounded by 9 α-helices. The amino acid residues involved in the active site have been identified and it is located on one of the ß-strands. The pyrG DNA sequence will be used for the complementation of a pyrG auxotroph mutant of A. oryzae.

Keywords: Aspergillus oryzae; orotidine 5-monophosphate dehydrogenase; pyrG

ABSTRAK

Dalam kajian ini, gen pyrG yang mengekod orotidina 5-monofosfat dekarboksilase (OMP dekarboksilase) Aspergillus oryzae strain S1 telah diklon dan dianalisis. Gen pyrG ~1.8 kb A. oryzae ini merangkumi kawasan pengawalaturan 5' (465 pb), rangka bacaan terbuka (899 pb) dan kawasan pengawalaturan 3' (475 pb). Gen pyrG ini mempunyai satu intron pada kedudukan 623-687 pb berdasarkan kepada analisis AUGUSTUS dan FGENESH (SoftBerry) bersamaan dengan kedudukan intron yang hadir dalam gen pyrG A. oryzae (Nombor Aksesi: Y13811). Analisis in silico menunjukkan bahawa enzim yang dikodkan oleh pyrG A. oryzae strain S1 mempunyai berat molekul teori sebanyak 30.28 kDa dan nilai pI teori bernilai 5.92. Enzim ini bersifat hidrofilik, berada di kawasan luar transmembran dan ia berfungsi di dalam sitoplasma sel. Lima motif telah dikenalpasti dalam jujukan asid amino pyrG iaitu tapak N-glikosilasi, tapak pemfosfatan protein kinase C (PKC), tapak pemfosfatan kasein kinase II (CK-2), tapak N-Miristolasi dan tapak aktif orotidina 5-monofosfat dekarboksilase. Struktur tiga dimensi enzim ini yang dijanakan menggunakan pendekatan pemodelan homologi protein melalui perisian bioinformatik Swiss Model menunjukkan bahawa OMP dekarboksilase adalah protein yang mempunyai struktur α/ß barrel dengan 8 kepingan struktur ß yang dikelilingi oleh 9 struktur heliks. Residu asid amino yang terlibat dalam tapak aktif telah dikenalpasti dan ia berada pada salah satu daripada kepingan struktur ß protein tersebut. Jujukan DNA pyrG ini akan digunakan untuk mengkomplementasikan mutan auksotrof pyrG A. oryzae.

Kata kunci: Aspergillus oryzae; orotidina 5-monofosfat dehidrogenase; pyrG

INTRODUCTION

Aspergillus oryzae is an important filamentous fungus and has a long history of use in the traditional Japanese fermentation industry such as sake (rice wine), shoyu (soy sauce) and miso (soybean paste). Its use in the food industry has proven its safety and thus, *A. oryzae* has been listed as a Generally Recognized As Safe (GRAS) organism (Kobayashi et al. 2007). *A. oryzae* is also a natural host factory in the biotechnology industry for the production of homologous and heterologous proteins and metabolites. The examples of heterologous proteins produced by *A. oryzae* that have already been approved in the food industry are cellulase, α-galactosidase, lipase, phytase, protease and xylanase (Ward et al. 2006). The *A. oryzae* genome consists of eight chromosomes with the genome size of 37 megabase (Mb) containing a total of 12,074 genes encoding proteins longer than 100 amino acid residues (Machida et al. 2005).

In this paper, we report on the cloning and bioinformatic analysis of the *pyrG* gene from *A. oryzae*. The *pyrG* gene encodes for orotidine-5'-monophosphate (OMP) decarboxylase; this enzyme is important for uridine synthesis process in the pyrimidine biosynthesis pathway. Complementation of uridine/uracil auxotrophs using the OMP decarboxylase-encoding gene, whereby *pyrG* deficient strains (*pyrG* mutants) that lack the activity are auxotrophic for uridine and uracil, become resistant to 5-fluoro-orotic acid (5-FOA) which is converted to the toxic intermediate 5-fluoro-UMP in prototrophs (Boeke et al. 1984). Hence, the *pyrG* mutants can only grow on the medium containing uridine and uracil, and direct selection for resistance towards 5-fluoroorotic acid (5-FOA) whereas the wild type (prototroph) strain is 5-FOA sensitive (Long et al. 2008, Gellison 2005, Mattern et al. 1987). The *pyrG* gene is commonly used as an auxotrophic selection marker system because it allows selection for *pyrG* mutants and the *pyrG*$^{+}$ transformants. Yolanda et al. (1989) have described a homologous transformation system based on *A. oryzae* deficiency in OMP decarboxylase (*pyrG*) and a vector containing a functional *A. oryzae pyrG* gene as selection marker. Ultimately, the *pyrG* gene isolated in this study will be used to complement a *pyrG* auxotroph mutant of *A. oryzae* strain S1, a host to be further employed in the production of heterologous proteins.

Materials and Methods

FUNGAL STRAIN

A. oryzae strain S1 was cultivated by inoculating onto Potato Dextrose Agar (PDA) plate and incubated for 5 days at 30°C. Fungal mycelia were grown in Potato Dextrose Yeast Extract (PDYE) and incubated with shaking at 180 rpm, for 2 days at 30°C, prior to genomic DNA isolation. Genomic DNA extraction was carried out using a modified protocol first described by Pich & Schubert (1993).

PCR AMPLIFICATION, GENE CLONING AND SEQUENCING

The *pyrG* region was amplified by polymerase chain reaction (PCR) using Expand High Fidelity PCR System (Roche, USA) with forward primer 5'-GATTAAATATTCTAGACCCAAGCCG-3' and reverse primer 5'-CCTCGGAATAGTCCTCTCGG-3' based on the *pyrG* sequence from the GenBank (Accession No.: Y13811) and the *A. oryzae* strain S1 genome as template. The PCR reactions (50 µL) according to the manufacturer's instructions contained 100 ng of template DNA genome, 10 × Buffer, 0.4 mM (each) dNTPs, 0.4 µM (each) of forward and reverse primers and 2.6 U enzyme mix. The PCR amplification conditions consisted of denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 65°C for 30 s and 72°C for 5 min 30 s, followed by final elongation step at 72°C for 10 min.

The targeted amplicon obtained from PCR were gel purified using MEGAquick-spinTM Agarose Gel DNA Extraction System (Intron Biotechnology, Korea). The PCR product was ligated into pGEM®-T Easy vector (Promega Inc., USA) and transformed into *E. coli* DH5α. Plasmids carrying positive fragments were isolated with the Wizard® Plus SV Minipreps DNA Purification System (Promega Inc., USA) and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

BIOINFORMATIC ANALYSIS

The nucleotide sequence obtained was analysed using the BLAST program from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) to identify and confirm the identity of the amplified fragment with other *pyrG* gene sequences in the GenBank. Bioinformatic sequence analysis was carried out using several software such as gene structure prediction software from AUGUSTUS (http://augustus.gobics.de/) (Stanke et al. 2004) and SoftBerry (http://www.softberry.com/berry.phtml) software. The predicted amino acid sequence was obtained using the Expasy TRANSLATE program (http://www.expasy.ch/tools/dna.html). Amino acid alignment was carried out using ClustalW and BOXSHADE 3.21 (http://www.ch.embnet.org/) software.

Hydrophobicity analysis was carried out using ProtScale programme based on Kyte & Doolittle (1982) to determine the *pyrG* protein hydrophobicity characteristics. THMMM software was used to predict the segment and transmembrane topology of the protein. A few softwares were used to predict the presence of a signal peptide such as SignalP 3.0 (Nielsen et al. 1997) and PrediSi (Hiller et al. 2003). Protein motive analysis was carried out using PROSITE bioinformatic software (Bairoch et al. 1997). Swiss Model (Arnold et al. 2006), an automated protein structure homology-modeling software was used to predict the putative tertiary structure of the orotidine 5-monophosphate decarboxylase enzyme encoded by *pyrG* of *A. oryzae* strain S1 based on the tertiary structure of *Saccharomyces cerevisiae* orotidine 5-monophosphate decarboxylase enzyme (IDQW_A, PDB) as template. The putative 3-D ribbon protein structure was generated using ViewerLite 4.2 software.

Results and Discussion

Isolation of *pyrG* sequence was carried out by amplification using the forward and reverse primers and *A. oryzae* strain S1 genome as template. PCR product of about 1.8 kb *pyrG* amplicon (Figure 1a) was amplified and cloned into the cloning vector, pGEM-T® Easy. Plasmids that carry the target DNA were analysed via restriction enzyme analysis using *Eco*RI. Digestion with *Eco*RI showed one insert with the size of ~1.8 kb (Figure 1(b)). Verification of the *pyrG* sequence was done via sequencing. Sequence analysis showed that the *pyrG* sequence has 99% identity with *pyrG* of *A. oryzae* (Accession No.: Y13811) from the NCBI GenBank. The 1% difference may be due to strain variation.

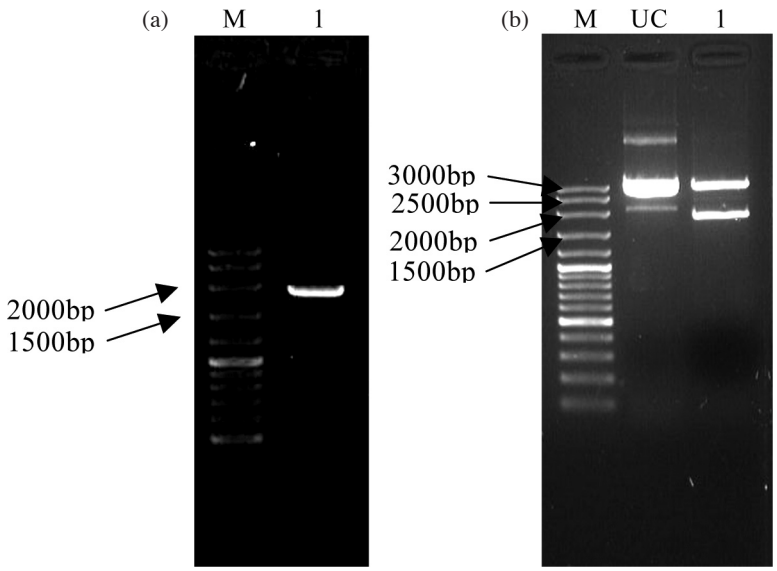

FIGURE 1. (a) Electrophoretic profile of the PCR amplicons of *pyrG* fragment encompassing the ORF and the 5' and 3'flanking regions (~1.8 kb) and (b) Digestion products of *pyrG* with *Eco*RI. Lanes M: 100 bp DNA ladder; Lane 1 (a): PCR amplicon; Lane 1(b): *Eco*R1 digested DNA; Lane UC: uncut plasmid

The 1.839 kb *A. oryzae pyrG* DNA fragment obtained represented the 5'-regulatory flanking region (465 bp), open reading frame (899 bp) and 3'-regulatory region (475 bp). The *pyrG* consists of one intron containing the consensus GT-AG splice junctions of 65 bp at position 623-687 bp based on the AUGUSTUS and FGENESH (SoftBerry) analysis. Using the FGENESH analysis too, the *pyrG* transcription start site (TSS) was predicted at position 314 bp from the ATG codon and polyadenylation site at position 1,420 bp (Figure 2). The *pyrG* sequence (1,839 bp) was translated to amino acid sequence using TRANSLATE tools and it consists of 277 amino acids encoded by 834 bp coding region. The amino acid alignment between *pyrG* of *A. oryzae* with *A. oryzae* (XP_001826440), *A. terreus* (XP_001218297), *Penicillium nalgiovense* (Q8J269), *Coccidioides immitis* (XP_001247091), *Phycomyces blakesleeanus* (P21593) and *S. cerevisiae* (IDQW_A) showed conserved sequences among species including the enzyme's active site (L/I/V/M/F/T/A)-(L/I/V/M/F)-x-D-x-K-x(2)-D-I-(G/P)-x-T-(L/I/V/M/T/A) (Jacquet et al. 1988) (Figure 3).

In silico analysis using Expasy tools (http://www.expasy.org/tools/) showed that this enzyme is deduced to have a molecular weight of 30.28 kDa with a pI value of 5.92. Hence, it is an acidic protein rich in alanine (9.7%) and serine (8.7%) amino acid residues. Analysis using the ProtScale and THMMM software showed that this enzyme is hydrophilic and located not within the transmembrane. The signal peptide analysis using the signal peptide prediction software showed that there is no signal peptides present within the sequence, indicating it is a non-secreted protein and functions in the cytoplasm. Five motives have been identified in the *pyrG* amino acid sequence using PROSITE software such as N-glycosylation site, protein kinase C (PKC) phosphorylation site, casein kinase II (CK-2) phosphorylation site, N-myristolation site and orotidine 5-monophoshate decarboxylase active site. The three dimensional structure of the OMP decarboxylase enzyme generated via protein homology modeling using the Swiss Model software showed that this protein has a TIM (triose phosphate isomerase) barrel or known as α/ß barrel structure possessing 8 ß-strands surrounded by 9 α-helices (Figure 4). The lysine 95 residue on the active site was identified and located on one of the ß-structure strands.

CONCLUSIONS

The *pyrG* gene of *A. oryzae* strain S1 of 1,839 bp encoding 277 amino acids was amplified, cloned and analysed. The *pyrG* gene was shown to have one intron. The *pyrG* sequence analysed via *in silico* confirmed that this gene encodes for orotidine 5-monophosphate decarboxylase enzyme. Analysis using protein homology modeling showed that OMP decarboxylase of *A. oryzae* strain S1 has an α/ß barrel structure possessing 8 ß-strands surrounded by 9 α-helices. Further study on the *pyrG* DNA sequence involves the complementation of a *pyrG* auxotroph mutant of *A. oryzae* strain S1.

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   1 GATTAAATATTCTAGACCCAAGCCGCTGCTGGAATTGACATTATTATGGCCGATAGGGTT
  61 GGGCTTATTGCTATGTCCCTGAAAGGATATCAAAAGCAGGCAAAAAGCCAGGCATAATCC
 121 CCGCGTGGACGGTACCCTAAGGATAGGCCCTAATCTTATCTACATGTGACTGCATCGATG
 181 TGTTTGGTCAAAATGAGGCATGTGGCTCACCCCACAGGCGGAGAAACGTGTGGCTAGTGC
 241 ATGACAGTCCCCTCCATAGATTCAATTTAATTTTTCGCGGCAATTGTCGTGCAGTTTGTA
 301 TCTACATTTCATTCCATATATCAAGAGTTAGTAGTTGGACATCCTGATTATTTTGTCTAA
 361 TTACTGAAAACTCGAAGTACTAACCTACTAATAAGCCAGTTTCAACCACTAAGTGCTCAT
   1                                                   M  S  S  K  S
 421 TTATACAATATTTGCAGAACCCCGCGCTACCCCTCCATCGCCAACATGTCTTCCAAGTCG
   6  Q  L  T  Y  S  A  R  A  S  K  H  P  N  A  L  V  K  K  L  F
 481 CAATTGACCTACAGCGCACGCGCTAGCAAGCACCCCAATGCGCTCGTAAAGAAGCTCTTC
  26  E  V  A  E  A  K  K  T  N  V  T  V  S  A  D  V  T  T  T  K
 541 GAGGTTGCCGAGGCCAAGAAAACCAATGTCACCGTTTCCGCCGACGTGACAACCACCAAA
  46  E  L  L  D  L  A  D  <-------------------- INTRON ----------
 601 GAGCTGCTGGATTTGGCTGACCgtatgcgcaccggggatgccacttacatgtgatctagt
  53  ---------->  R  L  G  P  Y  I  A  V  I  K  T
 661 aatggttaatggtggattatataacagGACTCGGTCCGTACATTGCCGTGATCAAAACTC
  64 H  I  D  I  L  S  D  F  S  E  E  T  I  T  G  L  K  A  L  A
 721 ACATCGATATCCTCTCCGATTTCAGCGAAGAAACCATCACCGGTCTGAAGGCCCTTGCAG
  84 E  K  H  N  F  L  I  F  E  D  R  K  F  I  D  I  G  N  T  V
 781 AGAAGCACAATTTCCTCATCTTCGAAGATCGCAAGTTCATCGATATCGGAAACACAGTCC
 104 Q  K  Q  Y  H  G  G  T  L  R  I  S  E  W  A  H  I  I  N  C
 841 AAAAGCAGTACCATGGCGGCACTCTGCGTATCTCTGAGTGGGCCCACATCATCAACTGCA
 124 S  I  L  P  G  E  G  I  V  E  A  L  A  Q  T  A  S  A  E  D
 901 GTATTCTGCCCGGTGAGGGTATCGTCGAGGCTCTGGCCCAGACTGCTTCGGCCGAGGACT
 144 F  P  Y  G  S  E  R  G  L  L  I  L  A  E  M  T  S  K  G  S
 961 TCCCCTACGGCTCCGAGAGGGGCCTTTTGATCCTTGCGGAGATGACCTCCAAGGGATCTT
 164 L  A  T  G  Q  Y  T  T  S  S  V  D  Y  A  R  K  Y  K  K  F
1021 TGGCTACCGGTCAATATACTACTTCTTCTGTTGACTATGCTCGGAAGTATAAGAAGTTTG
 184 V  M  G  F  V  S  T  R  H  L  G  E  V  Q  S  E  V  S  S  P
1081 TGATGGGATTCGTCTCGACACGTCACCTTGGCGAGGTTCAGTCTGAAGTTAGCTCGCCTT
 204 S  E  E  E  D  F  V  V  F  T  T  G  V  N  L  S  S  K  G  D
1141 CGGAGGAGGAAGATTTTGTCGTCTTCACGACAGGTGTCAACCTCTCCTCGAAGGGTGACA
 224 K  L  G  Q  Q  Y  Q  T  P  E  S  A  V  G  R  G  A  D  F  I
1201 AGCTGGGACAGCAGTACCAAACTCCTGAGTCGGCTGTTGGACGCGGTGCCGACTTTATTA
 244 I  A  G  R  G  I  Y  A  A  P  D  P  V  E  A  A  K  Q  Y  Q
1261 TTGCTGGCCGTGGAATTTATGCTGCTCCTGATCCCGTGGAGGCGGCGAAGCAGTACCAGA
 264 K  E  G  W  D  A  Y  L  K  R  V  G  A  Q
1321 AGGAGGGATGGGATGCATACCTGAAGCGTGTTGGTGCGCAATAAGTAGTGGTGGATACGT
1381 ACTCCTTTTATGGCAGTATGTCGCAAGTATGATGCGATTTATAAATTCAGCACTCGAAAT
1441 GACTACTACTATGTGTCTACGACAGATACCCTCTCCGTACGAATAAGACACCTGCCTCGA
1501 TATATGGACAAATTCAAAATCAGGGTCAAGGGTCATGTTTCAAAGTCACAACAATCTCCA
1561 ACATAGACGAGAATTTGTACCGGAGTGTCTGAAGGTGCAGCTGGAGATTGGTCTATTTTC
1621 TTAGAGTGGGGTATCACTAATGTACAGTCGGTCACTATCGTACAAACAATCACAATTATA
1681 TACAAGATTTCCCACCACCCCCTACTCTAACACGGCACAATTATCCATCGAGTCAGAGCC
1741 TAGCCACCATTTGGTGCTCTCGTAGAGACCAAAGTATAATCCTGATCCGACAGCGGCCAT
1801 AAACGTGTTGATAGCACACCCTCGGAATAGTCCTCTCGG
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FIGURE 2. Nucleotide and amino acid sequence of *A. oryzae pyrG* gene. The introns are represented with lowercase letters. Start (ATG) and stop (TAA) codons are shown in bold letters. The polyadenylation signal site is represented by boxed letters (grey). The forward and reverse primers are underlined

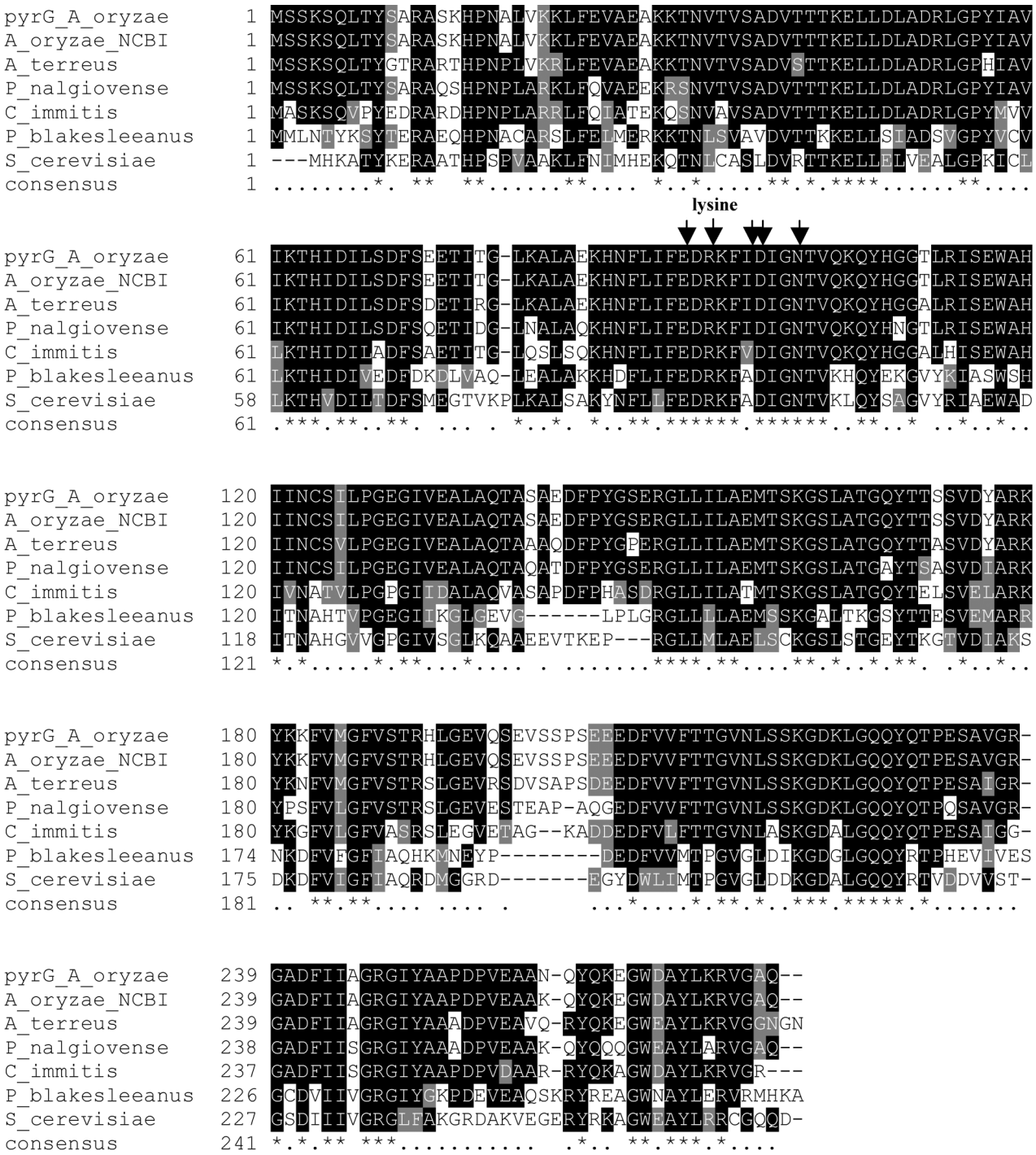

FIGURE 3. Multiple alignment of *pyrG* amino acid sequence between *A. oryzae* strain S1 with *pyrG A. oryzae* (XP_001826440), *A. terreus* (XP_001218297), *P. nalgiovense* (Q8J269), *C. immitis* (XP_001247091), *P. blakesleeanus* (P21593) and *S. cerevisiae* (1DQW_A). The alignment was constructed using the ClustalW and BOXSHADE software. The active site lysine residue is labeled (lysine) while the conserved active site residues are shown with arrows (D-K-D-I-T)

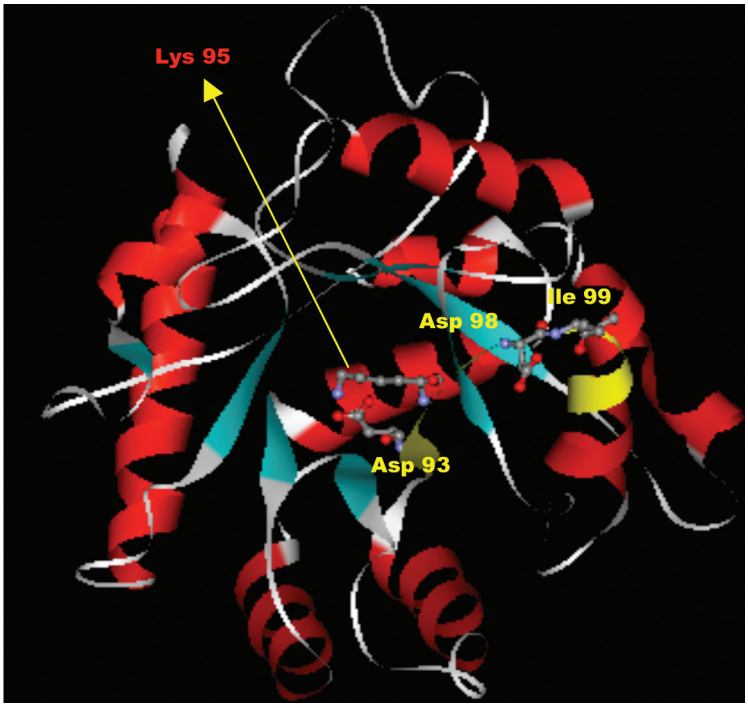

FIGURE 4. The three dimensional structure of *A. oryzae* OMP decarboxylase enzyme shown in ribbon structure using Swiss Model software. ß structure is shown in blue, whereas α-helix is shown in red. Residues on the enzyme's active site are shown in yellow. The residue (lysine 95) on the active site is shown with an arrow

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