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SHORT COMMUNICATION

Annotation and De Novo Sequence Characterization of Extracellular β-Fructofuranosidase from *Penicillium chrysogenum* Strain HKF42

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Abstract The genome of a fungal strain *Penicillium chrysogenum* strain HKF42, which can grow on 20% sucrose has been annotated for 7595 protein coding sequences. On mining of CAZymes, we could annotate a β-fructofuranosidase gene responsible for fructo-oligosaccharides (FOS) synthesis which is a known prebiotic. The enzyme activity was demonstrated and validated with the generation of FOS as kestose and nystose.

Keywords Prebiotic · Fructo-oligosaccharides · *Penicillium* · β-Fructofuranosidase

Prebiotics which can impart nutritional as well as health benefits to human population have recently gained the attention of the people worldwide [1]. Fructo-oligosaccharides (FOS) sometimes also mentioned as oligofructose come under the category of prebiotic food segment which selectively stimulates the beneficial colon bacteria and boosts the health of the host [2, 3]. Owing to their low caloric value, FOS are the most appropriate ingredients which have been incorporated in dairy and bakery products [4]. Hydrolysis of inulin by inulinase and transformation of sucrose by fructosyltransferase are the two widely used methods for the production of FOS [5]. Production of FOS from sucrose is an economical process as compared to FOS obtained by inulin hydrolysis because of low-cost of sucrose [6]. FOS synthesis from sucrose is mediated by two major enzymes Sucrase (sucrose fructosyltransferase, FTase, EC 2.4.1.9) and invertase (β-fructofuranosidase fructohydrolase, FFase, EC 3.2.1.26) [1]. Various FOS synthesized from sucrose include 1-kestose (GF_2), nystose (GF_3), and 1^F-β-fructofuranosylnystose (GF_4) where 1–3 units of fructose are attached in β-(2-1) linkage to the sucrose [4]. The recent increase in market-share of FOS among the prebiotics has propelled the need for exploring novel enzymes and microorganisms having the capacity for high FOS yields along with an increase in the cost effectiveness of the process [7].

Several studies have reported *Penicillium* sp. to be a potential producer of FFase which is utilised for the FOS production [8–10]. In the present study, *Penicillium chrysogenum* strain HKF42 isolated from effluent treatment plant (ETP) was screened for FFase enzyme activity and subsequent synthesis of FOS [11]. For a better understanding of the genes coding for required enzymes, de novo whole genome sequencing approach was followed. Genomic DNA was isolated with FastDNA SPIN Kit (MP Biomedicals, USA) followed by preparation of paired-end and mate-pair sequencing libraries with mean sizes 629 and 690 bp, respectively. The libraries were sequenced (2 × 150 bp) on Illumina HiSeq 2500 platform. High quality reads generated were used for de novo assembly using Soapdenovo2 assembler [12] resulting in a draft genome of 31.4 Mbp containing 160 contigs and 143 scaffolds (Table 1).

A total of 568,994 bp (1.81%) repeat sequences were masked using RepeatMasker and the RepBase library and afterwards, the repeat masked assembly was used for

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Table 1 Genome features of *Penicillium chrysogenum* strain HKF42

Features	*Penicillium chrysogenum* strain HKF42 genome
No. of reads	61,456,596
Pair end read length (bp)	150
Genome assembly size	31,410,885
Number of contigs	160
Number of scaffolds	143
Contigs N50	2,015,765
Gaps between scaffolds	0
Scaffold N50	2,810,928
No. of gene model	11,251
No. of exon per gene	3.14
Mean protein length	475.45
GC content (%)	53.22
No. of tRNA	188
NR annotated	11,184
SwissProt annotated	7595
KEGG annotated	2154
KOG annotated	5482
Pfam annotated	8154
CAZymes	600

coding gene prediction. Coding sequences (CDS) in the genome were predicted by GeneMarkES [13] which resulted in a total of 11,251 protein-coding genes. Later, NCBI's non-redundant (nr) database was used for similarity search of predicted CDS using the BLASTP algorithm. The total proteins were also searched for similarity against Swiss-Prot, Pfam and KOG databases using BLASTP, rpsblast and Hmmscan algorithms (via webMGA). Gene Ontology (GO) annotation obtained through nr database using blast2GO Pro and CDS associated with similar functions were assigned to the same GO functional groups. GO sequence analysis distributions revealed the presence of all three GO domains i.e. cellular components (3926), biological processes (3981) and molecular function (3348) (Fig. 1a). Proteins were mapped and orthologs were assigned to the biological pathways through KEGG automatic annotation server (KAAS) and compared with the KEGG database using BLASTP. For CAZymes analysis, CDS were annotated using dbCAN, the data generated in dbCAN was based on the family classification from CAZy database [14, 15]. Gene annotation disclosed the presence of an enzyme belonging to CAZymes family which was located on gene 6513 and corresponded to FFase with molecular weight of 72.8 kDa respectively. The nearest neighbour analysis of FFase gene 6513 carried out through BLASTP (version 2.7.1), amino acid sequence was searched for similarity against reference proteins (refseq_proteins) and 10 hits were chosen based on query coverage more than 80%. Sequences were aligned using ClustalW interface in MEGA 7 based on the neighbour joining method, and bootstrap values were based on 1000 replicates (Fig. 1b). Gene 6513 shares 78% similarity with *Penicillium digitatum* Pd1 protein, the nearest neighbor highlighted the diversity of the FFase enzyme in *Penicillium* and *Aspergillus* sp. We, further characterized the gene product using program MotifFinder, I-TASSER and COACH for binding and active site prediction as described in Table 2; Fig. S1 and S2 [16, 17]. Ancestral relationship of the isolate *P. chrysogenum* strain HKF42 with *P. chrysogenum* strain P2niaD18 was carried out

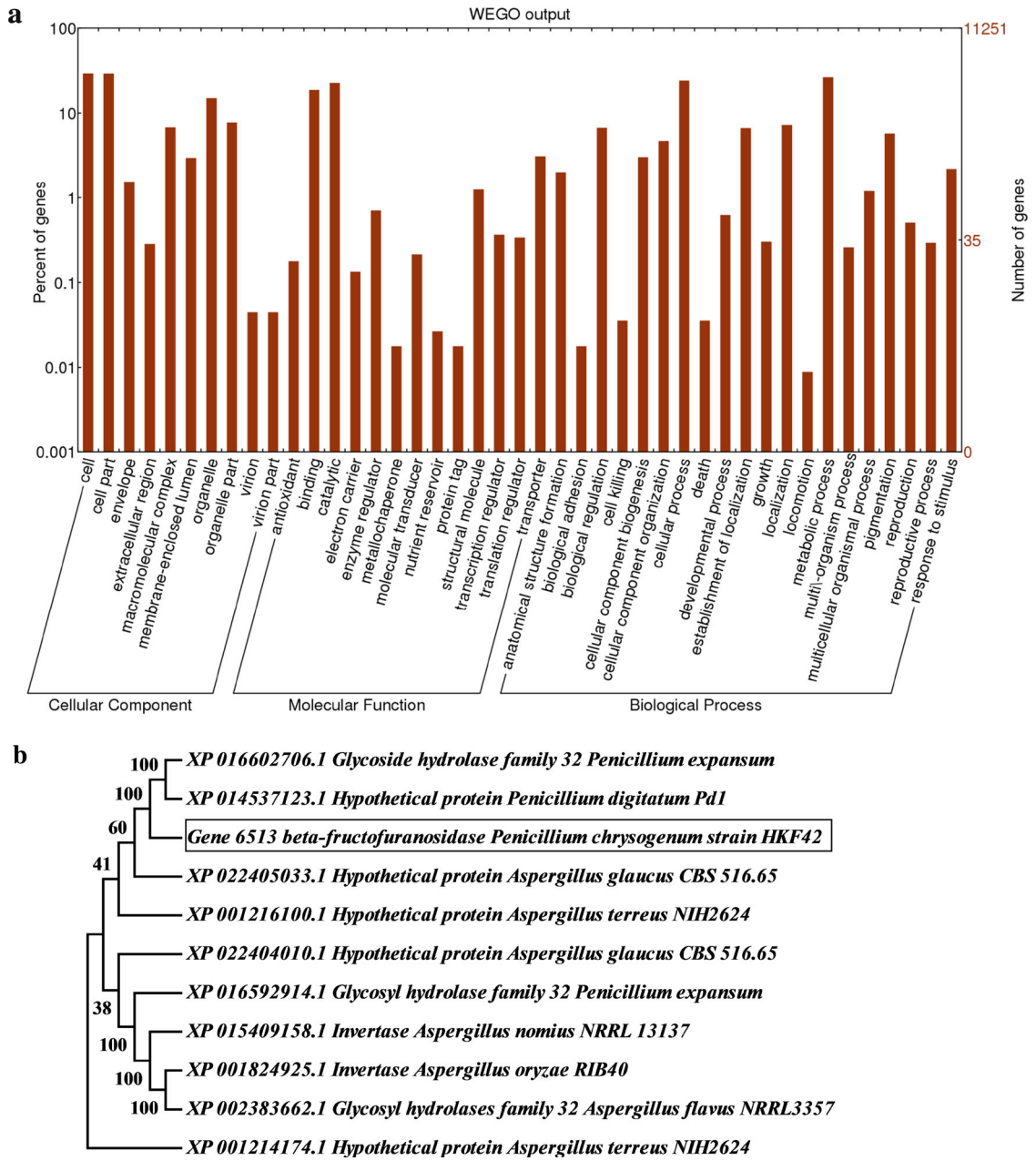

Fig. 1 **a** WEGO plot visualization of GO terms identified in *P. chrysogenum* strain HKF42. **b** Neighbor Joining tree calculated by ClustalW for β-fructofuranosidase gene from *Penicillium* sp. constructed by MEGA 7. Numbers displayed on the branches are the bootstrap support obtained through 1000 replications. **c** The synteny relationship between *Penicillium chrysogenum* strain P2niaD18 reference genome and *P. chrysogenum* strain HKF42 scaffolds. The right hand side of circle starting from P2niaD18_chrI to P2niaD18_chrIV represents the reference genome, and the *P. chrysogenum* strain HKF42 genome sample is displayed on the left side of circle represented as scaffolds. Different colors of lines connect the scaffolds of the *P. chrysogenum* strain HKF42 genome on the left, to the matched sections of the reference genome on the right

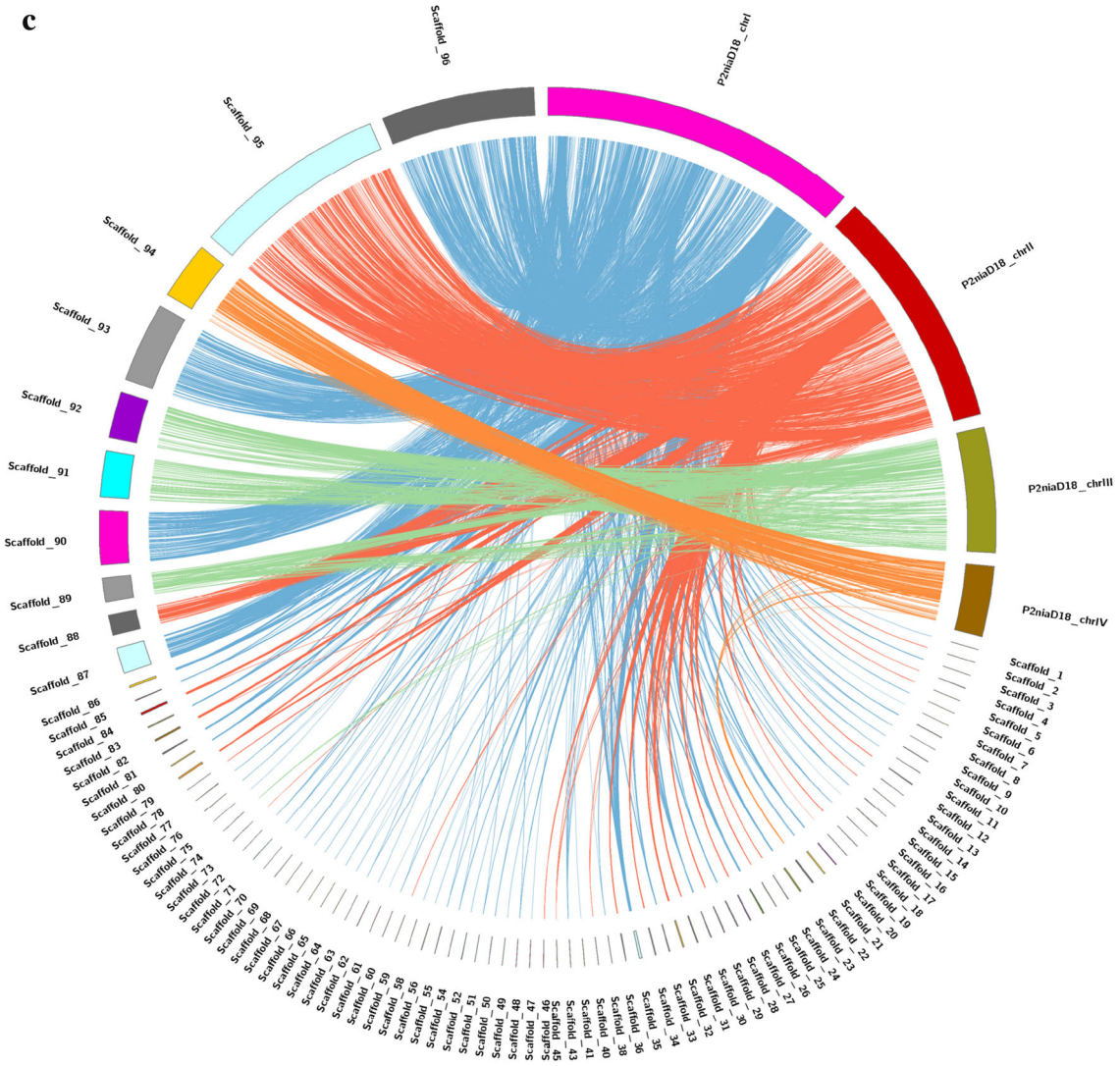

Fig. 1 continued

using synteny analysis through BLASTN and CIRCOS which gave a visual overview of the alignment (Fig. 1c) [18].

The genetic capacity of the isolate for FFase production and FOS synthesis was further validated by submerged fermentation studies. Inoculum development and submerged fermentation strategy was followed as per Prata et al. [19]. The samples were withdrawn at a regular interval of 24 h; centrifuged using a refrigerated centrifuge (4 °C, 10,000 RPM) and the supernatant without further purification was used as the source of FFase. Assay of FFase activity was carried out as per the method of Sangeetha et al. [20] wherein the quantitative analysis of the FOS was done using HPLC and retention time of products

Table 2 Characterization of active site motifs and domains identified in gene 6513 coding for β-fructofuranosidase (FFase)

Domain				Pfam		Predicted binding site residues using different tools			Active site residues predicted by I-Tasser
NCBI-CDD	214,757	185,737	185,718	PF00251	PF08244	TM-SITE	S-SITE	COACH	
Motif	Glycosyl hydrolases family 32	Glycosyl hydrolase family 32, beta-fructosidases	Glycosyl hydrolase families: GH43, GH62, GH32, GH68	Glycosyl hydrolases family 32 N-terminal	Glycosyl hydrolases family 32 C-terminal				
Gene 6513	Position 46–526	Position 52–397	Position 62–395	Position 46–340	Position 468–667	Ligands (fructose, sucrose, nystose) G55, D56, L74, T78, F114, D115, R186, D187, E264, T265, Y337	Ligands (glucose, kestose, sucrose) D56, L74, F114, D115, L137, P138, I139, H140, R186, E264, E289, S298, S300, Y337, W365, F371, G372	Ligands (fructose) G55, D56, L74, T78, F114, D115, R186, D187, E264, Y337, W365	D56 and E264

were compared with the FOS standards for identification. One unit of FFase activity was defined as the amount of enzyme required to produce 1 μmol of glucose per minute at 55 °C with 55% sucrose at pH 5.5 [8]. The consumption of sucrose by the fungal isolate and concomitant production of FFase is shown in Fig. 2a. A rapid reduction in sucrose concentration was observed with 85% sucrose being hydrolyzed in 48 h followed by complete hydrolysis in 72 h. FFase activity increased gradually to 8.3 U/mL in 72 h which doubled to 15.4 U/mL in 96 h resulting in complete hydrolysis of sucrose. HPLC analysis of the assay reaction mixture confirmed the hydrolysis of sucrose to glucose and fructose by FFase along with the formation of two types of FOS viz., kestose and nystose as indicated in Fig. 2b.

In conclusion, *P. chrysogenum* strain HKF42 can be considered a promising candidate for the production of extracellular β-fructofuranosidase and synthesis of short chain FOS and the genome data further can be utilized for mining of other prebiotic synthesizing enzymes.

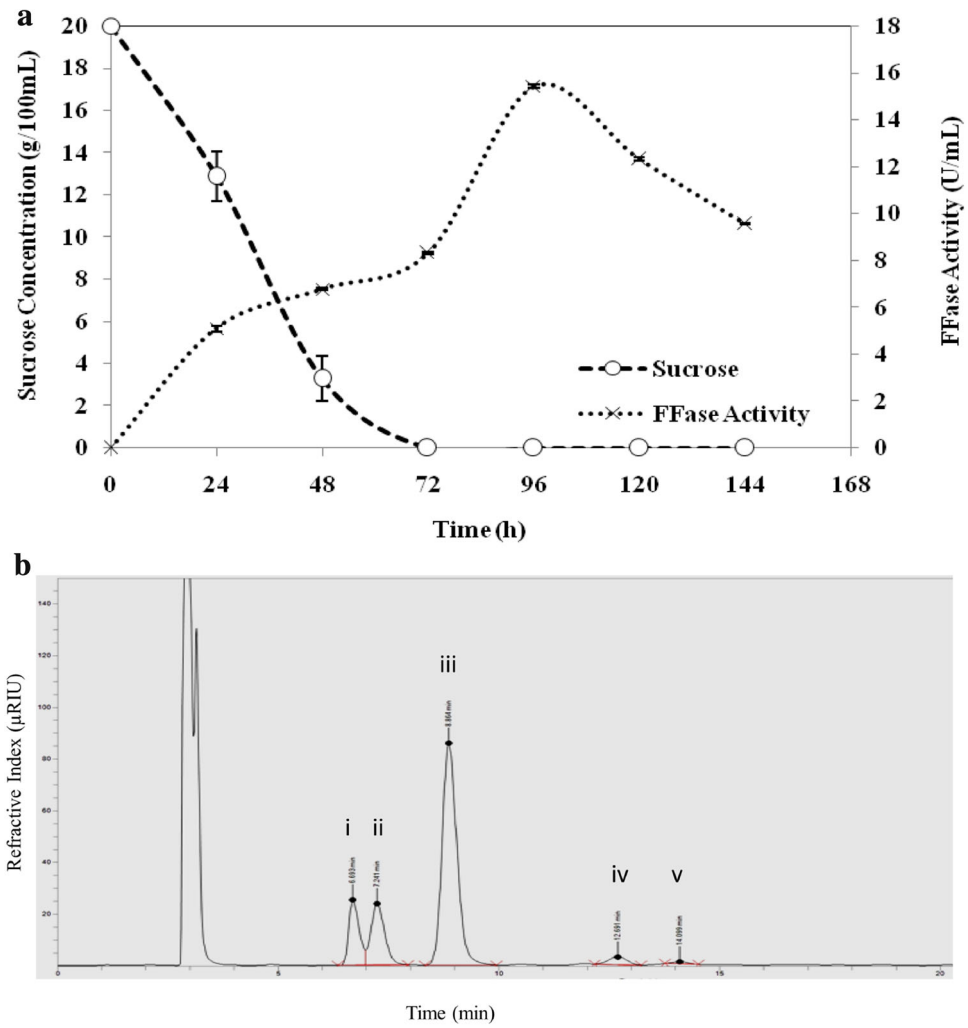

Fig. 2 **a** Sucrose utilization and concomitant production of extracellular β-fructofuranosidase (FFase) from *P. chrysogenum* strain HKF42. **b** HPLC chromatogram of the short chain FOS synthesized by crude β-fructofuranosidase (FFase) from *P. chrysogenum* strain HKF42 (i) fructose; (ii) glucose; (iii) sucrose; (iv) kestose; (v) nystose

This Whole Genome Shotgun project has been deposited in GenBank under the accession number MWKT00000000. The version described in this paper is MWKT01000000.

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References

1. Vega R, Zuniga-Hansen ME (2014) A new mechanism and kinetic model for the enzymatic synthesis of short-chain fructooligosaccharides from sucrose. Biochem Eng J 82:158–165. https://doi.org/10.1016/j.bej.2013.11.012
2. Huang MP, Wu M, Xu QS, Mo DJ, Feng JX (2016) Highly efficient synthesis of fructooligosaccharides by extracellular fructooligosaccharide-producing enzymes and immobilized cells of *Aspergillus aculeatus* M105 and purification and biochemical characterization of a fructosyltransferase from the fungus. J Agric Food Chem 64:6425–6432. https://doi.org/10.1021/acs.jafc.6b02115
3. Lorenzoni ASG, Aydos LF, Klein MP, Rodrigues RC, Hertz PF (2014) Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from *Aspergillus aculeatus*. Carbohydr Polym 103:193–197. https://doi.org/10.1016/j.carbpol.2013.12.038
4. Flores-Maltos DA, Mussatto SI, Contreras-Esquivel JC, Rodrigues-Herrera R, Teixeira JA, Aguilar CN (2014) Biotechnological production and application of fructooligosaccharides. Crit Rev Biotechnol 36:259–267. https://doi.org/10.3109/07388551.2014.953443
5. Marin-Navarro J, Talens-Perales D, Polaina J (2015) One-pot production of fructooligosaccharides by a *Saccharomyces cerevisiae* strain expressing an engineered invertase. Appl Microbiol Biotechnol 99:2549–2555. https://doi.org/10.1007/s00253-014-6312-4
6. Aguiar-oliveira E, Maugeri F (2012) Effects of lyophilization on the catalytic properties of extracellular fructosyltransferase from *Rhodotorula* sp. LEB-V10. Int Res J Biotechnol 3:96–111
7. Bali V, Panesar PS, Bera MB, Panesar R (2013) Fructooligosaccharides: production, purification and potential applications. Crit Rev Food Sci Nutr 55:1475–1490. https://doi.org/10.1080/10408398.2012.694084

8. Mussatto SI, Prata MB, Rodrigues LR, Teixeira JA (2012) Production of fructooligosaccharides and β-fructofuranosidase by batch and repeated batch fermentation with immobilized cells of *Penicillium expansum*. Eur Food Res Technol 235:13–22. https://doi.org/10.1007/s00217-012-1728-5
9. Nascimento AKC, Nobre C, Cavalcanti MTH, Teixeira JA, Porto ALF (2016) Screening of fungi from the genus *Penicillium* for production of β-fructofuranosidase and enzymatic synthesis of fructooligosaccharides. J Mol Catal B Enzym 134:70–78. https://doi.org/10.1016/j.molcatb.2016.09.005
10. Xu Q, Zheng X, Huang M, Wu N, Yan Y, Pan J, Yang Q, Duan CJ, Liu JL, Feng JX (2014) Purification and biochemical characterization of a novel β-fructofuranosidase from *Penicillium oxalicum* with transfructosylating activity producing neokestose. Process Biochem 50:1237–1246. https://doi.org/10.1016/j.procbio.2015.04.020
11. Deshmukh R, Mathew A, Purohit HJ (2014) Characterization of antibacterial activity of bikaverin from *Fusarium* sp. HKF15. J Biosci Bioeng 117:443–448. https://doi.org/10.1016/j.jbiosc.2013.09.017
12. Luo R, Liu B, Xie Y, Li Z, Huang W, Yuan J, He G, Chen Y, Pan Q, Liu Y, Tang J (2012) SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. Gigascience 1:18. https://doi.org/10.1186/2047-217X-1-18
13. Borodovsky M, Lomsadze A (2011) Eukaryotic gene prediction using GeneMark.hmm-E and GeneMark-ES. Curr Protoc Bioinforma 33:6494–6506. https://doi.org/10.1002/0471250953.bi0406s35
14. Hu L, Taujale R, Liu F, Song J, Yin Q, Zhang Y, Guo J, Yin Y (2016) Draft genome sequence of *Talaromyces verruculosus* ("*Penicillium verruculosum*") strain TS63-9, a fungus with great potential for industrial production of polysaccharide-degrading enzymes. J Biotechnol 219:5–6. https://doi.org/10.1016/j.jbiotec.2015.12.017
15. Yin Y, Mao X, Yang J, Chen X, Mao F, Xu Y (2012) DbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic Acids Res 40:445–451. https://doi.org/10.1093/nar/gks479
16. Yang J, Roy A, Zhang Y (2013) Protein–ligand binding site recognition using complementary binding-specific substructure comparison and sequence profile alignment. Bioinformatics 29:2588–2595. https://doi.org/10.1093/bioinformatics/btt447
17. Yang J, Zhang Y (2015) I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res 43:W174–W181. https://doi.org/10.1093/nar/gkv342
18. Specht T, Dahlmann TA, Zadra I, Kürnsteiner H, Kück U (2014) Complete sequencing and chromosome-scale genome assembly of the industrial progenitor strain P2niaD18 from the penicillin producer *Penicillium chrysogenum*. Genome Announc 2:4–5. https://doi.org/10.1128/genomeA.00577-14
19. Prata MB, Mussatto SI, Rodrigues LR, Teixeira JA (2010) Fructooligosaccharide production by *Penicillium expansum*. Biotechnol Lett 32:837–840. https://doi.org/10.1007/s10529-010-0231-y
20. Sangeetha PT, Ramesh MN, Prapulla SG (2004) Production of fructo-oligosaccharides by fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. Process Biochem 39:755–760. https://doi.org/10.1016/S0032-9592(03)00186-9