Cloning, characterization, and heterologous expression of a dextranase gene from *Penicillium pinophilum* SMCU3-14

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ABSTRACT: A DNA fragment encoding dextranase was cloned from *Penicillium pinophilum* SMCU3-14 using the genome walking approach. Sequence analysis of the gene (*SMCU-DEX*) revealed a putative CAAT box at −165 (CCAAT), a putative TATA box at −93 (TATAA) in the 5′-noncoding region, and a polyadenylation signal (AATAAG) in the 3′-noncoding region. A cDNA sequence analysis revealed no evidence of introns. The deduced open reading frame is 1824 bp in length and encodes a predicted protein of 608 amino acids (molecular weight (MW) of ∼66 kDa), with a putative N-terminal 20-amino acid signal peptide, giving a predicted mature protein of 588 amino acids (MW of ∼64 kDa) that belongs to glycosyl hydrolase family 49, as with other fungal dextranases. This is the first report of a dextranase gene sequence from *P. pinophilum*. The cDNA was cloned and expressed in *Escherichia coli*, and the transformants showed dextranase activity on a dextran-containing agar medium. Crude extracts from the transformants analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis containing blue dextran revealed a distinct specific band of dextranase activity at an MW of approximately 66 kDa. This recombinant dextranase is likely to have valuable and cost-effective applications in medicine and industry.

KEYWORDS: genome walking, *Escherichia coli*

INTRODUCTION

Dextran is a homopolysaccharide composed of D-glucose units connected by α-1,6 glucosidic linkages and various numbers of side branches joined to the backbone via α-1,2, α-1,3, or α-1,4 linkages. Dextran is typically synthesized from sucrose by the action of dextransucrase or glucosyltransferase enzymes. In the sugar industry, sugarcane juice, the raw material for sugar production is always contaminated with bacteria of the genera *Lactobacillus*, *Leuconostoc*, or *Streptococcus*, which produce an extracellular dextransucrase that synthesizes dextran from the sucrose present in the juice[1,2]. This formation of dextran therefore causes decreases in the yield and quality of the sugar produced; clogs and blocks the pipelines, tanks, strainers, and filters; increases the viscosity of the juice; results in a poor clarification and filtration ability; and further inhibits sucrose production (extraction) by decreasing the rate of sucrose crystallization[3]. For these reasons, dextran production is a considerable problem resulting in severe economic loss to the sugar industry.

In addition, dextran is also a problem for dental health. Oral streptococci, which colonize the tooth surface, synthesize dextran from sucrose using glucosyltransferases[4]. Dextran is a structural component of dental plaque, a component of closely packed bacteria and noncellular materials, which causes the development of dental caries[5].

Physicochemical techniques to remove dextran have proven to be unsuccessful. Enzymes that specifically hydrolyse dextran are therefore employed to overcome some of the problems associated with the removal of dextran[6].

Dextranase (EC 3.2.1.11, α-1,6 glucan 6-glucanohydrolase) is an inducible enzyme that specifically hydrolyses the α-1,6-glucosidic linkages within the dextran molecule to release smaller oligosaccharides[7]. As this process reversibly reduces the stickiness caused by dextran, it has been suggested as a mean to solve the problem of microbe-produced dextran in the cane sugar industry[8]. The same rationale has also been used for the prevention of caries by inhibiting

biofilm formation on teeth[9, 10], whereby dextranase retards plaque deposition on the tooth surface, thus repressing the adherence of oral streptococci to the tooth surface and inhibiting the formation of dental caries. Moreover, dextranase has been used in degrading dextran to an appropriate smaller size range suitable for use as a synthetic blood volume expander in patients suffering from hypovolaemic shock[11]. The use of dextranase has also been proposed as a method to increase antibiotic efficacy in infective endocarditis and a universal targeting method for therapeutic agents[12]. Sources of dextranases are widespread: the enzyme has been found in mammalian tissues and in various microorganisms, such as fungi, yeasts, bacteria, and *Actinomyces*[13, 14]. Fungi are the most important commercial source of dextranases as they produce high levels of enzyme[15]. Although several fungi have been reported to produce dextranase, only a few nucleotide sequences of fungal dextranases are available in public databases.

Conserved regions in the amino acid sequences of dextranases from different sources have been reported[16]. On the basis of their amino acid sequence similarity, dextranases have been classified into two glycosyl hydrolase families (GH), GH49 and GH66, among which there is no significant sequence similarity. The bacterial dextranases from *Streptococcus* species belong to the GH66 family[17], whereas the bacterial dextranases from *Arthrobacter* species, fungal dextranases from *Penicillium* species, and the dextranase from the yeast *Lipomyces starkeyi* all belong to the GH49 family[18, 19].

In addition to their high levels of enzyme, fungi are also the most important source for commercial dextranase production due to the stability of the enzymes at high temperatures. However, because fungi also produce various antibiotics and metabolites, there has been difficulty in obtaining consumer approval[20]. To solve this problem, a fungal dextranase gene can be heterologously expressed in an alternate host, such as a non-harmful bacterial species. Previous studies have shown that *Penicillium pinophilum* SMCU3-14 produces a high level of dextranase activity, up to 600 units/ml[21]. In the present report, cloning and sequence characterization of a dextranase-encoding genomic DNA and cDNA from this fungus and its expression in *Escherichia coli* were described.

MATERIALS AND METHODS

Strains and vectors

P. pinophilum SMCU3-14 (EU635728), previously isolated from a local soil sample[21, 22], was used as

Table 1 Primers used in this study.

Primers	Sequences (5′ to 3′ direction)
Dex1F	TCTGTACCTGGTGGCATGATTC
Dex1R	TCGCTAATTTGACTTGAGATGC
Dex5R	ACGCAGTACCATGTTTGACCACCCCCAAGG
Dex6R	TGCAACATAGTTGTCGCCGGCATTGGCTTG
Dex3F	GCTGCGCCAATCTCACAAGTATTCCGTCCGTGC
Dex4F	CTTTGTATATGAATCGATCCCCCGGAACG
Dex8F	TAATTGACGCCTTGACAGGTGATG
Dex10R	CATATCGTCCACGTCTTTTGAAAG
Dex14F	CTCAAA<u>CATAT</u>GCCCACAATG†
Dex14R	AGCTGGA<u>GGATCC</u>CTAATCTG†

† Underlined sequences of Dex14F and Dex14R indicate the *Nde*I and *Bam*HI restriction sites, respectively.

a source for the isolation of the dextranase gene. Vectors pDrive (Qiagen, USA) and pJET1/blunt (Fermentas, USA) were used to clone the PCR products. pETHIS, kindly provided by National BioResource Project (NIG, Japan), was used as an expression vector. *E. coli* isolate DH5α was used for all the cloning experiments whereas *E. coli* Rosetta-gami B (DE3)pLysS (Novagen, USA) was used to express recombinant dextranase protein. All the standard molecular genetic techniques were performed according to Sambrook and Russell[23].

Cloning of the dextranase gene from *P. pinophilum* SMCU3-14

Chromosomal DNA of *P. pinophilum* SMCU3-14 was isolated according to Reader and Broda[24]. A segment of the dextranase gene was amplified based on the conserved sequences of different dextranase genes among *Penicillium*. To this end, the Dex1F/Dex1R primers (Table 1) were designed and used to amplify the central part of the dextranase gene using *P. pinophilum* SMCU3-14 genomic DNA as a template. The PCR product was commercially sequenced bidirectionally by 1st Base Laboratory, Malaysia.

Gene-specific primers were designed based on the obtained sequence to amplify the 5′ and 3′ sequences flanking the conserved sequence using the BD Genome Walker Universal kit (BD Biosciences Clontech, USA). To amplify the 5′ flanking sequence, outer and nested reverse primers, Dex5R and Dex6R (Table 1), specific to the dextranase conserved sequences were designed; and outer and nested forward primers Dex3F and Dex4F (Table 1) were used to amplify the 3′ flanking sequence. These primers were used together with the outer adaptor primer 1 (AP1) and nested adaptor primer 2 (AP2) provided in the kit in accordance with the manufacturer's protocol. Briefly, *P. pinophilum* SMCU3-14 genomic DNA was separately digested with the *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I blunt-ending restriction enzymes and then sep-

arately ligated to the BD Genome Walker Adaptor to form four restriction libraries. The upstream and downstream flanking fragments of the conserved dextranase sequence (via PCR amplification using the Dex1F/Dex1R primer pair) were then amplified by primary PCR using the Dex5R/AP1 and Dex3F/AP1 primer pairs, respectively. The primary PCR reaction cycles using a GeneAmp PCR System 2400 (Perkin Elmer, USA) thermal cycler were as follows: 7 cycles at 94 °C for 2 s and 70 °C for 3 min, followed by 32 cycles at 94 °C for 2 s and 69 °C for 3 min, and a final extension at 67 °C for 4 min.

The primary PCR products were then diluted 50-fold and used as the template for the secondary nested PCR. This secondary amplification step served to specifically amplify the desired product prior to cloning and utilized the nested reverse (Dex6R) and adaptor (AP2) primers and the nested forward (Dex4F) and adaptor (AP2) primers to amplify the 5' and 3' flanking fragments, respectively. The secondary PCR reaction cycles using a DNA Thermal Cycle 2400 (Perkin Elmer) instrument were as follows: 5 cycles at 94 °C for 2 s and 70 °C for 3 min, followed by 20 cycles at 94 °C for 2 s and 60 °C for 3 min, and a final extension at 67 °C for 4 min.

After the secondary amplification, the PCR products were purified and cloned into the pDrive Cloning Vector (Qiagen, USA). The presence of the insert was confirmed by restriction enzyme digestion, and the recombinant plasmids were extracted and commercially sequenced in both directions, as above. The sequenced fragments were assembled and characterized using DNASIS (Hitachi Software Engineering, USA). Sequence alignments were used to search GenBank public database sequences with the online BLASTN algorithm (www.ncbi.nlm.nih.gov).

To obtain the complete dextranase gene in a single fragment by PCR amplification, the Dex8F/Dex10R primers (Table 1) were used with *Pfu* DNA polymerase (Fermentas, USA) and *P. pinophilum* SMCU3-14 genomic DNA as the template. After amplification, the PCR product was purified and cloned into the pJET/blunt-cloning vector (Fermentas, USA). The recombinant plasmid harbouring the PCR product encoding the complete dextranase gene was then sequenced and analysed as described before.

Cloning of the dextranase cDNA of *P. pinophilum* SMCU3-14 (SMCU-DEX)

P. pinophilum SMCU3-14 was cultivated in 50 ml of modified Fukumoto's medium[25], with or without 1% (w/v) dextran (industrial grade; Sigma, USA) to induce dextranase production, at room temperature with shaking at 200 rpm. Mycelium samples were harvested by filtration every three days, lyophilized until completely dry, and kept at −70 °C until use. Total RNA was extracted using the e-Zi RNA Extraction kit (Sunolin, Thailand) according to the instructions provided.

Total RNA from each sample was separated by formaldehyde agarose gel electrophoresis and transferred to a Hybond N membrane according to the manufacturer's protocol (Amersham Biosciences, USA). The conserved dextranase fragment obtained by PCR amplification with the Dex1F/Dex1R primers was used as a probe in a Northern hybridization assay using the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche Biochemical Products, Germany), as described by the manufacturer.

Poly(A)$^+$ RNA was isolated from dextran-induced mycelia by the PolyATtract mRNA Isolation System III (Promega, USA), and cDNA was synthesized with *Pfu* DNA polymerase using the Universal RiboClone cDNA Synthesis System (Promega, USA) according to the manufacturer's specifications. The DNA fragment encoding the full-length dextranase gene from the start to stop codons was obtained by PCR using the cDNA and Dex14F/Dex14R primer pair (Table 1), containing a 5' flanking *Nde*I or *Bam*HI restriction sites (Table 1, underlining indicates the *Nde*I and *Bam*HI restriction sites, respectively). The PCR product was blunt-end cloned into the pJET1/blunt Cloning Vector (Fermentas, USA), and the recombinant plasmid (pNA-1) was commercially sequenced, as detailed above.

Sequence analyses were performed using DNA-SIS, BLASTN, BLASTX, and CLUSTALX. For the purpose of comparison, the sequences of dextranases and related proteins from *Arthrobacter* sp. (accession number P39652), *Arthrobacter globiformis* (accession number BAA13598), *Brevibacterium fuscum* var. *dextranlyticum* isomaltotriodextranase (accession number BAA76382), *Penicillium minioluteum* (accession number AAB47720), and *Aspergillus niger* (accession number BAA18971) were used.

Southern hybridization of the dextranase gene

Aliquots (10 µg) of *P. pinophilum* SMCU3-14 genomic DNA were digested with *Bam*HI, *Eco*RI, *Hin*dIII (NEB Biolabs, USA), *Pst*I (Fermentas, USA), *Kpn*I, or *Xba*I (Promega, USA) restriction enzymes. Each digestion was resolved on a 1% (w/v) agarose-TBE gel and then transferred to a Hybond N membrane (Amersham Biosciences, USA). The complete dextranase gene (∼3 kb PCR product) was used as the probe for Southern hybridization using the DIG

High Prime DNA Labelling and Detection Starter Kit I (Roche Biochemical Products, Germany), as described by the manufacturer. Genomic DNA from *Lentinus* sp. was used as a negative control.

Expression of recombinant dextranase protein

To construct the plasmid for the heterologous expression of recombinant dextranase in *E. coli*, pNA-1 was digested with *Nde*I and *Bam*HI. The released dextranase gene fragment was gel purified and directionally cloned into the corresponding *Nde*I and *Bam*HI sites of the pETHIS expression vector. The obtained recombinant plasmid (pNAT2) was transformed into *E. coli* Rosetta-gami B (DE3)pLysS, and the transformants screened on LB agar supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. Protein expression was induced with 4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Fifteen colonies of transformants and the host strain carrying the empty pETHIS vector as a control were spotted onto Luria-Bertani (LB) agar plates containing 1% (w/v) dextran and 10 mM IPTG to test for the production of extracellular dextranase activity. After 18 h of incubation at 37 °C, the test plate was overlaid with 95% (v/v) ethanol to reveal the cloudy dextran and observe any dextranase activity as a clear halo zone around the positive colonies.

Dextranase activity was also zymographically assayed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) containing blue dextran (D5751; Sigma, USA). Briefly, *E. coli* Rosetta-gami B (DE3)pLysS cells transformed with pNAT2 and the host strain carrying the empty vector were cultured with and without IPTG induction. The crude protein extracts were resolved by SDS-PAGE (10% (w/v) acrylamide resolving gel)[26] containing 0.5% (w/v) blue dextran. The resolved gel was incubated overnight at 55 °C in 0.05 M sodium acetate buffer (pH 4.5) containing 1% (v/v) Triton X-100 to remove the SDS and allow the renaturation of the enzyme and the dextranase cleavage of blue dextran[27]. Dextranase activity was observed as a clear band against a blue background. Duplicate samples were separated by SDS-PAGE without blue dextran and then stained with Coomassie brilliant blue to visualize the protein and allow an estimate of the (subunit) molecular weight.

RESULTS

Cloning of the genomic dextranase gene

A 1-kb fragment of the dextranase gene was isolated using PCR amplification (Dex1F/R primers)
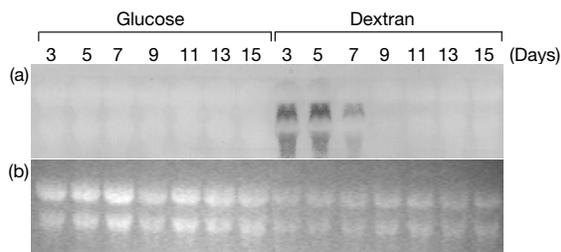
Fig. 1 (a) Northern hybridization of the total RNA isolated from *P. pinophilum* SMCU3-14 grown in a medium containing 3% (w/v) glucose with or without 1% (w/v) dextran at the time points indicated using *SMCU-DEX* as a probe. (b) Total RNA visualized as an ethidium-bromide stained gel prior to transfer and Northern blotting.

from *P. pinophilum* SMCU3-14 and commercially sequenced. The consensus DNA sequence was then used to BLASTN search against the GenBank database and showed 100% DNA sequence identity to five reported dextranase genes from *Penicillium* spp. Thus it was concluded that a portion of the dextranase gene of *P. pinophilum* SMCU3-14 (*SMCU-DEX*) had been isolated.

The complete genomic DNA encoding the dextranase and the 5′ and 3′ flanking sequences were then obtained by genome walking using the BD Genome-Walker universal kit. A fragment of approximately 3.0 kb was cloned and sequenced. The sequence was verified as a dextranase gene by homology searching (BLASTN) of the GenBank database, with the highest DNA sequence identity (99%) being found to the dextranase gene of closely related *P. minioluteum* (accession number L41562). The complete nucleotide sequence of *SMCU-DEX* was deposited in the GenBank database under the accession number EU635729.

Northern hybridization and cDNA cloning of the dextranase gene

Total RNA extracts from *P. pinophilum* SMCU3-14 grown in Fukumoto's media with or without 1% (w/v) dextran (to induce dextranase production) were harvested every two days, and the samples were subjected to a Northern analysis using a complete *SMCU-DEX* fragment as the probe. The result revealed that dextranase was only expressed in the dextran-induced culture; the expression was transient, starting as early as after 3 days of incubation, reaching the highest level at day 5, and then decreasing until it was undetectable by day 9 and thereafter (Fig. 1). Accordingly, the total RNA sample from the 5-day post-

dextran-induced culture was selected for subsequent cDNA amplification by PCR using the gene-specific DEX14F/R primers. The obtained cDNA was cloned and commercially sequenced.

Sequence characterization of the complete dextranase gene of *P. pinophilum* SMCU3-14

The genomic and cDNA sequences (Fig. 2) were analysed using the DNASIS program. Both sequences were verified as a dextranase gene by homologous analysis with other fungal dextranase genes available in GenBank. No evidence of any intron in *SMCU-DEX* was found.

Four potential initiation codons (ATG_1, ATG_2, ATG_3, and ATG_4 in Fig. 2) were found, all of which coded for the same protein that differed only in the length of the N-terminus (12, 31, and 34 amino acids smaller for ATG_2, ATG_3, and ATG_4, respectively, than for ATG_1). Translation from the first ATG codon (ATG_1), for a dextranase gene (*SMCU-DEX*) sequence from position 1450–3273, would generate a deduced 1824-bp open reading frame (ORF) that encodes a protein of 608 amino acids (SMCU-DEX) with a predicted molecular weight (M_W) of approximately 66 kDa. This polypeptide contained a predicted (by SIGNALP version 3.0; www.cbs.dtu.dk/ services/SignalP) N-terminal sequence of 20-amino acid residue signal peptide (positions 1–20 in the ORF) (Fig. 2), resulting in a predicted mature protein of 558 amino acids and an M_W of ∼64 kDa.

Compared to other dextranases obtained from GenBank by BLASTX and BLASTP searches, SMCU-DEX revealed 97% amino acid sequence identity to the dextranase from *P. funiculosum* (accession number AJ272066) and 99.5%, 90%, and 87% identity to the enzymes from three isolates of *P. minioluteum* (accession numbers L41562, AF020619, and DQ394070; see Garcia et al[28], Li et al[19]).

The deduced amino acid sequence was compared to the GenBank conserved domain and amino acid databases, which revealed that SMCU-DEX belongs to the GH49 family. Furthermore, SMCU-DEX shared the same seven conserved amino acid regions as other GH49 dextran-hydrolysing enzymes, although the sequence in region III exhibited a higher degree of identity (12/16 residues, 75%) than the other six regions (39–58% identity) (Fig. 3).

Neural network promoter prediction version 2.2 (www.fruitfly.org/seq_tools/promoter.html) was used to identify any putative promoter sequence upstream of ATG_1. A CAAT Box-like sequence (CCAAT) at position 1281–1285, a TATAA box at position 1353–1357, and a putative transcription start site at

position 1385 were found (Fig. 2). In addition, the Hamming clustering polyA prediction in eukaryotic genes (HC polyA) program (http://bioinfo4.itb.cnr.it/ ~webgene/wwwHC_polya.html) revealed a putative polyA signal-like sequence (AATAAG) at position 3490–3495 (Fig. 2).

Determination of the *SMCU-DEX* gene copy number in *P. pinophilum* SMCU3-14

To determine the likely copy number of the *SMCU-DEX* gene in the genome of *P. pinophilum* SMCU3-14, Southern hybridization was performed using the full-length *SMCU-DEX* sequence as the hybridization probe (Fig. 4). All the restriction-digested DNA preparations (*Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Kpn*I, and *Xba*I) produced a hybridization band, whereas no signal was observed with *Lentinus* sp. genomic DNA (negative control). Furthermore, all samples showed only a single hybridization band, except for the *Xba*I restricted DNA, which yielded two smaller fragments. The most likely explanation was that there is a single copy of *SMCU-DEX* in the *P. pinophilum* SMCU3-14 genome but that it contains an internal *Xba*I site; an analysis of the *SMCU-DEX* sequence revealed a *Xba*I site in the 5′ flanking region (see 1218–1224 bp in Fig. 2).

Heterologous expression of recombinant dextranase in *E. coli*

The *SMCU-DEX* gene was subcloned from pNA-1 into pETHIS at the *Nde*I and *Bam*HI sites to yield the recombinant plasmid pNAT2. The plasmid was propagated in *E. coli* DH5α and then transformed into *E. coli* Rosetta-gami B (DE3)pLysS for dextranase expression under the control of the strong T7 promoter with IPTG induction. Transformants were plated onto LB plates with 1% (w/v) dextran; after 18 h of growth, the plate was overlaid with 95% (v/v) ethanol to visualize the dextran and screen for dextranase activity. All the screened transformants bearing pNAT2 revealed a clear halo zone around the colony demonstrating that they produced dextranase (Fig. 5), whereas the *E. coli* transformed with the empty pETHIS vector did not produce any halo zone.

In-gel dextranase activity assays

To confirm the expression and activity of dextranase in the transformant, an in-gel zymograph for dextranase activity was performed. Supernatants from IPTG-induced and non-induced cultures of one selected transformant (pNAT2-transformed *E. coli* Rosetta-gami B (DE3)pLysS) and the host strain transformed

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1     ATCGGTTTCCAGCTTCATTGCTCCCGCTTCGACATGGGCCGAATGAGCCTTGGAATCCATCTTTGGATAGGGTGGCAAAGGAAGATGTCCTCTGCAGAAT   100
101   GAATGAATAACGAGGAGGTAATGAATGAATTGTCCGTAACAATTTGTAAAAGGTAAAGGTCAACGACAACTCAGAGGCAGATCAAGCAATATATGTAGAT   200
201   GCCGAAAAACAGAGCGCTTGAATTAGGGCAAGAGGTTCGAAAGTCTCTTCCTCCGTACGGGGTTACTTCCATCACTGTTGATTTTCCGCAGTCGTACGTT   300
301   TATTCTCTTCTCCATCAGAATTATTGGCATAGTAATCCGCACAGCCGAGTATGATGGAGCTTCTTCGGATAATGATAGCGCCACCAGACCTTGCTTGAGC   400
401   TGGAGAGCTAAACATTAAACGCCACACGACCAACACTCTCATTAGTTGCGATAGATGATGCTCGGAGCTGTTGAAACTCAGAAATTCCTTCTATGCGGGG   500
501   TCTCCAAGATCGATCCTGGGGGATGTGAATCACCTACGGCGGACCTAATTGACGCCTTGACAGGTGATGTTAAGCGAACCAAGGAAGAAATAATCTGGGG   600
601   CTAGATGAAGATGTTGAGCTGTAAGGTACGGTCGTTCCTATTGGCTTTATCGGCGCTTCTCCGGGGTTACTCAGTCTTTCCGGGAGCATGATCATTTTTG   700
701   TATTGTCCAATAGTAAGCAGAAACTGAGAGCCACCACAAACTCAAAACCTCGGTAGCGAAGTTTCACGGAACCAGTCAGGATTCTCAGAAACTGTGCTCG   800
801   TGTTGCGGGGAATCCGCATTCTACGTCGTCTGGAGCAAGGAAATGTTCGTGCGGGATTGAGGAGGATAGGTAGGTTGGAGAATCTCTTCGCTAACCAATC   900
901   TATAAGCATGCTCCGGTAACCTTTAGAGTTTCACGTCCAACGTAATTTCCAAGATAGCCAGAGCGTACTTGAATTACTATGTAGAAATCCTAAAATTTCC   1000
1001  CCTCTAAAACGCAAGTCAACGAGATGCGTGCCCTCAATGTCTCTCGGCGCTACCCCGGAAATGATGCATAAGGCCAAGAATGTCACCCGGTAACTTTTTC   1100
1101  TTCAGAATATCCTAAGTTTCCATCAAACACAGTCGAATAGGTCAATGCTCGCGAGAGACTTTCTGCCTTCACTCTACGTCCTACTGATAGAAGTTCAACG   1200
1201  GCTCAATTCCGGGGTAATCTAGAGTTTGGACCTCAAGTGAGATGTTGCAACAAATTGTACTAGAACGATGCGCTTGCTTT|CCAAT|ACAGTAGTTAGCTTC   1300
1301  ATATAGCTTCCAACAAAAGGGATGGGGGATGAAGGCTCTATAGCGAGAAGTC|TATAA|GAAAGTGTCCTCATACCTGTATCTCTC**A**GTCGTTCGAGAACAA   1400
1401  TCCCGGAAACTATCTTATCTTGCGAGAAAGAAGACAATATCTCAAACTT   ATG₁ CCC ACA ATG₂ CTA AAG CTA CTT GCG TTG ACC CTT   1485
                                                          **M   P   T   M   L   K   L   L   A   L   T   L**   12
1486  GCA ATT AGC GAG TCC GCC ATT GGA GCA GTC ATG₃ CAC CCT GGC AAT TCT CAT CCC GGT ACC CAT ATG₄ GGC ACT   1560
13    **A   I   S   E   S   A   I   G** A   V   M   H   P   P   G   N   S   H   P   G   T   H   M   G   T   37
1561  ACG AAT AAT ACC CAT TGC GGC GCC GAT TTC TGT ACC TGG TGG CAT GAT TCA GGG GAG ATC AAT ACG CAG ACA CCT   1635
38    T   N   N   T   H   C   G   A   D   F   C   T   W   W   H   D   S   G   E   I   N   T   Q   T   P   62
1636  GTC CAA CCA GGG AAC GTG CGC CAA TCT CAC AAG TAT TCC GTG CAA GTG AGC CTA GCT GGT ACA AAC AAT TTT CAT   1710
63    V   Q   P   G   N   V   R   Q   S   H   K   Y   S   V   Q   V   S   L   A   G   T   N   N   F   H   87
1711  GAC TCC TTT GTA TAT GAA TCG ATC CCC CGG AAC GGA AAT GGT CGC ATC TAT GCT CCC ACC GAT CCA CCC AAC AGC   1785
88    D   S   F   V   Y   E   S   I   P   R   N   G   N   G   R   I   Y   A   P   T   D   P   P   N   S   112
1786  AAC ACA CTA GAT TCA AGT GTG GAT GAT GGA ATC TCG ATT GAG CCT AGT ATC GGC CTT AAT ATG GCA TGG TCC CAA   1860
113   N   T   L   D   S   S   V   D   D   G   I   S   I   E   P   S   I   G   L   N   M   A   W   S   Q   137
1861  TTC GAG TAC AGC CAC GAT GTA GAT GTA AAG ATC CTG GCC ACT GAT GGC TCA TCG TTG GGC TCG CCA AGT GAT GTT   1935
138   F   E   Y   S   H   D   V   D   V   K   I   L   A   T   D   G   S   S   L   G   S   P   S   D   V   162
1936  GTT ATT CGC CCC GTC TCA ATC TCC TAT GCG ATT TCT CAG TCT GAC GAT GGT GGG ATT CTC ATC CGG GTC CCA GCC   2010
163   V   I   R   P   V   S   I   S   Y   A   I   S   Q   S   D   D   G   G   I   V   I   R   V   P   A   187
2011  GAT GCG AAC GGC CGC AAA TTT TCA GTT GAG TTC AAA ACT GAC CTG TAC ACA TTC CTC TCT GAT GGC AAT GAG TAC   2085
188   D   A   N   G   R   K   F   S   V   E   F   K   T   D   L   Y   T   F   L   S   D   G   N   E   Y   212
2086  GTC ACA TCG GGA GGC AGC GTC GTC GGC GTT GAG CCT ACC AAC GCA CTT GTG ATC TTC GCA AGT CCG TTT CTT CCT   2160
213   V   T   S   G   G   S   V   V   G   V   E   P   T   N   A   L   V   I   F   A   S   P   F   L   P   237
2161  TCT GGC ATG ATT CCT CAT ATG ACA CCC GAC AAC ACG CAG ACC ATG ACG CCA GGT CCT ATC AAT AAC GGC GAC TGG   2235
238   S   G   M   I   P   H   M   T   P   D   N   T   Q   T   M   T   P   G   P   I   N   N   G   D   W   262
2236  GGC GCC AAG TCA ATT CTT TAC TTC CCA CCA GGT GTA TAC TGG ATG AAC CAA GAT CAA TCG GGC AAC TCG GGG AAG   2310
263   G   A   K   S   I   L   Y   F   P   P   G   V   Y   W   M   N   Q   D   Q   S   G   N   S   G   K   287
2311  TTA GGA TCT AAT CAT ATA CGT CTA AAC TCG AAC ACT TAC TGG GTC TAC CTT GCC CCC GGT GCG TAC GTG AAG GGT   2385
288   L   G   S   N   H   I   R   L   N   S   N   T   Y   W   V   Y   L   A   P   G   A   Y   V   K   G   312
2386  GCT ATA GAG TAT TTT ACC AAG CAG AAC TTC TAT GCA ACT GGT CAT GGT ATC CTA TCG GGT GAA AAC TAT GTT TAC   2460
313   A   I   E   Y   F   T   K   Q   N   F   Y   A   T   G   H   G   I   L   S   G   E   N   Y   V   Y   337
2461  CAA GCC AAT GCC GGC GAC AAC TAT GTT GCA ATG AGC GAT TCA ACC AGC CTC CGG ATG TGG TGG CAC AAT AAC   2535
338   Q   A   N   A   G   D   N   Y   V   A   V   K   S   D   S   T   S   L   R   M   W   W   H   N   N   362
2536  CTT GGG GGT GGT CAA ACA TGG TAC TGC GTT GGC CCG ACG ATC AAT GCG CCA CCA TTC AAC ACT ATG GAT TTC AAT   2610
363   L   G   G   G   Q   T   W   Y   C   V   G   P   T   I   N   A   P   P   F   N   T   M   D   F   N   387
2611  GGA AAT TCT GGC ATC TCA AGT CAA ATT AGC GAC TAT AAG CAG GTG GAA GCC TTC TTC TTC CAG ACG GAT GGG CCA   2685
389   G   N   S   G   I   S   S   Q   I   S   D   Y   K   Q   V   G   A   F   F   F   Q   T   D   G   P   412
2686  GAA ATA TAT CCC AAT AGT GTC GTG CAC GAC GTC TTC TGG CAC GTC AAT GAT GAT GCA ATC AAA ATC TAC TAT TCG   2760
413   E   I   Y   P   N   S   V   V   H   D   V   F   W   H   V   N   D   D   A   I   K   I   Y   Y   S   437
2761  GGA GCA TCT GTA TCG CGG GCA ACG ATC TGG AAA TGT CAC AAT GAC CCA ATT ATC CAG ATG GGA TGG ACG TCT CGG   2835
438   G   A   S   V   S   R   A   T   I   W   K   C   H   N   D   P   I   I   Q   M   G   W   T   S   R   462
2836  GAT ATC AGT GGA GTG ACA ATC GAC ACA TTA AAT GTT ATT CAC ACC CGC TAC ATC AAA TCG GAG ACG GTG GTG CCT   2910
463   D   I   S   G   V   T   I   D   T   L   N   V   I   H   T   R   Y   I   K   S   E   T   V   V   P   487
2911  TCG GCT ATC ATT GGG GCC TCT CCA TTC TAT GCA AGT GGG ATG AGT CCT GAT TCA AGA AAG TCC ATA TCC ATG ACG   2985
488   S   A   I   I   G   A   S   P   F   Y   A   S   G   M   S   P   D   S   R   K   S   I   S   M   T   512
2986  GTT TCA AAC GTT GTT TGC GAG GGT CTT TGC CCG TCC CTA TTC CGC ATC ACA CCC CTA CAG AAC TAC AAA AAT TTT   3060
513   V   S   N   V   V   C   E   G   L   C   P   S   L   F   R   I   T   P   L   Q   N   Y   K   N   F   537
3061  GTT GTC AAA AAT GTG GCT TTC CCA GAC GGG CTA CAG ACG AAT AGT ATT GGC ACA GGA GAA AGC ATT ATT CCA GCC   3135
538   V   V   K   N   V   A   F   P   D   G   L   Q   T   N   S   I   G   T   G   E   S   I   I   P   A   562
3136  GCA TCT GGT CTA ACG ATG GGA CTG AAT ATC TCC AAC TGG ACT GTT GGT GGA CAA AAA GTG ACT ATG GAG AAC TTT   3210
563   A   S   G   L   T   M   G   L   N   I   S   N   W   T   V   G   G   Q   K   V   T   M   E   N   F   587
3211  CAA GCC AAT AGC CTG GGG CAG TTC AAT ATT GAC GTC AGC TAT TGG GGG GAG TGG CAG ATT AGC TGA ATTCCAGCTCTC   3288
588   Q   A   N   S   L   G   Q   F   N   I   D   V   S   Y   W   G   E   W   Q   I   S   *
3289  GGAGCGCGTGAGTGCTTCTACCCGCTCCTTTACCCTTGTCGAGGGATAAAGGCATAAGTTAGCTCATGTGAAGGCGATTTCAGTTCATTCTCTCTTTTTG   3388
3389  GAGCTTATTTCCTGTTCGACCAATTGTGACACCAATTGCCTTTCAAAAGACGTGGACGATATGTGTACGGTAATCAGTCAAATGAACGTCAACATTCATT   3488
3489  TAATAAGGACATTTCCAGGTTTCCTTACTCTGTCTAACTCGGGTTGATGTCTTGTCAGGATGGAAAATCTCGTTATGTACTTCCAGTGAAA   3588
3589  TGGGCAGGGCTAAGCCCTAAACCCTAACGCATACAATTTGTAGGCACCTACCCGTGTAAGTTCACACCAGTCGACTTATAAGTCTAGATATTTATGCTAT   3688
3689  GCAGGCTCTGGAATGATTTACAGTCCATGCTATACATAGTTATTTGCAAGAATTTGCAGACGAGATAAAAATCAATGGACGAATAATCACGCATTACTCC   3788
3789  ACAGGCTCATGCCACGGAGCAAGGGTTCCCCCGAATCTAGGCCAGACCGGGATGATATTCAACCGATTCTTTTTGCAGTAACAATCTCGTACGAGCTGCA   3888
3889  CGAGCTAAACGGCATTATATAAAGGTGCTGACCCAGACTTGGATCCGTACGTTATATGAAATGACTTTTAATCTCGCAGATTTGGACCGTAATCATATTG   3988
3989  AAGCGGCCGCTAGCGCCAAACCTACGGTACGTTCA                                                                   4023
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Fig. 2 The nucleotide sequence of the *SMCU-DEX* gene and its deduced amino acid sequence. Four putative initiation codons (ATG₁₋₄) are shown. The putative CAAT-box and TATA-box of the promoter are boxed. The **A̲**, in bold and underlined, indicates the potential transcription start site. The amino acid sequence at positions 1–20 (from ATG₁) in bold are the putative N-terminal signal peptide residues, with the signal peptidase cleavage site marked with an arrow (↑). After the stop codon (TGA; ⋆), the underlined A̲A̲T̲A̲A̲G̲ nucleotide sequence indicates the putative polyA signal sequence. This sequence has been submitted to GenBank database under accession number EU635729.

Source	Region I	Region II	Region III	Region IV	Region V	Region VI	Region VII
Arthro-1	TWWHDNGV	GYRFSVEFEPQLYT	WVYLAPGAYVKGAFRF	TGYGVLSGEQYVY	YKQVGSWYWQTDGIELYKG	NDDVLKMYHSDVT	PVIQWGWTPRNI
Arthro-2	TWWHDNGV	GYRFSVEFEPQLYT	WVYLAPGAYVKGAFRF	TGYGVLSGEQYVY	YKQVGSWYWQTDGIELYQG	NDDVLKMYHSDVS	PVVQWGWTPRNI
IMTD	TWWHDNAV	GYRFSVEFDPQLYT	WVYLAPGAYVKGAFRF	TGYGVLSGEQYVY	YKQVGSWYWQTDGIELYQG	NDDVLKMYHSDVT	PVIQWGWTPRNI
SMCU-DEX	TWWHDSGE	GRKFSVEFKTDLYT	WVYLAPGAYVKGAIEY	TGHGILSGENYVY	YKQVGAFFFQTDGPEIYPN	NDDAIKIYYSGAS	PIIQMGWTSRDI
pUDEX	TWWHDSGE	GRKFSVEFKTDLYT	WVYLAPGAYVKGAIEY	TGHGILSGENYVY	YKQVGAFFFQTDGPEIYPN	NDDAIKIYYSGAS	PIIQMGWTSRDI
IPUA	TWWHNTGE	GYRFSVEFDDDLIS	WVYFAPGAYVKGAVEF	SGHGVLSGEQYVW	YKQVGAFYGQTDGLEMYPG	DDDGLKMYYSNVT	PVVEFGWTPRNT

Fig. 3 The seven conserved regions (I-VII) of dextranases, as reported by Aoki and Sakano [16], are indicated. Arthro-1, *Arthrobacter* sp. CB-8 endodextranase (P39652); Arthro-2, *A. globiformis* endodextranase (BAA13598); IMTD, *B. fuscum* var. *dextranlyticum* isomaltotriodextranase (BAA76382); SMCU-DEX, *P. pinophilum* SMCU3-14 dextranase; pUDEX, *P. minioluteum* HI-4 endodextranase (AAB47720); IPUA, *A. niger* isopullulanase (BAA18971).
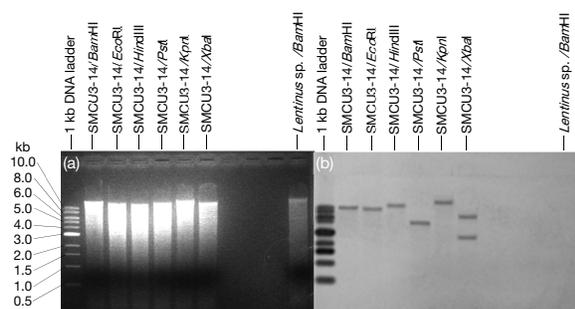
Fig. 4 (a) Agarose gel electrophoresis of the genomic DNA of *P. pinophilum* SMCU3-14 following complete restriction digestion with the indicated enzymes. The first lane is a 1-kb DNA ladder, and the last lane is the genomic DNA from *Lentinus* sp. digested with *Bam*HI as a negative control. (b) Southern hybridization with the digoxigenin-labelled full-length *SMCU-DEX* probe. The positions of the molecular markers (in bp) are indicated on the left.

with the empty pETHIS vector as a control were harvested and subjected to SDS-PAGE and blue dextran-SDS-PAGE. SDS-PAGE stained with Coomassie brilliant blue R250 revealed an extra band with an estimated M_W of approximately 66 kDa in the IPTG-induced culture of the pNAT2 transformant. A corresponding dextranase activity band was also observed in the SDS-PAGE containing blue dextran zymograph (Fig. 6). Thus *E. coli* cells harbouring pNAT2 produced an active dextranase with a molecular mass of approximately 66 kDa.

DISCUSSION

Dextranases are found in a wide variety of fungi, bacteria, yeast, and *Actinomyces*, with fungi being the most important commercial sources [15, 29]. However, only eight dextranase genes from five species of filamentous fungi plus one species of yeast have been reported to date, namely the dextranase genes from *P. funiculosum* (AJ272066), *Penicillium marneffei* ATCC 18224 (XM_002152550), *P. minioluteum*
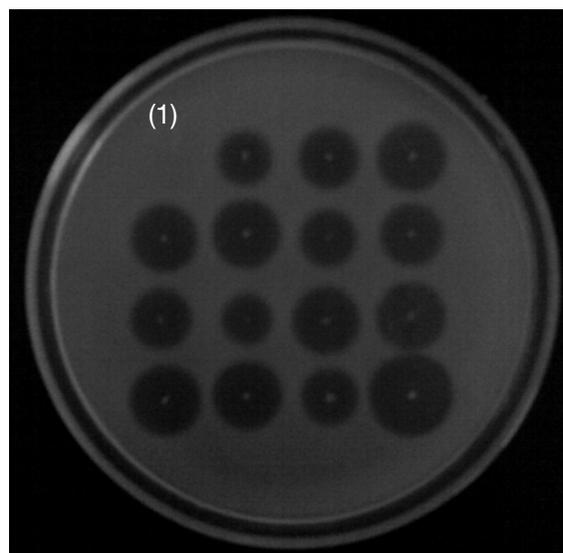
Fig. 5 LB agar plates with 1% (w/v) dextran after 18 h of culture with pNAT2- or the empty pETHIS vector-transformed *E. coli* colonies and then overlaid with 95% (v/v) ethanol. All the pNAT2-transformed *E. coli* colonies showed a large clear zone of dextran hydrolysis, whereas colonies of the empty vector transformant (indicated by (1)) did not.

(L41562, DQ394070 and AF020619), *Verticillium alboatrum* VaMs.102 (XM_003005140), *V. dahliae* VdLs.17 (DS572701), and the yeast *L. starkeyi* (AY520537). These fungal dextranases all belong to the GH49 family [19]. In this study, the cloning and sequencing of a GH49 dextranase from *P. pinophilum* SMCU3-14 and its heterologous expression in an alternative bacterial host were reported. The dextranase from *P. pinophilum* SMCU3-14 was found to be quite similar in amino acid sequence to all other currently known fungal dextranases. This is the first report of a dextranase gene sequence from *P. pinophilum*.

Sequence analysis of the complete dextranase gene (*SMCU-DEX*) revealed a single ORF of 1824 bp without any intron. Four putative initiation codons
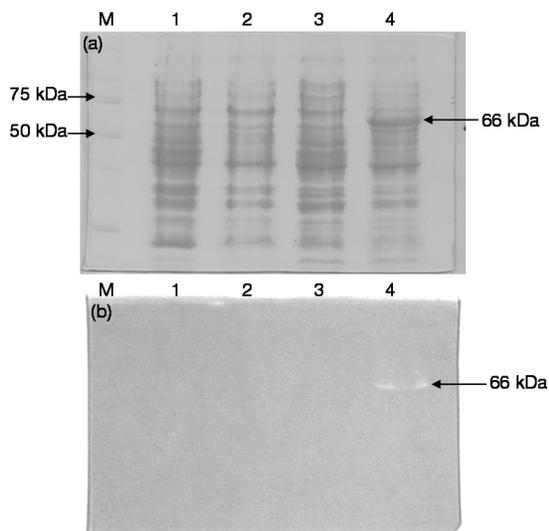
Fig. 6 SDS-PAGE analysis of the total protein and SDS-PAGE-blue dextran zymograph analysis of the dextranase activity in the total protein sample. (a) Protein samples were separated by SDS-PAGE (10% (w/v) acrylamide resolving gel) and then stained with Coomassie brilliant blue R250. (b) Protein samples were separated by SDS-PAGE containing 0.5% (w/v) blue dextran and incubated for ∼18 h at 55 °C for renaturation and dextranase activity. Dextranase activity is observed as a white band on a blue background. M: standard of the indicated molecular masses; lanes 1–4: protein samples from Rosetta-gami B (DE3)pLysS transformed with (lanes 1, 2) pETHIS or (lanes 3, 4) pNAT2 and (lanes 1, 3) non-induced or (lanes 2, 4) induced with IPTG.

were found in the ORF, all in the same frame and coding for the same protein that (before maturation) would differ only in the length of the N-terminus (by up to 34 amino acids) and the presence ($ATG_{1/2}$) or absence ($ATG_{3/4}$) of an N-terminal signal sequence. Although most fungal genes have several potential initiation codons, translation typically begins at the first ATG[28]. The ORF of *SMCU-DEX* starting from the first ATG (ATG_1) encodes a predicted protein of 608 amino acids (SMCU-DEX) with a predicted M_W of approximately 66 kDa. However, it is also possible that the translation of the dextranase gene might begin from the second ATG (ATG_2) because the sequence preceding that codon (CACA*ATG$_2$*) resembles the consensus sequence for translation initiation identified in 99 genes of filamentous fungi (5′ CAC/AA/C*ATG$_2$* 3′)[30]. If the translation of the dextranase gene begins at the second ATG (ATG_2) codon, this gene would encode a protein of 605 amino acids with an M_W of approximately 65 kDa. Regardless, both trans-

lational products would be predicted to have an N-terminal signal sequence and would yield the same mature protein of 588 amino acids and a molecular weight of ∼64 kDa upon cleavage. The predicted presence of the signal peptide was expected given that dextranase is a secreted protein. Thus although not conclusive, our results suggest that the dextranase protein from *P. pinophilum* SMCU3-14 is synthesized from the first (or perhaps second) ATG codon as a preproenzyme, as the other two codons would not include the secretory signal peptide in the polypeptide sequence. The predicted mature polypeptide (M_W of ∼64 kDa) is smaller than that observed in the present study by SDS-PAGE (M_W ∼66 kDa), suggesting post-translational modification in the bacterial host or a failure to cleave the signal peptide.

We found that *E. coli* Rosetta-gami B (DE3)pLysS cells harbouring pNAT2 likely secreted active dextranase into the medium. Although heterologous protein expression in *E. coli* is mainly transported into the periplasmic space[31], the potential secretion of recombinant dextranase observed in this study may occur due to the native secretion signal of *SMCU-DEX* present in the construct being operatively functional in *E. coli*. Regardless, the activity of dextranase from *E. coli* was confirmed in both agar diffusion assays and blue dextran-SDS-PAGE zymographs. Future studies will seek to optimize the production and secretion of dextranase. Additionally, the subsequent optimization of large-scale expression and purification would be required for the enzyme to be of commercial interest.

Since the application of native-produced fungal dextranases in the food and dental health industries might create some difficulty for obtaining approval from consumer or regulatory agents, the heterologous production of recombinant dextranase, such as in bacteria, as in this work, or in acceptable yeasts, may be cost-effective in medical and industrial applications. Another research is ongoing to express the enzyme in the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, which are publicly acceptable for use in the food industry and may offer higher production yields and/or specific activities.

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