MYCOBIOLOGY
2023, VOL. 51, NO. 4, 239–245
https://doi.org/10.1080/12298093.2023.2247221

RESEARCH ARTICLE

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Isolation and Characterization of Xylanase from a Novel Strain, *Penicillium menonorum* SP10

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ABSTRACT

Xylanase has been applied in various sectors, such as biomass conversion, paper, pulp, textiles, and pharmaceutical industries. This study aimed to isolate and screen potential xylanase-producing fungi from the soil of Suphan Buri Province, Thailand. Fifteen fungi were isolated, and their xylanase activities were tested by the qualitative method. The result showed that isolate SP3, SP10 and SP15 gave high xylanase activity with potency index (PI) of 2.32, 2.01 and 1.82, respectively. These fungi were selected for the xylanase quantitative test, isolate SP10 performed the highest xylanase activity with 0.535 U/mL. Through molecular methods using the *β-tubulin* gene, isolate SP10 was identified as *Penicillium menonorum*. The xylanase characteristics from *P. menonorum* SP10 were determined, including the xylanase isoforms and the optimum pH and temperature. The xylanase isoforms on SDS-PAGE indicated that *P. menonorum* SP10 produced two xylanases (45 and 54 kDa). Moreover, its xylanase worked optimally at pH 6 and 55 °C while reaching 61% activity at 65 °C. These results proposed *P. menonorum* SP10 as a good candidate for industrial uses, especially in poultry feed and pulp industries, to improve yield and economic efficiency under slightly acidic and high-temperature conditions.

ARTICLE HISTORY
Received 1 May 2023
Revised 28 July 2023
Accepted 29 July 2023

KEYWORDS
Hemicellulose; *Penicillium menonorum*; xylanase; xylanase-producing fungi

1. Introduction

Hemicellulase is an enzyme group in the glycoside hydrolases (GHs) family that degrades the hemicellulose in the lignocellulose structure. Xylanase is the main enzyme in the hemicellulase group because it can degrade 70% of hemicellulose [1]. The xylanase application has been attractive since the circular bioeconomy (CBE) was processed [2]. In this trend, xylanase, cellulase and other GHs are required to break down biomass and release monosaccharides for bio-products, biochemicals, and bioenergy production. This provides the economic approach for environmental treatment [3]. Moreover, xylanase also has been used in other sectors, such as paper, pulp, textiles and pharmaceutical industries [4,5].

Xylanase can produce from various sources, such as microorganisms (e.g., fungi, bacteria, yeast), protozoa and crustaceans [6,7]. Among these, fungi were dominant because they can produce extracellularly xylanase in large amounts [8]. *Trichoderma*, *Aspergillus* and *Penicillium* genera have been well-known as good xylanase producers. The xylanase from *Trichoderma reesei* has been produced at the industrial level, while *Aspergillus* spp. and *Penicillium* spp. could produce multiple xylanase isoforms, some were characterized as thermophilic xylanases [2,7].

However, the industrial application of xylanase has not been extended because of its low activity in harsh environments (e.g., extremely acidic/alkalic or high-temperature conditions) [6]. Most fungal xylanases worked at pH 4 to 6 and about 50 °C [8], and they mainly became denatured at above 60 °C [9]. This resulted in a high cost due to adding xylanase to maintain the yield during the enzymatic process [8]. In this circumstance, the finding of potential xylanase producers for industrial uses has still drawn much interest. Along this stream, the present study aimed to isolate and screen xylanase-producing fungi from soil and determine xylanase characterization to propose good candidates for xylanase production.

2. Materials and methods

2.1. Isolation and screening of xylanase-producing fungi

2.1.1. Fungal isolation

The soil samples were collected in Suphan Buri Province, Thailand. One gram of the sample was

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added into a flask containing 24 mL of distilled water. Then, the mixture was diluted as the standard dilution technique. After that, spread 100 µL of each dilution level onto xylan agar medium (g/L: $NaNO_3$ 2.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5, peptone 0.2, xylan 2.0, agar 1.8). The fungi were purified by the hyphae tip technique and maintained on potato dextrose agar (PDA) for further experiments.

2.1.2. The xylanase qualitative test

The qualitative test was carried out on the xylan medium as Neethu described [10]. A plug of 7-day-old fungal mycelium (5 mm in diameter) was inoculated on a plate containing xylan medium and then incubated for 4 days. After incubation, the inoculated plate was stained with 5 mL of Gram's iodine solution for 5 min to show the clear zone. The xylanase activity in the qualitative test was evaluated by the potency index (PI).

$$PI = \frac{The\ diameter\ of\ clear\ zone\ (mm)}{The\ diameter\ of\ colony\ (mm)}$$

2.1.3. The xylanase quantitative test

In the quantitative test, the selected fungi with high xylanase activity were cultured in potato dextrose broth (PDB) to produce xylanase. One plug of 7-day-old mycelium (5 mm in diameter) was transferred into a bottle containing 5 mL of PDB. Every seven days, the culture broth was centrifuged at 13,000 rpm, 4 °C, for 10 min. The supernatant was obtained and used as the crude enzyme. The xylanase activity was determined by the xylanase assay method.

Xylanase assay (U/mL) was conducted based on the dinitrosalicylic acid (DNS) method to determine the reducing-sugar concentration release [11]. Fifty microliters of the crude enzyme and 50 µL of substrate solution (1% beechwood xylan) were mixed on a thermomixer. The reaction was done at 50 °C for 30 min. Then, stop the reaction with 300 µL of DNS reagent. Followed by boiling for 5 min at 100 °C and cooling. The mixture was diluted 5-fold with H_2O before measuring the absorbance at 540 nm. The standard curve of xylose concentration was built to determine the reducing sugar concentration released from the reaction. One xylanase activity (U/mL) was the enzyme required to catalyze the substrate and release 1 µmol of xylose for 1 min at the reaction conditions.

2.2. Morphological and molecular identification

The morphological and molecular methods were used to identify the fungus that gave the highest xylanase activity in the quantitative test.

2.2.1. Morphological identification

The fungus was cultured on a PDA medium. After seven days, the colony characters were recorded, including color, texture, and margin. Moreover, the microcharacters (e.g., the hyphae and conidiospore) were also examined under a light microscope.

2.2.2. Molecular identification

The total DNA genome was extracted using the thermolysis method described by Tangthirasunun & Poeaim [12]. The *β-tubulin* genes were amplified with primer pair T1/T22 [13]. The polymerase chain reactions (PCR) were done with the protocol described by Tangthirasunun [14]. The PCR products were checked the quality and size by electrophoresis technique using 1% agarose gel. Then, PCR products were sequenced following the Barcode Taq sequencing (BTSeq) technique based on Next-Generation sequencing (NGS), Illumina Hiseq by Celemics, Inc. Korea. Comparing the DNA sequences in this work with other sequences on the GenBank database by BLAST analysis on NCBI (http://www.ncbi.nlm.nih.gov). Analyzing the phylogenetic relationship was also regarded. The neighbor-joining method in the Kimura 2-parameter model on MEGA11 software built the phylogenetic tree. The bootstrap analysis was done with 1,000 replications.

2.3. Xylanase characterization

2.3.1. Xylanase isoforms

The xylanase isoforms were conducted on SDS-PAGE following the method of Cano-Ramírez [15]. The resolving gel was prepared with 10% acrylamide gel adding 0.1% beechwood xylan. The stacking gel was prepared with 4% acrylamide gel. The crude enzyme was mixed with SDS sample buffer (the ratio of 2: 1), and the sample was treated neither with boiling nor adding β-mercaptoethanol. The supply power was conducted at 100 V under 20 ± 2 °C. After running, the gel would be rinsed with 0.05 M sodium citrate buffer pH 4.8 for 60 min at 20 °C and then incubated at 50 °C for 2 h. The gel was stained with two sequential steps: (1) staining with Coomassie blue G-250 [16]; and then (2) staining with 0.1% Congo red solution. The "Perfect Protein Markers" (Merck), ranging from 10 to 225 kDa, estimate the molecular weight of xylanase isoforms.

2.3.2. Optimum pH and temperature

The optimum pH was investigated, ranging from pH 4 to 10, following the method Prajapati [17]. The buffers were used in this experiment, including sodium citrate buffer (pH 4–5.5), sodium phosphate

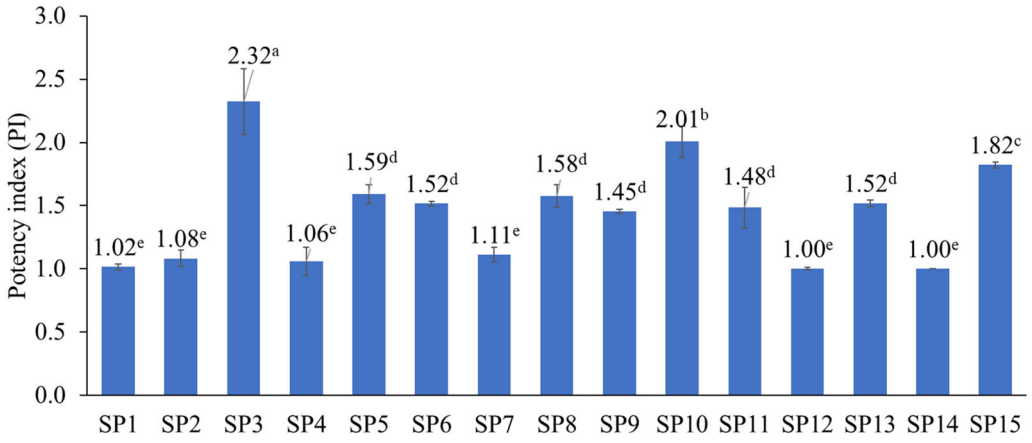

Figure 1. Xylanase activity of 15 fungal isolates in the qualitative test; a, b, c, d and e: homogeneous groups by statistics analysis.

(pH 6–6.5), Tris-HCl buffer (pH 7–9), and glycine-NaOH buffer (pH 9.5–10). The 1% beechwood xylan solutions were prepared in the corresponding buffer. The xylanase assay was conducted at 50 °C with various pH values.

The optimum temperature was investigated from 40 to 80 °C (5 °C distance). The xylanase assay was done with optimum pH under various temperatures.

2.4. Statistical analysis

All the experiments were conducted with three independent replicates. The data in the chart was the average of three replicates. SPSS statistics software (IBM, version 26.0) was used for ANOVA analysis.

3. Results

3.1. Isolation and screening of xylanase-producing fungi

Fifteen isolates were picked up from soil samples, and all were tested for their xylanase activities by the qualitative method. The PI of 15 isolates ranged from 1.00 to 2.32 (Figure 1). Among these, isolates SP3, SP10 and SP15 gave high xylanase activity with PI of 2.32, 2.01 and 1.82, respectively. These three isolates visualized a large clear zone on the xylan medium (Figure 2), and they were selected for the xylanase quantitative test.

In the quantitative test, isolate SP3, SP10 and SP15 were cultured in PDB to produce the enzyme, and the xylanase activity was determined by xylanase assay (U/mL). The result shows that all three isolates could produce xylanase in PDB (Figure 3). The xylanase production of isolate SP3 and SP10 reached a peak on the 14th day of incubation, while isolate SP15 was on the 21st day. Over the investigated period, isolate SP10 produced the highest xylanase compared with isolate SP3 and SP15. It reached maximum activity with 0.535 U/mL.

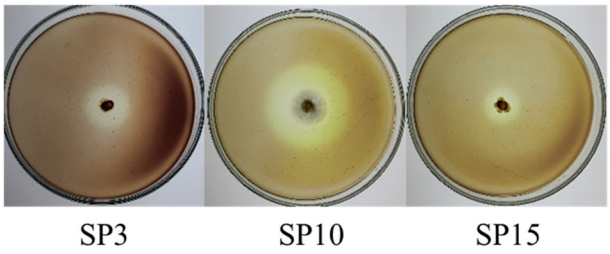

Figure 2. The clear zone of isolate SP3, SP10 and SP15 visualized on xylan medium on the 4th incubation after staining with gram's iodine solution.

Isolate SP10 performed as a good xylanase producer in qualitative and quantitative tests. Thus, it was selected for the identification and determination of xylanase characterization.

3.2. Morphological and molecular identification of isolate SP10

3.2.1. Morphological identification

The morphology of isolate SP10 was observed on the PDA medium after seven days of culture. Its central colony was dark grey, while the around area was white (Figure 4). Moreover, the rosy exudate appeared around the central colony on the 7th day. Conidiophores arise from aerial hyphae, and the spore bear with the phialides. The above morphological characters indicated that SP10 belonged to the *Penicillium* genus.

3.2.2. Molecular identification

The molecular identification of isolate SP10 was conducted using the *β-tubulin* gene (TUB) as a barcode locus. The result showed that the TUB sequence of SP10 was 98.15% identical to the sequence of *P. menonorum* NRRL 50410 (HQ646573) and *P. menonorum* NK 65 (OL652650). Moreover, phylogenetic analysis of isolate SP10 was done by distance tree

construction. The result indicated that SP10 was nested in the same clade with two reference isolates of *P. menonorum*, while distanced from other *Penicillium* species (Figure 5). Therefore, isolate SP10 was identified as *P. menonorum*.

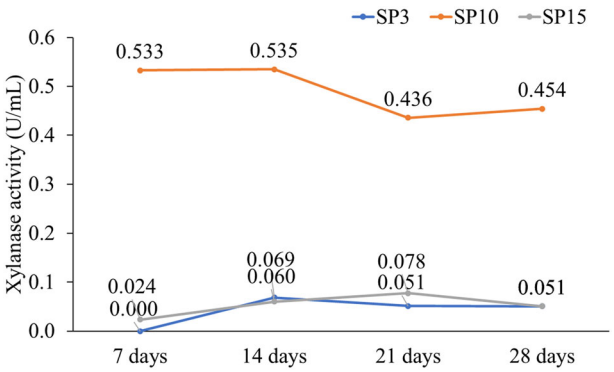

Figure 3. Xylanase activity of isolate SP3, SP10 and SP15 by quantitative test.

3.3. Xylanase characterization

The crude enzyme of *P. menonorum* SP10 on the 14th day of cultivation was used to determine the xylanase characterization, including xylanase isoforms and optimum pH and temperature.

3.3.1. Xylanase isoforms

The xylanase isoforms were done on SDS-PAGE containing 0.1% beechwood xylan. The result showed that *P. menonorum* SP10 produced two xylanase isoforms: approximately 45 kDa and 54 kDa (Figure 6).

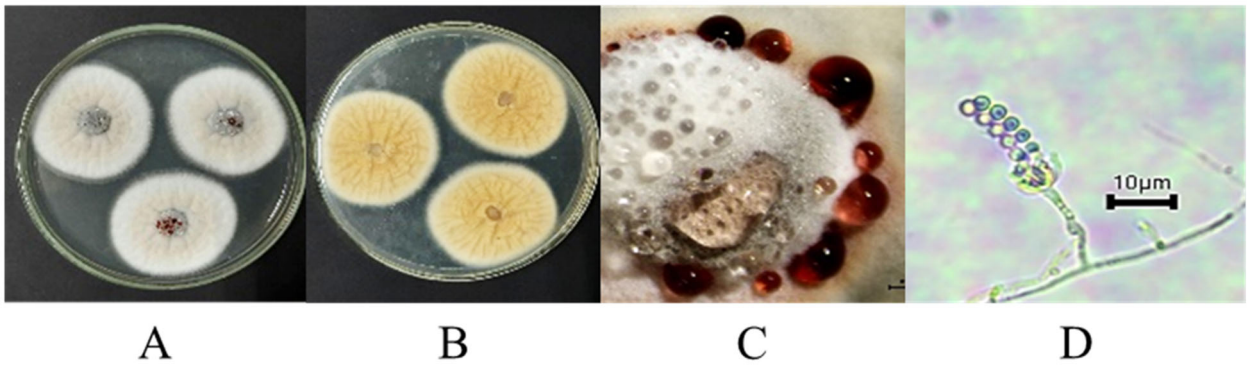

Figure 4. The morphology of isolate SP10 on PDA at 7 days of cultivation. A and B: the colony in front and reverse side; C: the rosy exudate around the central colony; D: the conidiophore under the microscope (40X magnification).

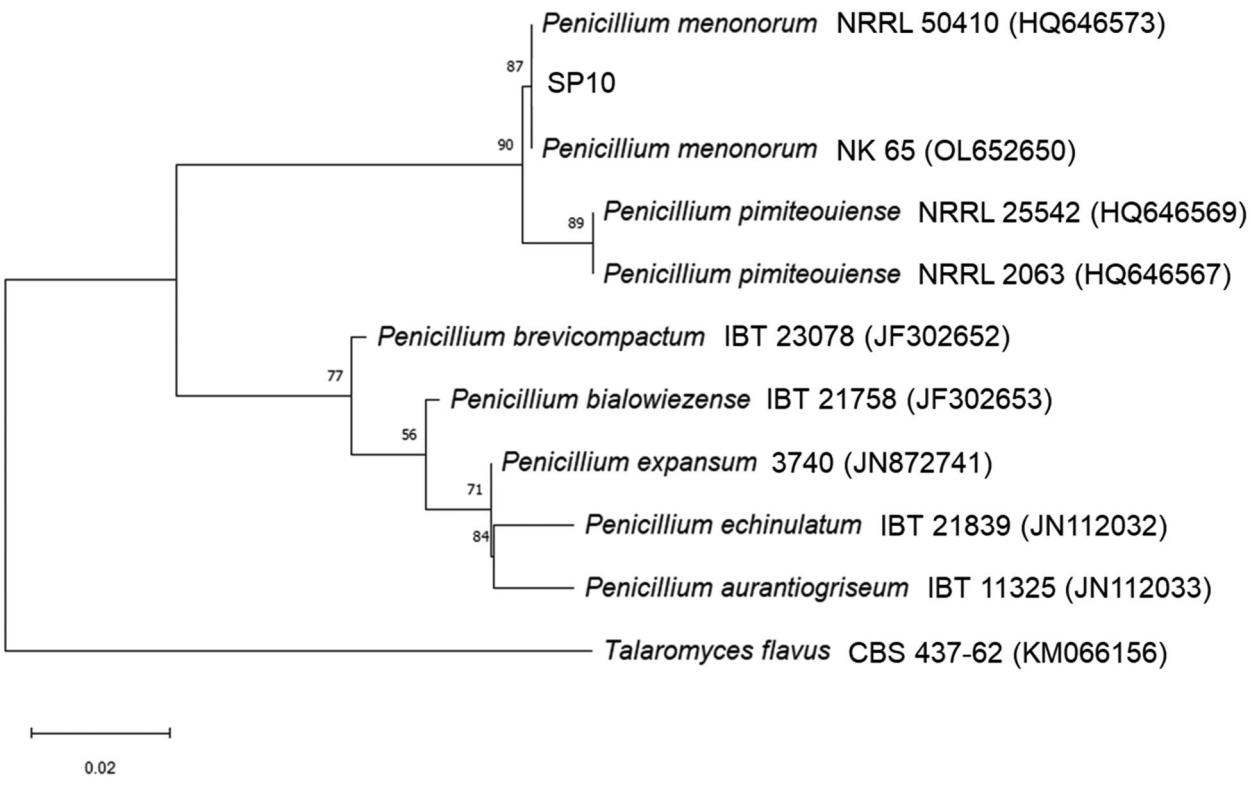

Figure 5. Phylogenetic tree of *β-tubulin* gene of a fungal isolate SP10. The number of branches is the bootstrap value.

3.3.2. Optimum pH and temperature

The effect of pH on the xylanase activity of *P. menonorum* SP10 was investigated in pH 4 to pH 10. The xylanase worked optimally at pH 6 (100% activity). However, it also reached a second peak at pH 5 (93% activity) (Figure 7A).

Besides, the effect of temperature on activity was conducted from 40 °C to 80 °C (Figure 7B). The xylanase *P. menonorum* SP10 reached a maximum activity at 55 °C (100% activity). Moreover, it could reach 61% activity at 65 °C.

4. Discussion

This study regarded the isolation and screening of the potential xylanase-producing fungi from soil. During the qualitative and quantitative tests, we proposed *P. menonorum* SP10 as a good candidate for xylanase production. Along with *Trichoderma* and *Aspergillus* species, the xylanase from *Penicillium* species has drawn much attention. The xylanase from *Penicillium* genus was preferred over *Trichoderma* and *Aspergillus* genera because it could work in acidic and high-temperature conditions [2]. Thus, many *Penicillium* species were published for their xylanase activity, such as *P. crustosum* [18], *P. janczewskii* [19], *P. ramulosum* [20], *P. rolfsii* [21], *P. funiculosum* [22], *P. sclerotiorum* [23], *P. citrinum* [24,25] and *P. chrysogenum* [26]. To our knowledge, the xylanase-producing ability of *P. menonorum* has not been explored since it was reported for taxonomy in 2015 [27].

P. menonorum SP10 produced two xylanase isoforms with 45 kDa and 54 kDa. The multiple xylanases were also found in other *Penicillium* species, such as *P. oxalicum* GZ-2 [28], *P. ramulosum* N1 [20], *P. janczewskii* [19] and *P. crustosum* FP 11 [18]. This may be due to *Penicillium* species containing many xylanase genes. For example, *P. oxalicum* GZ-2 had at least six xylanase genes [28]. Similarities were also found in *T. reesei* with 16 hemicellulase genes and *Aspergillus* sp. with more than 200 polysaccharide-degrading genes [29]. The multiple xylanase isoforms help these fungi to deal with the hemicellulose complex and degrade it more efficiently [28]. The molecular weight of *P. menonorum* SP10's xylanase differed from most *Penicillium* species which were reported under 35 kDa [18]. Only some produced xylanase of more than 40 kDa, such as *P. verruculosum* (65 kDa) [21], *P. funiculosum* (46 kDa) [30] and *P. oxalicum* GZ-2 (43 kDa) [28].

The xylanase of *P. menonorum* SP10 worked optimally at a slightly acidic condition (pH 6). This property was similar to other *Penicillium*'s xylanases, which were active at pH 6.0, including *P. rolfsii* c3-2(1) IBRL [21], *P. chrysogenum* [26] and *P. janczewskii* [19]. On mycoCLAP website (https://mycoclap.fungalgenomics.ca/mycoCLAP/), most fungal

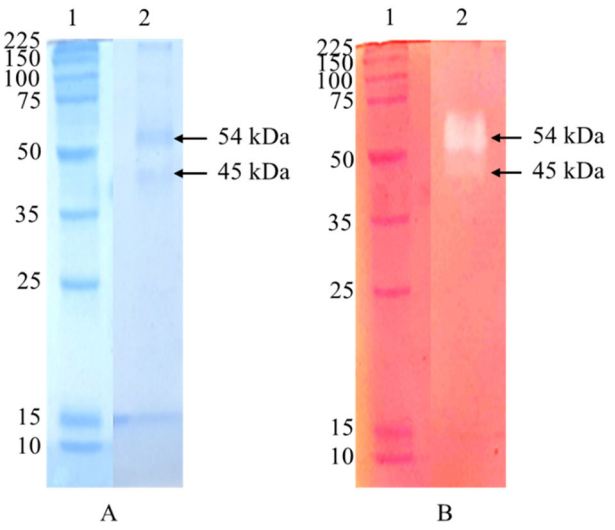

Figure 6. The xylanase isoforms of *Penicillium menonorum* SP10 on SDS-PAGE containing 0.1% beechwood xylan. A: the gel was stained with Coomassie blue G-250; B: the gel was stained with Congo red solution. Lane 1: protein marker (10–225 kDa); lane 2: the crude enzyme of *P. menonorum* SP10.

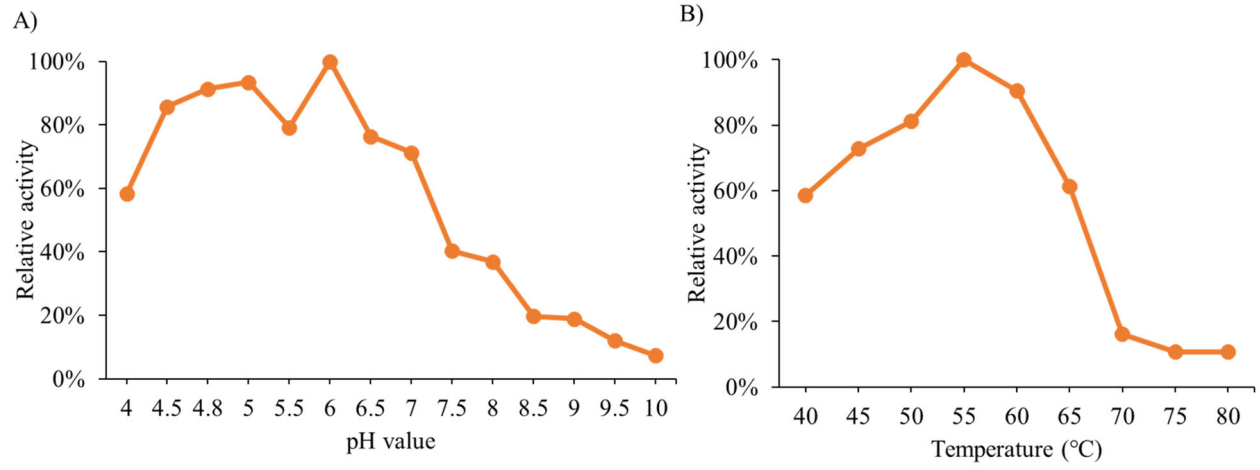

Figure 7. The effect of pH (A) and temperature (B) on the activity of *Penicillium menonorum* SP10 xylanase.

xylanases were also reported with optimum pH at 3.5 to 6.0, including *Aspergillus* and *Trichoderma* genera. Besides, *P. menonorum* SP10's xylanase had a second peak at pH 5. This indicated that two xylanase isoforms worked at different pH conditions. The similarities were found in previous studies, such as *P. sclerotiorum* produced two xylanase isoforms that work optimally at pH 2.5 and 4.5 [31]; the crude xylanase of *T. harzianum* containing two isoforms also worked at different pH conditions (pH 5 and pH 8) [8]. Besides, *P. menonorum* SP10's xylanase reached the maximum activity at 55 °C. Other fungi, including *Aspergillus niger*, *Trichoderma* sp. and *Fusarium* sp., also acted at 40 °C to 60 °C (https://mycoclap.fungalgenomics.ca/mycoCLAP/). Interestingly, the xylanase from *P. menonorum* SP10 could reach 61% activity at 65 °C, while most fungal xylanases became inactive at above 60 °C [9]. For example, xylanase of *P. rolfsii* c3-2(1) IBRL reached only 18% activity at 65 °C [21].

The properties of *P. menonorum* SP10 xylanase, including working at slightly acidic and high-temperature conditions, are beneficial for industrial applications. Normally, xylanase is expected to work or retain its function in harsh environments. In the poultry feed industry, for example, xylanase is required to work at the acid and neutral pH to boost the digestibility of broiler chickens [6,21]. In the pulp industry, xylanase is desired to deal with elevated temperatures to reduce the cost of the cooling process [6,8]. The xylanase from *P. menonorum* SP10 may be applicable to improve the yield and economic efficiency in both poultry feed and pulp industries.

5. Conclusion

Fifteen fungi were isolated from soil located in Suphan Buri, Thailand. Through qualitative and quantitative tests, we proposed *P. menonorum* SP10 as a good candidate for xylanase production. It produced xylanase with 0.535 U/mL. The xylanase isoforms on SDS-PAGE indicated that *P. menonorum* SP10 produced two xylanases (45 kDa and 54 kDa). Besides, its xylanase worked optimally at pH 6 and 55 °C while reaching 61% activity at 65 °C. The xylanase from *P. menonorum* SP10 can be used in poultry feed and pulp industries where slight acidic and thermos- treatment is required.

Acknowledgments

The authors thank King Mongkut's Institute of Technology Ladkrabang for financial support (grant no. KDS2020/048).

Disclosure statement

The authors declare no competing interests.

Funding

This research was financially supported by King Mongkut's Institute of Technology Ladkrabang (KMITL) under Grant number KDS2020/048.

Data availability statement

The data that support the findings of this study are available in the article

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