

Isolation of filamentous fungi exhibiting high endoxylanase activity in lignocellulose hydrolysate

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Abstract: For complete degradation of hemicellulose into its monomers from lignocellulose biomass, the synergistic action of a broad range of hydrolytic enzymes is needed. Therefore, production of enzymes from their natural producer is desirable. To obtain a powerful β -1, 4-endoxylanase producing fungus, 304 environmental samples were collected from various locations in Singapore, leading to 603 isolates. Among them, 71 exhibiting β -1, 4-endoxylanase activity were identified belonging mainly to the genera of *Aspergillus*, *Penicillium* and *Trichoderma*. Further analysis revealed *A. niger* DSM 26641 as a potential and stable β -1, 4-endoxylanase producer, being able to grow in hydrothermal lignocellulose hydrolysate exhibiting its maximal β -1, 4-endoxylanase activity at pH 4 and 60°C. This strain is thought to be very suitable for lactic acid production in a simultaneous saccharification and fermentation at pH values below 5.

Keywords: β -1, 4-endoxylanase, on-site enzyme production, filamentous fungi, lignocellulose, *Aspergillus niger*, phylogeny

1. Introduction

Lignocellulose, the widely abundant biomass, is mainly composed of cellulose (20-50%), hemicellulose (15-35%) and lignin (15-35%) [1, 2]. It is seen as a promising resource for production of value-added chemicals such as ethanol and lactic acid [3]. Degradation of the sugar polymers to their monomers is essential for microbial fermentation.

The complete degradation of hemicellulose needs the synergistic action of a group of hydrolytic enzymes [4]. Even though these enzymes can be produced in larger quantities individually by recombinant DNA technology [5-8], full and fast degradation of hemicellulose can only be achieved by the synergistic action of all these enzymes [9]. The compositions of hemicellulose differ between plants and even between different parts of a single plant. Therefore, a process based on whole cell enzyme production would provide flexibility for efficient enzymatic hydrolysis of hemicellulose.

Filamentous fungi are natural degraders of lignocellulose and therefore also potential producers of complex enzyme mixtures. Their growth occurs naturally on solid substrates but they are also able to grow in submerged cultures forming various morphological structures like pellets which are tightly associated with enzyme production [10]. As submerged fermentation has well established protocols, it is favored industrially over solid state fermentation which is often more difficult to control.

In general, the usability of an enzyme mixture is dependent on its cost-effectiveness as the success of production of bulk chemicals is cost-driven. Process costs need to be reduced by measures such as on-site enzyme production [11], recycling of fungal pellets and avoidance of genetic modifications which lowers wastewater treatment costs. On the other hand, the selected fungal strain is of uttermost importance. In addition to a high production of key enzymes like β -

1, 4-endoxylanases which are responsible for hydrolysis of xylan, the major component of hemicellulose, the enzymes need to have suitable pH and temperature optima for the intended applications.

Here we report the isolation of a filamentous fungus *Aspergillus niger* DSM 26641 from the Singaporean environment exhibiting high β -1, 4-endoxylanase activity in submerged culture of lignocellulose hydrolysate with desired pH and temperature optima for simultaneous saccharification and fermentation of lignocellulose.

2. Materials and Methods

2.1 Sample collection, strain isolation and storage

For obtaining filamentous fungi secreting β -1, 4-endoxylanases, samples were collected from the Singaporean environment and processed depending on their origins. Solid samples like soil, plant parts and rotten materials were submerged, vigorously mixed and diluted with autoclaved water while liquid samples were directly diluted. Fungal fruiting bodies were partially sterilized by submersion in technical ethanol. Subsequently, the treated samples were transferred to primary pre-dried PDA plates (potato extract 4 g l⁻¹, dextrose 20 g l⁻¹, agar 20 g l⁻¹, 50 mg l⁻¹ chloramphenicol dissolved in methanol). The addition of chloramphenicol excluded the growth of bacteria while the extensive drying of the agar plates before usage made it unlikely for yeasts to emerge. After incubation of the primary PDA plates for 4-5 days at 30°C, single colonies of morphologically unique filamentous fungi were repeatedly transferred to fresh secondary PDA plates until morphological uniformity was observed. For mid-term storage, the obtained isolates were stored at 4°C and transferred to new PDA plates every 6 months. For long-term storage, fungal mycelium and spores from fresh PDA plates were stored in 20% glycerol at -80°C.

2.2 Screening for β -1, 4-endoxylanase activity

For β -1, 4-endoxylanase activity screening, enzymes were produced in submerged cultures using a defined broth (beechwood xylan 2 g l⁻¹, yeast extract 0.1 g l⁻¹, peptone 0.1 g l⁻¹, 0.1 g l⁻¹ xylose). Broth (5 ml) in 15 ml tubes were inoculated with spores or mycelium from the prior obtained isolates and incubated horizontally for 4 days at 30°C and 200 rpm. Subsequently, tubes were centrifuged to separate mycelium and culture supernatant.

Remazol Brilliant Blue R dyed xylan (RBB-Xylan) prepared following an earlier developed protocol [12] was used to determine β -1, 4-endoxylanase activity in the obtained culture supernatants according to a modified protocol [13]. The absorbance was measured at 590 nm and used directly or set as a percentage to the highest measured absorbance value during the respective screening. Due to the initial large amount of fungal isolates, the first screening of all isolates was done in batches to exclude isolates which did not significantly produce β -1, 4-endoxylanase.

2.3 Identification and phylogenetic classification of isolates

Fungal mycelium of isolates identified as potential β -1, 4-endoxylanase producers was grown in 15 ml tubes as described earlier. After centrifugation, the mycelium was washed with autoclaved water to remove possibly formed spores and residual culture supernatant. The mycelium was transferred into 2 ml thick-walled reaction tubes and a ceramic ball with 100 mg quartz sand (50-70 mesh) was added. Additionally, 1 ml extraction buffer (SDS 5 g l⁻¹, EDTA 7.3 g l⁻¹, NaCl 14.61 g l⁻¹, TRIS 24.23 g l⁻¹, pH 8.5) was supplied.

The prepared samples were homogenized at 4.5 m s⁻¹ for 20 s using the FastPrep24-Homogenizer (MP Biomedicals) and subsequently centrifuged for 10 min at 20,800 × g at 4°C to

remove non-homogenized biomass. The supernatants were transferred into new reaction tubes and phenol : chloroform : isoamylalcohol (1 ml) was added. After vigorous mixing, the samples were centrifuged for 15 min at $20,800 \times g$ and 4°C . The aqueous phases (0.5 ml) were transferred to new 1.5 ml reaction tubes. Ethanol (1 ml, 100%) and sodium acetate (0.15 ml, 3 M) was added followed by vigorous mixing. After precipitation overnight at -80°C , samples were centrifuged at $20,800 \times g$ for 30 min at 4°C . The supernatants were discarded and pellets were washed twice with 70% (v/v) ethanol. Subsequently, pellets were air-dried and dissolved in 50 μl DNase free water. Nucleic acid concentrations were estimated using a Nanodrop 2000 spectrophotometer.

For amplification by Polymerase Chain Reaction (PCR), a mixture containing 5 μl Taq buffer (10X), 3 μl MgCl_2 (25 mM), 1 μl dNTP Mix (10 mM), 1 μl primer ITS1 (10 mM) (5'-TCC GTA GGT GAA CCT GCG G-3'), 1 μl primer ITS4 (10 mM) (5'-TCC TCC GCT TAT TGA TAT GC-3') and 0.3 μl Taq polymerase (5 U/ml) per reaction was prepared. A volume of DNA sample containing an estimated amount of up to 1 μg template DNA each was added. Reactions were filled up to 50 μl total volume by addition of PCR grade water. After completion of the PCR (3 min at 95°C ; 35 cycles of 1 min at 95°C , 1 min at 55°C , 1 min at 72°C ; 72°C for 10 min), 2 μl electrophoresis buffer (6X) was added to 10 μl PCR product and run at 6 V cm^{-1} for 30 min on a 1% agarose gel. When a clear band around 700 bp was observed, 40 μl of the corresponding PCR product left was purified using a PCR purification kit according to its protocol. Samples were sequenced by capillary sequencing using the ITS 1 and ITS 4 primers. The two obtained sequences were aligned to a combined sequence and compared with the NCBI Nucleotide collection databases using the blast algorithm [14]. The best hit was used as an identifier for its corresponding fungal isolate.

For creation of a phylogenetic tree, established protocols were used [15]. The pre-labeled sequences were aligned to the first multiple sequence alignment (MSA) by GUIDANCE [16] using the FFT-NS-1 MAFFT algorithm [17]. A set of Neighbor-joining bootstrap trees were calculated by GUIDANCE from the first MSA [18, 19] followed by the creation of MSAs from the set of trees. Additional to GUIDANCE scores, Head-or-Tail (HoT) scores were calculated. Sequences with low GUIDANCE or HoT scores were excluded from the final MSA to improve the overall confidence in the final tree. MEGA5 [20] was used to identify the best suitable substitution model and distribution pattern for the final MSA through the Bayesian Information Criterion score and used subsequently to construct a hypothetical phylogenetic tree to identify promising clusters and to exclude clusters of pathogenic filamentous fungi.

2.4 Identification of the most suitable filamentous fungus

The potential β -1, 4-endoxylanase producers were screened in a common screening to establish a rank order sorted by activity. Subsequently, isolates were screened again independently for testing the robustness of the established rank order in comparison with the activity of a reference strain (*A. niger* ATCC 16888). Both screenings were done without replicates.

The enzyme mixtures of the 5 fungal isolates with robust and higher or comparable β -1, 4-endoxylanase activity than the reference strain were tested regarding their activity depending on pH and temperature. Supernatant was prepared and β -1, 4-endoxylanase activity was measured as described earlier. Instead of using sodium acetate buffer (pH 5) at a reaction temperature of 50°C, phosphate-citrate buffer (pH 3 - 7; 1 pH step) and phosphate buffer (pH 8) were used over a broad temperature range (30°C-80°C; 10°C steps). The absorbance values

reflecting the β -1, 4-endoxylanase activity were set as percentages to the maximal measured absorbance of each isolate.

The 3 most suitable isolates regarding their pH and temperature dependences of β -1, 4-endoxylanase activity were compared with 4 reference strains (*A. niger* ATCC 16888, *T. reesei* ATCC 56765, *T. reesei* ATCC 26921 and *D. dictyoides* ATCC 60575) in defined xylan broth and tested for their ability to grow and produce enzymes in lignocellulose hydrolysate. For production of lignocellulose hydrolysate, empty fruit bunch powder (1 mm) was prior treated in a ratio of 1:10 with water in a Parr reactor for 10 min at 150°C as described earlier [13]. Lignocellulose hydrolysate (40 ml) and defined xylan broth (40 ml), respectively, were inoculated in conical flasks with spores of the isolates and the reference strains. Cultures were incubated for 8 days at 30°C and 200 rpm and the β -1, 4-endoxylanase activity was measured on a regular basis.

3. Results and Discussion

3.1 Isolation and screening of β -1, 4-endoxylanase producing fungi

Totally 304 environmental samples were collected all over Singapore leading to 603 isolates of filamentous fungi. All isolates were recorded and are publically available for further usage (www.domse.org). The screening for β -1, 4-endoxylanase activity was done in 5 batches with an overall distribution of 12% active isolates, 9% possibly active isolates and 79% clearly inactive isolates. The 71 clearly active isolates were screened in a common screening to establish a rank order. YOPX-FS04 could be identified at a level of 100% as the most active isolate with all other isolates at a level of below 75%.

For identification of the stability of the rank order, the isolates were screened again and all isolates at a level of below 40% were excluded from further consideration leading to 21 isolates with a clearly strong β -1, 4-endoxylanase activity and a stable rank order (**Fig.1**). When compared to the reference strain, the first 5 isolates were of the highest interest with 2 isolates of slightly lower activity (BBTP-FS01, YOPX-FS01), 2 isolates of slightly higher activity (KRPX-FS08, WTPE-FS01) and one isolate exceeding the activity of the reference strain at almost 180% (YOPX-FS04). Therefore, all 5 isolates were selected for further experiments.

3.2 Phylogenetic analysis

For identification of clusters showing high β -1, 4-endoxylanase activity and possible pathogenic clusters which need to be reviewed more carefully before industrial usage, a phylogenetic tree based on 65 isolates (after exclusion of 6 isolates as suggested by GUIDANCE and HoT scores) was created (**Fig. 2**). The best fitting substitution model was determined to be a Tamura 3-parameter model following a discrete gamma distribution. The best approach was identified as a Neighbor-joining tree and the Maximum Likelihood method with a complete deletion of gaps based on 100 bootstrap replicates and a collapse of branches which could not be reproduced in at least 69% of the replicates.

Three main genera (*Aspergillus*, *Trichoderma* and *Penicillium*) could be identified with 32, 24 and 10 isolates, respectively. *Aspergillus* section *nigri* [21] was with 21 isolates the biggest cluster showing in average very high β -1, 4-endoxylanase activities (**Fig.3**), followed by *Aspergillus* section *flavi* [22] with 8 isolates. As the section *flavi* is known to contain strong aflatoxin producers and human pathogens like *A. flavus* [23], members of this group should not be used without further investigation. Within the genus *Penicillium*, *P. simplicissimum* was with 8

isolates the largest cluster showing a similar high average activity as the *Aspergilli*. Even though members of the genus *Trichoderma* showed considerable activity levels, only *T. erinaceum* (5 isolates) could be considered of similarly high activity.

From the 5 most active isolates, isolate YOPX-FS01 (*A.flavus/oryzae*) was identified as a member of the section *flavi*, while the other three (YOPX-FS04, KRPX-FS08, BBTP-FS01) were members of the section *nigri*. WTPE-FS01 was identified as *P. simplicissimum*.

3.3 Identification of the most suitable filamentous fungus

The measurement of the pH and temperature dependent β -1, 4-endoxylanase activity of all five isolates revealed a similar profile for YOPX-FS04, KRPX-FS08, BBTP-FS01 and WTPE-FS01 with an maximal activity at pH 4 and 60°C (**Fig. 4a**). YOPX-FS01 in opposite exhibited its maximal activity at pH 6 (**Fig. 4b**). Considering lactic acid production in a simultaneous saccharification and fermentation process preferably at pH 5 and below [24], the profile of YOPX-FS01 would not be suitable. Therefore, it was excluded from further investigation as it was also found belonging to a pathogenic cluster.

After excluding WTPE-FS01 from further examinations due to poor growth, the 3 isolates left were grown together with 4 reference strains over 8 days on defined xylan broth (**Fig. 5a**) and lignocellulose hydrolysate (**Fig. 5b**), while β -1, 4-endoxylanase activity was measured in regular intervals. While no strong differences between all isolates and reference strains were observed in defined xylan media (overall activity below 40%), *A. niger* KRPX-FS08 and *A. niger* BBTP-FS01 were found having the strongest β -1, 4-endoxylanase activity at 100% after 6 days on lignocellulose hydrolysate while all other isolates and reference strains

remained below 40% similar to the observation on defined xylan media. Isolate BBTP-FS01 was subsequently deposited as *A. niger* DSM 26641.

4 Conclusions

From over 600 isolates of filamentous fungi from Singaporean environment, *A. niger* DSM 26641 was found to be a potential and stable β -1, 4-endoxylanase producer. *A. niger* DSM 26641 is capable of growing in hydrothermal lignocellulose hydrolysate exhibiting its maximal enzyme activity at pH 4 and 60°C. This makes the strain suitable for simultaneous saccharification and fermentation at pH below 5 in submerged cultures.

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Figure legends

Fig. 1 Comparison of β -1, 4-endoxylanase activity of the 21 most active and stable isolates with that of *A. niger* ATCC 16888

Fig. 2 Phylogenetic tree for 65 isolates exhibiting β -1, 4-endoxylanase activity (Numbers on branches reflect the amount of positive bootstrap replicates)

Fig. 3 Distribution of β -1, 4-endoxylanase activity amongst the 71 active isolates sorted on genus and species levels. *The isolate with maximal activity (100%) is not shown.

Fig. 4 β -1, 4-endoxylanase activity dependence on pH and temperature for isolates BBTP-FS01 (A) and YOPX-FS01 (B)

Fig. 5 Growth of 3 selected isolates (YOPX-FS04, KRPX-FS08, BBTP-FS01) and 4 reference strains (*A. niger* ATCC 16888, *T. reesei* ATCC 56765, *T. reesei* ATCC 26921 and *D. dictyoides* ATCC 60575) on defined xylan broth (A) and lignocellulose hydrolysate (B) for 8 days (Error bars are representing standard deviation values)

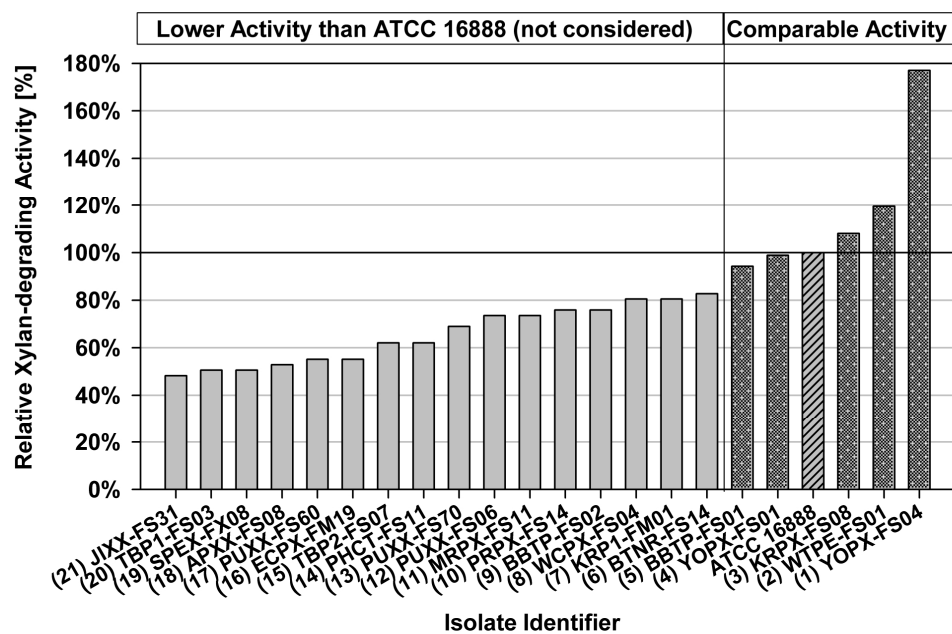


Fig. 1

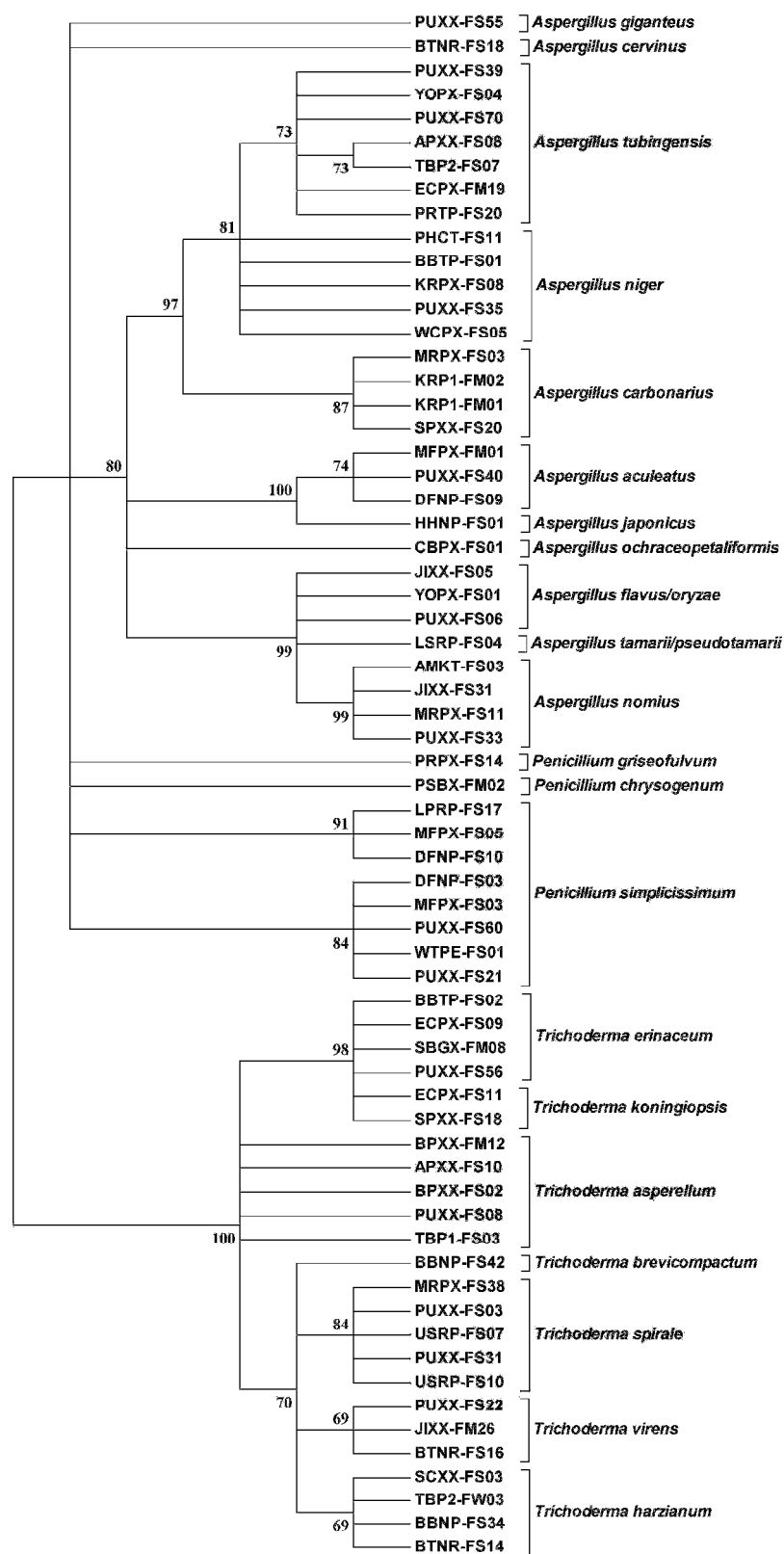


Fig.2

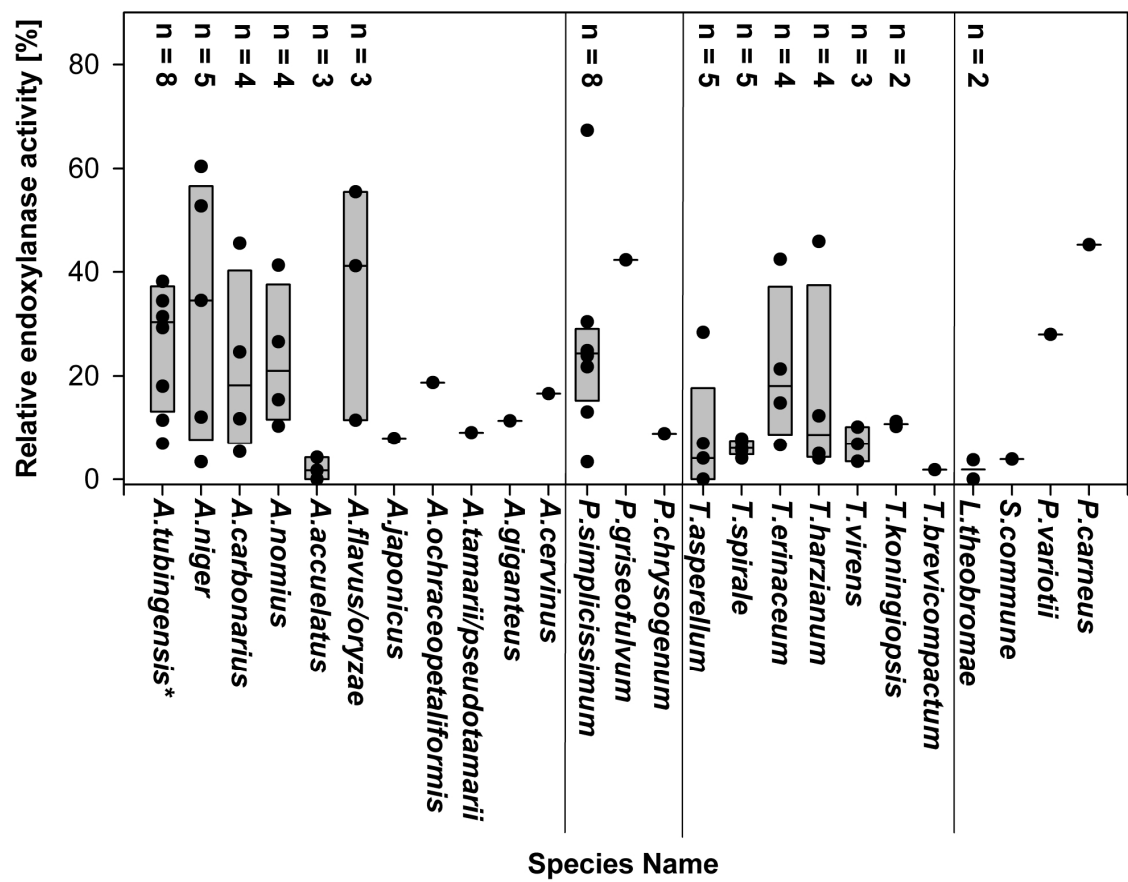


Fig. 3

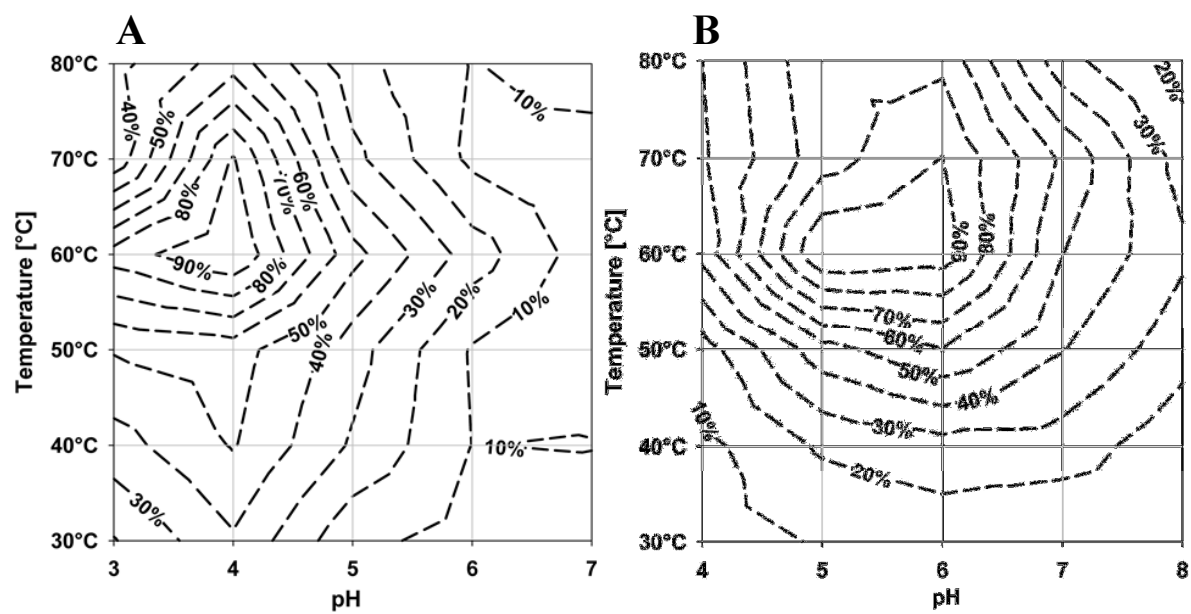


Fig. 4

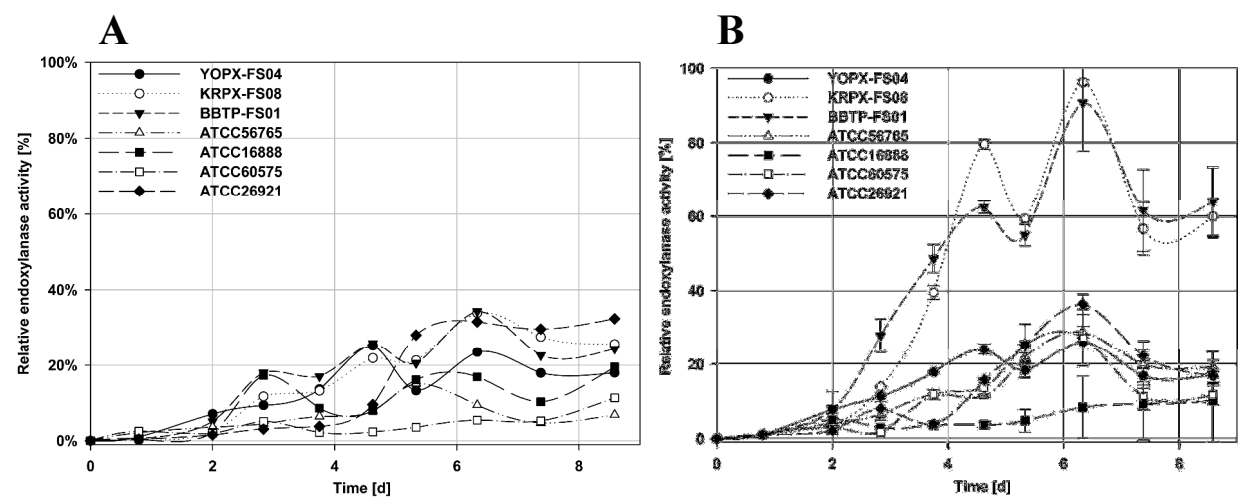


Fig. 5