Improved endoxylanase production and colony morphology of *Aspergillus niger* DSM 26641 by γ-ray induced mutagenesis

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Abstract: *Aspergillus niger* DSM 26641 was exposed to ^{60}Co γ-radiation to enhance the β-1, 4-endoxylanase activity, restrict colony growth and improve robustness of pellets. The first promising mutant obtained after γ-radiation of the fungal spores at 50-2000 Gy showed a restricted colony growth and an 82% enhancement in β-1, 4-endoxylanase activity. The mutant was subjected to a second round of γ-radiation at 1400 Gy generating a mutant with double the β-1, 4-endoxylanase activity compared to the native strain. The selected final mutant, deposited as *Aspergillus niger* DSM 28712, showed a maximal saccharification activity of 26 U ml^{-1} on xylan based broth, 48 U ml^{-1} on lignocellulose hydrolysate and 375 U ml^{-1} on lignocellulose hydrolysate supplemented with yeast extract and mineral salts.

Keywords: Filamentous Fungi, γ-Radiation Mutagenesis, Enzymes, Endoxylanase, Microbial Growth, Optimisation.

1. Introduction

Filamentous fungi are a good source for xylanotic enzymes owing to their naturally inhabiting as lignocellulose decomposers. Individual fungal xylanases have been successfully overexpressed in various organisms such as bacteria [1], filamentous fungi [2], yeasts [3] and transgenic plants [4]. For complete degradation of hemicellulose, however, at least two backbone-degrading enzymes (β-1,4-endoxylanase and β-xylosidase) and three accessory enzymes (arabinofuranosidase, α-glucuronidase and acetyl xylan esterase) are needed [5]. Therefore, production of enzymes from filamentous fungi as enzyme cocktails is more suitable for industrial applications in breaking down hemicellulose to fermentable sugars. Native filamentous fungi are usually not ideal for producing industrial enzymes due to the low enzyme activities or un-favored morphology. In most cases, the activity of the backbone-degrading enzyme, β-1, 4-endoxylanase, needs to be enhanced for more efficient hydrolysis of xylan, the major component of hemicellulose. In addition, filamentous fungi cannot be easily optimized by using well-developed agar plate supported screening methods due to their widespread and fast-growing morphology, so colony restriction needs to be developed for easier screening. Although a limited colony restriction can be achieved by adding chemicals like oxgal [6, 7], a permanent change to a more restricted morphology would be more desirable to achieve easier recycling and

handling of fungal pellets in submerged cultures, which is the preferred modus for enzyme production in industry rather than the natural growth modus of filamentous fungi. For instance, it has been shown that a hard and smooth pellet surface favored the production of enzymes and organic acids like citric acid [8].

The complete degradation of hemicellulose needs the synergistic action of a group of enzymes, so a random approach followed by suitable screening methods is most promising for improving the enzyme cocktails produced by fungi. The enzyme activity levels are not only influenced by their coding genes but also by control elements. Some important control elements include repression by carbon sources [9], expression of transcription factors [10] and expression of kinases and phosphatases for sensing different carbon sources or controlling the cellular energy states [11]. The use of γ-radiation for introducing random mutations has the advantages of deep penetration and fast induction of various mutations such as single and double strand breaks, creation of base-free sites and modification of bases [12]. γ-radiation from ^{60}Co radiation sources is a low linear energy transfer radiation (LET radiation) with a LET value of 1 keV μm^{-1} and below and tends to create clustered non-double strand breaks which can be repair-resistant or at-least more difficult to repair than non-clustered DNA damages as shown by using synthetic oligonucleotides [13]. In addition, it enhances non-DNA related cell

stress by creation of free radicals [14]. γ-radiation has been successfully used for 2-fold enhancement of cellulase and galactosidase production in fungi [15,16].

Here we report the introduction of random mutations in *Aspergillus niger* DSM 26641 by γ-radiation to enhance its β-1, 4-endoxylanase activity and restrict its colony and pellet morphology for favorable industrial applications. This is the first report on improving an *Aspergillus niger* strain in both its β-1, 4-endoxylanase activity and cellular morphology.

2. Materials and Methods

2.1 β-1, 4-endoxylanase assays

The β-1, 4-endoxylanase activity was assayed by using either Remazol Brilliant Blue dyed xylan (RBB-Xylan) or pure xylan as substrates.

For the activity assay using RBB-Xylan as the substrate, RBB-Xylan was prepared following the developed protocol [17]. Spores or mycelia from selected mutants were used to inoculate 10 ml broth containing 2 g l^{-1} xylan, 0.1 g l^{-1} yeast extract, 0.1 g l^{-1} peptone and 0.1 g l^{-1} xylose in 15 ml tubes. The tubes were incubated at 30°C and 200 rpm for 4 days. After successful growth, the tubes were centrifuged for 10 min at 3,220×g at 4°C to separate the mycelia from the supernatant. The culture supernatant was used to determine the β-1, 4-

endoxylanase activity following the protocol as described previously [18]. The absorbance of the native strain *Aspergillus niger* DSM 26641 was set as 100%.

For the activity assay using xylan as the substrate, the produced sugars in the supernatant were detected by the 3, 5-dinitrosalicylic acid (DNS) method [19].

2.2 γ-Ray irradiation procedures

Aspergillus niger DSM 26641, which was isolated from a soil sample in Singapore and exhibited high β-1, 4-endoxylanase activity, was subjected to γ-radiation for improving its performances. After mycelial growth for 3 days at 30°C on Potato Dextrose Agar (PDA, potato extract 4 g l^{-1}, dextrose 20 g l^{-1}, agar 20 g l^{-1}), black spores were formed by the fungus. The PDA plates were submerged in 10 ml sterile ultrapure water each and the spores were brought into suspensions.

A γ-ray irradiation chamber 4000a containing a ^{60}Co radiation source inside a shielded compartment was used for irradiation of the spores at a dose rate of 88 Gy h^{-1}. For estimation of γ-radiation sensitivity, the spore solution was exposed to radiation doses of 50-2000 Gy. Subsequently, the irradiated spore samples were spread on PDA plates at different dilutions to reach a countable amount of colony forming units (CFU). Afterwards, morphologically different colonies were selected from suitable plates and transferred to fresh PDA plates for further observation.

The obtained CFU values were used to estimate its D_{10} value. The D_{10} value represents the radiation dose at which the viable inoculum is reduced by 90%.

After screening the morphological different colonies for β-1,4-endoxylanase activity, spore solutions from three selected mutants each (different colony morphology; higher β-1,4-endoxylanase activity) were directly irradiated with a total dose of 1400 Gy (as estimated by the sensitivity experiments) and streaked out on PDA plates. Not only morphologically different but also similar colonies were isolated and screened for higher β-1, 4-endoxylanase activity.

2.3 Validation of mutants and final strain selection

For final validation of β-1,4-endoxylanase activity produced by the selected mutants, spores of the respective mutants and the original wildtype strain were used to inoculate 40 ml xylan broth in 50 ml shaking flasks in duplicate. The shaking flasks were incubated at 30°C and 200 rpm. In regular intervals, β-1, 4-endoxylanase activity was measured using RBB-Xylan as described above.

For final strain selection, spores of the selected mutant were spread on 10 PDA plates in a fitting dilution to obtain single colonies. Before spores were observed, colonies exhibiting restricted colony growth were picked and transferred into a well plate half filled with PDA. After observation of spores with the desired morphology, the wells were filled up with sterile water. The obtained spore

solutions were pooled and mixed. The mixed spore solution was streaked out in a fitting dilution on 10 new PDA plates and the same procedure was repeated. For investigating the changes in β-1, 4-endoxylanase activity during final strain selection, the spore solutions were stored and assayed using RBB-Xylan. In addition, the initial xylan broth, the hydrothermal palm oil empty fruit bunch fiber (EFB) hydrolysate, a lignocellulose derivate, and hydrothermal hydrolysate of EFB supplied with 0.05% yeast extract, mineral salts and 3% EFB were used for production of enzymes and the total saccharification activity of the enzyme solutions were assayed using the DNS method. The final mutant was deposited as *Aspergillus niger* DSM 28712. All other mutants were preserved at -80°C in a 20% glycerol stock. Details and pictures were recorded and stored in a publically available database (www.domse.org).

3. Results and Discussion

3.1 Strain selection

Aspergillus niger is an attractive target for whole cell mutagenesis as it is generally recognized as safe and even used for producing food additives [20]. It is also one of the most important fungi used for industrial production of citric acid [21] and various enzymes. For hydrolytic enzymes, an improvement in production of β-1, 4-endoxylanase is not only favorable for more efficient hydrolysis of xylan but is also beneficial to cellulose decomposition by cellulases due to the removal of

xylan [22, 23]. The mutants created by γ-ray irradiation are not considered as genetically modified organisms, so their release into the environment after usage would be permitted, making the on-site enzyme production more cost-effective [24].

3.2 γ-Radiation sensitivity and mutant screening

To achieve a high mutation probability and avoid extensive radiation damages at the same time, two strategies were adapted. First, using spores instead of mycelia for the γ-ray irradiation to achieve a higher level of stress resistance and minimize the undesired side effects [25]. Second, a γ-radiation dosage of 1400 Gy was applied, which equals 3-4 D_{10}, leading to a balance of damage and mutation rates, similar to the established protocols [26]. The D_{10} value was estimated to be 400 Gy in the first round of γ-ray irradiation (Fig.1), similar to that estimated for other strains of *Aspergillus niger* reported in literature [27, 28].

The first round of screening led to mutants that showed changes in spore formation, spore color, spore quantity, mycelial growth rate, colonial growth rate and formation of pigments (Fig.2). Totally, 40 mutants were isolated and tested for their β-1, 4-endoxylanase activity, from mutant Pre_04 which completely lost its β-1, 4-endoxylanase activity to the mutant Pre_M36 which increased its β-1, 4-endoxylanase activity to 125% compared to the wild type strain. Totally 15 mutants showed an obviously higher β-1, 4-endoxylanase activity than the wild

type strain. In 7 out of 10 of the most active mutants, a loss or a very strong reduction in spore formation was observed. The fungal sporulation system is considered to be very sensitive, therefore easily susceptible to mutation damages [29]. Sporulation is a very disturbing process for submersion production of enzymes, but it is still useful for optimization, storage and easy multiplication and thus should not be completely abolished. A delayed or weaker sporulation would be more desirable. Therefore, in the second round of γ-ray irradiation, 3 mutants that showed higher β-1, 4-endoxylanase activities beside morphological alterations but still maintained the ability for spore formation were selected (Pre_M30: 82% enhancement in β-1,4-endoxylanase activity, fewer spores and restricted colonial growth; Pre_M15: 57% enhancement in β-1, 4-endoxylanase activity and slightly slower mycelial growth; Pre_M40: 56% enhancement in β-1, 4-endoxylanase activity and a change to a brownish spore pigmentation). The second round of γ-ray irradiation of the spore solutions of the 3 selected mutants resulted in 101 new mutants. All mutants showed a broad variation in β-1, 4-endoxylanase activity similar to the mutants obtained in the first round.

3.3 Validation of mutants and final strain selection

For final validation, the best mutants from the second round of γ-ray irradiation and their predecessors, the best two non-spore-forming mutants from

the first round of γ-ray irradiation and the wild type strain were tested for their β-1, 4-endoxylanase production for 89 h (Fig.3).

Pre_M15, Pre_40 and Pre_M30 showed roughly the same activity level at 110% (wild type 100%) when cultivated in 50 ml flasks. Pre_26 and Pre_36 showed a clearly higher activity (130%) than the wild type strain, but they were not selected for further study due to the undesired loss in spore formation. Almost all mutants from the second screening (M26, M20, M12, M09) round gave lower activities, except the descendants of Pre_M30 (M42, M43) which showed an activity level at 180%, almost double that of the wild type strain.

The obvious differences between observed activities in different screening rounds and the final validation experiment might reflect the susceptibility of filamentous fungi to the changes of environmental conditions such as the change of culture volume and shape of the incubators [30].

Mutant M42 was selected for the final strain selection as it showed almost double β-1, 4-endoxylanase activity of the wild type strain with restricted colony growth. The occurrence of the widespread colony morphology could be found initially in up to 8% of the colonies after streaking a suitably diluted solution on PDA plates. After selecting colonies with restricted growth followed by pooling and re-plating, the occurrence of widespread colony growth dropped from 8%

(initial mutant) to 3.8% (first round), 2.7% (second round), 0.6% (third round) and below 0.04% (final round) of all counted colonies. Not only the desired colony morphology was observed but the β-1, 4-endoxylanase activity enhanced significantly in each round (Fig.4). The repeated selection and pooling of spores from restricted colonies stabilized the obtained mutant strains. Almost no occurrence of the wild type growth modus was observed. Additionally, after each selection round, an obvious increase in β-1, 4-endoxylanase activity was observed, suggesting a possible relation between the observed restricted colony growth and the enhanced β-1, 4-endoxylanase activity. The relation between morphology and enzyme activity was reported earlier but is still poorly understood [31]. Therefore, one of the possible explanations lies within the ability of the mutant to form smaller pellets, lowering the amount of undersupplied or even dead mycelia, increasing the pellet density and subsequently the enzyme production. However, possibilities like the changes of enzyme activities or metabolic pathways are other possible explanations.

When assayed for the saccharification activity using the DNS method, the wild type and mutant strain exhibited similar activities (27 U ml^{-1} and 26 U ml^{-1}) on xylan. This stands in opposite to the different β-1, 4-endoxylanase activities which were observed earlier using the RBB-Xylan assay. The main reason might be the fundamental differences of the two assays. The RBB-Xylan assay measures

solely the liberated dyed xylan oligomers and thus only β-1, 4-endoxylanase activity, while the DNS assay measures the liberation of reducing carbohydrate units directly. Therefore other enzyme activities such as β-xylosidase activity might be higher in the supernatant of the wild type strain leading to similar overall reducing carbohydrate units. The wild type strain showed a higher activity than the mutant (59 U ml^{-1}vs 48 U ml^{-1}) on hydrothermal hydrolysate of EFB. For the EFB hydrolysate supplied with additional carbon source, nitrogen source and salts, the mutant produced a more active supernatant than the wild type strain (375 U ml^{-1} vs 337 U ml^{-1}). The saccharification activity of 375 U ml^{-1} of the mutant is higher than the reported 210 U ml^{-1} of a wild type strain [32] but lower than that of a genome shuffled strain (427.5 U ml^{-1}) [33].

3.4 Morphological comparison between wild type and mutant strains

In comparison with the wild type strain *Aspergillus niger* DSM 26641, the finally stabilized mutant M42, deposited as *Aspergillus niger* DSM 28712, showed all desired morphological attributes. When growing on PDA plates (Fig.5a-b), colonies of the mutant were well separated from each other at higher colony density. The spore formation of the mutant was delayed for 1 day and a yellow pigment was formed in excess after 4 days. Under the microscope (Fig.5c-d), the wild type strain revealed a more widespread colony growth than the mutant. The mutant grew on PDA in a dense and more uplifted way with no obvious growth

after 4 days. When growing in submerged cultures (Fig.5e-f), the mutant formed small, restricted, robust and regular pellets, while the wild type strain exhibited the opposite growth pattern. The pellets of the mutant were easier to handle for recycling purposes.

3.5 Conclusions

Aspergillus niger DSM 26641, a native β-1, 4-endoxylanase-producing filamentous fungus, was successfully improved by several rounds of γ-radiation and subsequent strain stabilization. The finally selected mutant, *Aspergillus niger* DSM 28712, showed double β-1,4-endoxylanase activity compared to the wild type strain and a restricted widespread filamentous growth on agar plates and in submerged cultures. This makes it easier to be picked up from agar plates for screening for further improvement by γ-radiation and other mutagenesis methods and more suitable for submerged fermentation for industrial applications.

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Figure legends

Fig. 1. Relation between γ-radiation dose and survival rate

Fig. 2. Comparison of selected colony morphologies after the first round of γ-radiation. The wild type strain *Aspergillus niger* DSM 26641 is marked red

Fig.3. Time courses of β-1, 4-endoxylanase activity of selected mutants. The activity of the wildtype strain at 65 h was set as 100% (Error bars = standard deviation values)

Fig.4. Change of β-1, 4-endoxylanase activity of mutant Pre_M30 during 3 generations of final strain selection of mutant M42. The initial activity was set as 100% (Error bars = standard deviation values)

Fig.5. Comparison of colony morphologies between the wild type *Aspergillus niger* DSM 26641 and the final mutant *Aspergillus niger* DSM 28712. a and b: colonies on agar plates; c and d: colonies on agar plates in magnification; e and f: pellets in submerged cultures

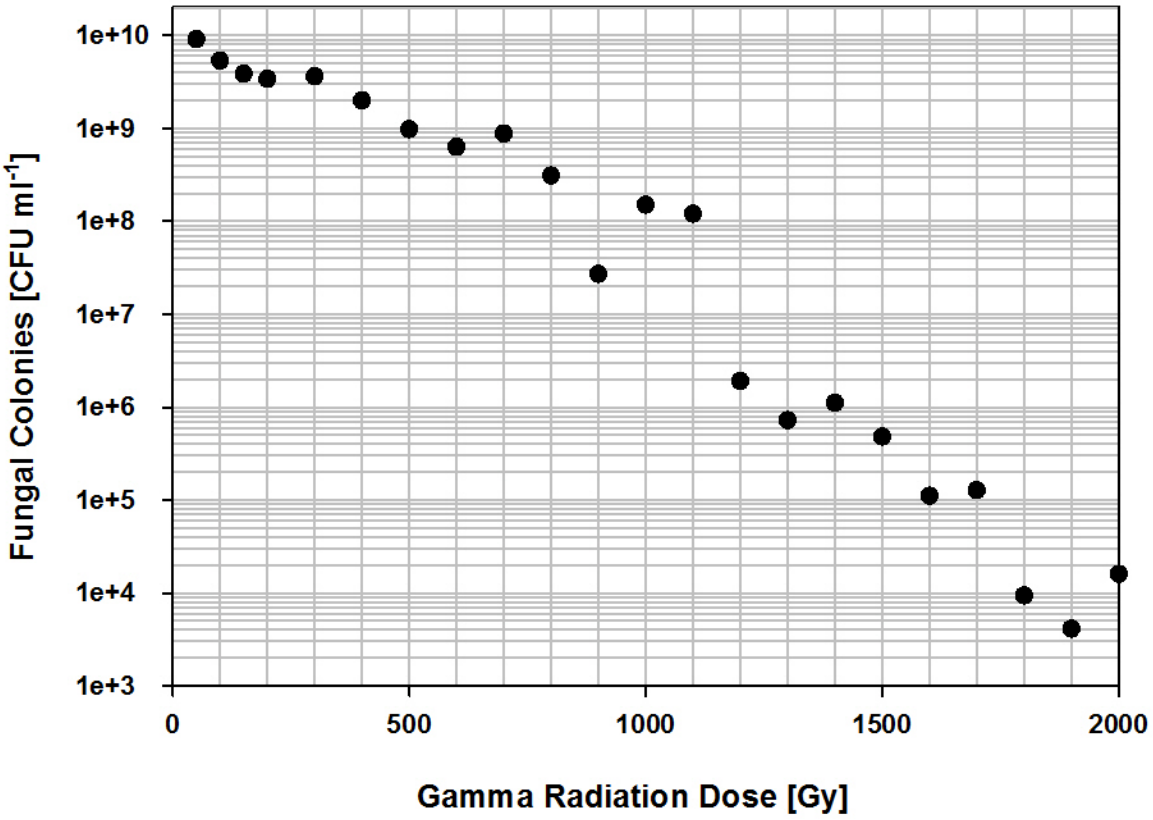
Fungal Colonies [CFU ml-1]
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
500
1000
1500
2000
Gamma Radiation Dose [Gy]

Fig.1

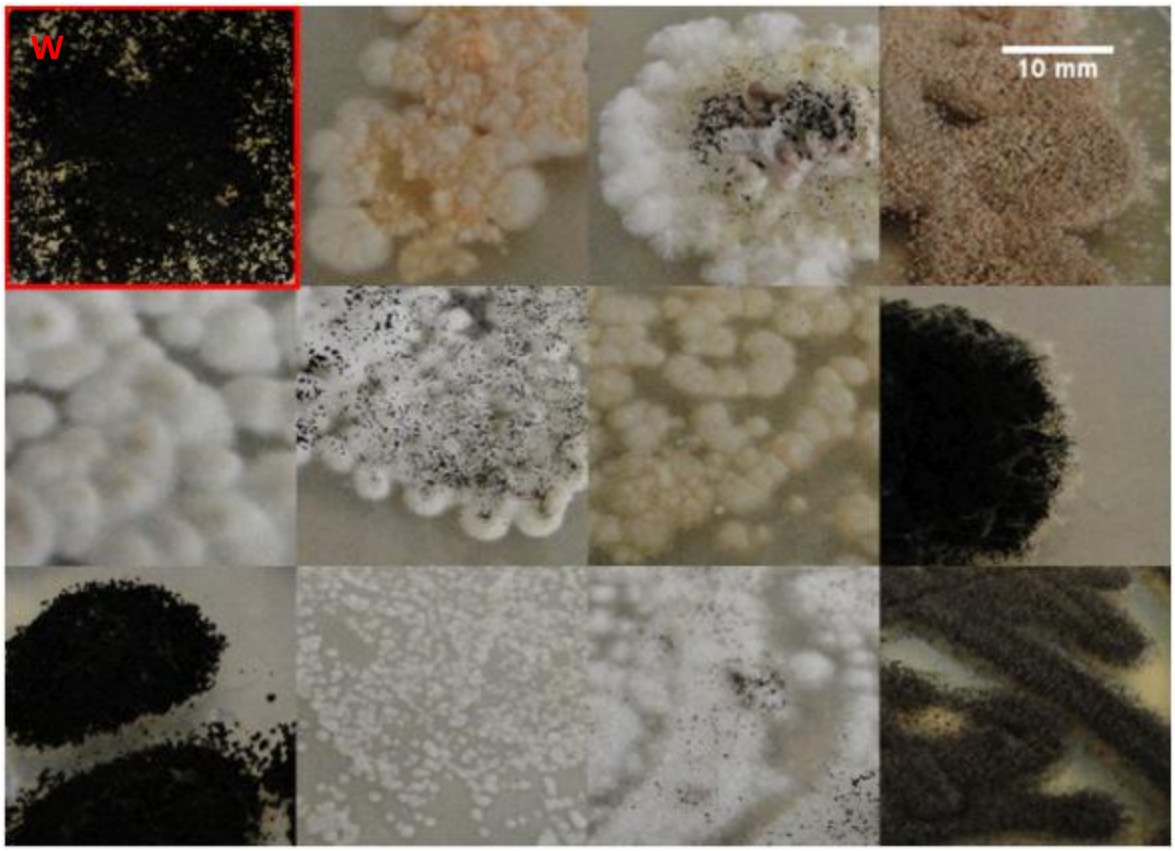

Fig.2

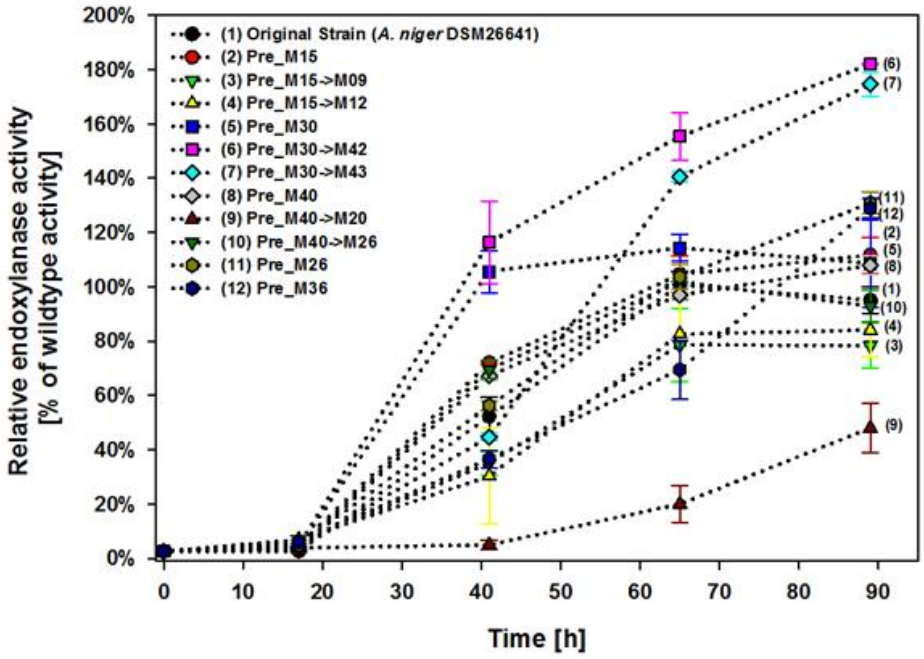
Relative endoxylanase activity
[% of wildtype activity]
Time [h]
(1) Original Strain (A. niger DSM26641)
(2) Pre_M15
(3) Pre_M15->M09
(4) Pre_M15->M12
(5) Pre_M30
(6) Pre_M30->M42
(7) Pre_M30->M43
(8) Pre_M40
(9) Pre_M40->M20
(10) Pre_M40->M26
(11) Pre_M26
(12) Pre_M36
200%
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
0
10
20
30
40
50
60
70
80
90

Fig.3

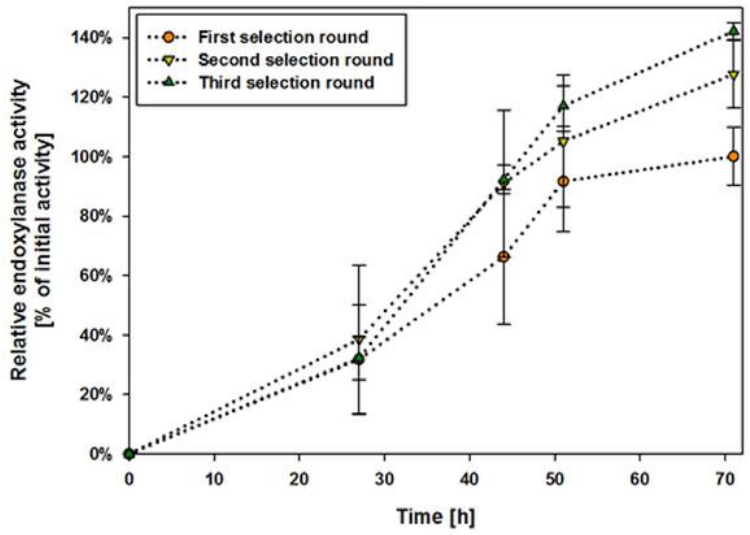
First selection round
Second selection round
Third selection round
Relative endoxylanase activity
[% of initial activity]
140%
120%
100%
80%
60%
40%
20%
0%
0
10
20
30
40
50
60
70
Time [h]

Fig.4

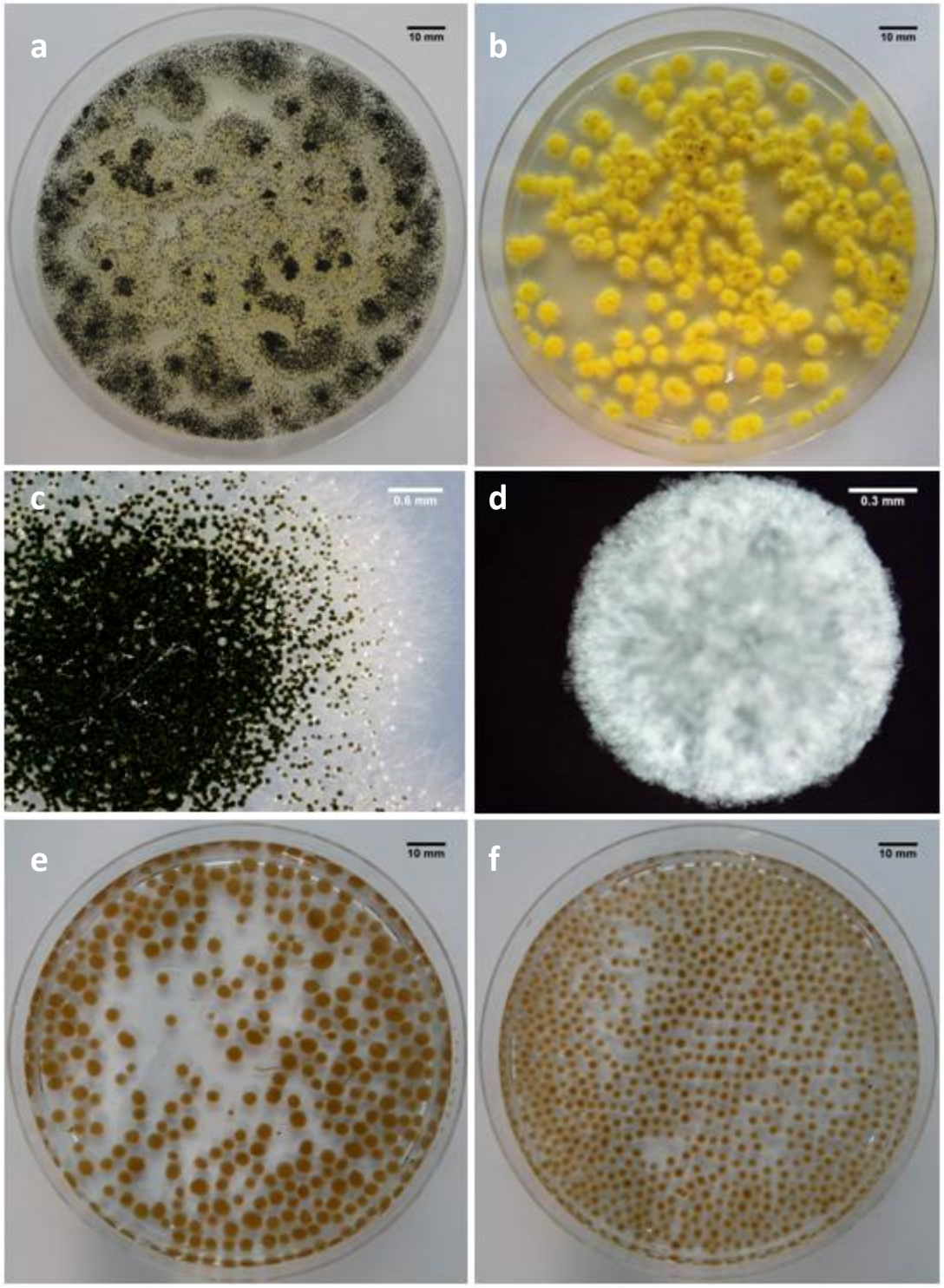

Fig.5