# Improvement of ligninolytic properties by recombinant expression of glyoxal oxidase gene in hyper lignin-degrading fungusPhanerochaete sordidaYK-624

Regular paper

# Improvement of ligninolytic properties by recombinant expression of glyoxal oxidase gene in hyper lignin-degrading fungus *Phanerochaete sordida* YK-624

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Running title: Ligninolytic improvement by expression of GLOX gene

**Abstract**

Glyoxal oxidase (GLOX) is a source of the extracellular $H_2O_2$ required for the oxidation reactions catalyzed by the ligninolytic peroxidases. In the present study, the GLOX-encoding gene (*glx*) of *Phanerochaete chrysosporium* was cloned, and *bee2* promoter of *P. sordida* YK-624 was used to drive the expression of *glx*. The expression plasmid was transformed into a *P. sordida* YK-624 uracil auxotrophic mutant (strain UV-64), and 16 clones were obtained as GLOX-introducing transformants. These transformants showed higher GLOX activities than wild-type *P. sordida* YK-624 and control transformants harboring marker plasmid. RT-PCR analysis indicated that the increased GLOX activity was associated with elevated recombinant *glx* expression. Moreover, these transformants showed higher ligninolytic activity than control transformants. These results suggest that the ligninolytic properties of white-rot fungi can be improved by recombinant expression of *glx*.

## Introduction

Lignin is one of the most abundant and recalcitrant natural polymers, consisting of various nonphenolic phenylpropanoid units complexed into a three-dimensional network. Therefore, lignin degradation is a key step for carbon recycling in forest ecosystems, and the process is a central issue in lignocellulose-based industries (*e.g.*, in paper pulp manufacture and production of bioethanol and other renewable chemicals).[1)] In nature, white-rot fungi (members of the basidiomycete group) are able to degrade recalcitrant lignin efficiently [2)]. The biodegradation process is initiated via a one-electron oxidation of lignin units, a step that is mediated by extracellular heme peroxidases (including lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase), enzymes that are of keen interest as industrial biocatalysts.[3, 4)] Activation of molecular oxygen to hydrogen peroxide by extracellular oxidases represents a common step in the fungal decay strategy, as shown by biochemical [2, 5)] and genomic [6, 7)] evidence. White-rot decay is based on peroxide activation of high-redox-potential fungal heme peroxidases that depolymerize lignin, leaving a cellulose-rich residue.[8)] The mechanism of enzymatic attack on lignin by these fungi has been extensively investigated because of its biotechnological interest.[1, 9)]

The white-rot fungus *Phanerochaete chrysosporium* secretes three known classes of extracellular enzymes under ligninolytic (secondary metabolic) conditions in defined glucose medium: glyoxal oxidases (GLOXs), LiPs, and MnPs.[3)] GLOX represented a family of radical copper oxidases produced by filamentous fungi, mainly organisms belonging to the group of white-rot fungi; the GLOX from *P. chrysosporium* is the most extensively studied of this enzyme family. GLOX acts as a source of the extracellular hydrogen peroxide that is required for the oxidations catalyzed by the LiPs and MnPs involved in lignin degradation.[10, 11)] The enzyme catalyzes the oxidation of aldehydes to the corresponding carboxylic acids by coupling the reaction to the reduction of dioxygen to $H_2O_2$. Although GLOX activity was detected under ligninolytic conditions,[10, 11)] there is no evidence for GLOX involved in lignin degradation.

The white-rot fungus *Phanerochaete sordida* YK-624, which was isolated from rotted wood, exhibits greater ligninolytic activity and selectivity than either *P. chrysosporium* or *Trametes versicolor*.[12)] In our previous study, we demonstrated that *P. sordida* YK-624 produces MnP [12, 13)] and LiP [14, 15)] as ligninolytic enzymes. Moreover, we previously identified a protein (BUNA2) that accumulated to high levels in cultures of *P. sordida* YK-624 under wood-rotting conditions. The promoter region of the BUNA2-encoding gene, designated *bee2*, was successfully cloned and demonstrated to

be a useful regulator for the high expression of genes under ligninolytic conditions.[16)] More recently, we have reported that the production of MnP was increased by the recombinant expression of a 5-aminolevulinic acid synthase-encoding gene.[17)]

In the present study, we report the transformation of *P. sordida* YK-624 with a recombinant *glx* gene under regulation of the *bee2* promoter, yielding elevated transcript accumulation and GLOX activity under wood-rotting conditions. Moreover, we demonstrate that these transformants exhibit improved ligninolytic properties.

## Materials and Methods

### Strains

*P. sordida* strain YK-624 (ATCC 90872), uracil auxotrophic *P. sordida* strain UV-64,[18)] and *P. chrysosporium* ME-446 (ATCC 34541) were used in this study. All strains and transformants were maintained on potato dextrose agar (PDA) slants at 4 °C.

### Construction of a GLOX-encoding gene expression vector, co-transformation of UV-64, and screening of regenerated clones

Genomic DNA was isolated from *P. chrysosporium* ME-446 mycelium using

ISOPLANT II (Nippon Gene, Tokyo). Gene sequences for the GLOX-encoding gene were obtained from the DOE Joint Genome Institute and used to design PCR primers. The full-length *glx* (1907 bp) gene was amplified using the primer pair PcGLOX F1-PcGLOX R1. The procedure for constructing the *glx* expression plasmid, pBUNA2pro-GLOX, is described in Fig. 1. The plasmid pBUNA2pro, which was generated in our previous study,[16] was used for the construction of the *glx* expression plasmid. Primers GLOX F1 and Pc GLOX R1 were designed to amplify the *glx* gene and to introduce a *Bgl* II site just before the first methionine codon and to add a *Bgl* II site just after the stop codon, respectively (step 1). The amplified DNA fragment was digested with *Bgl* II (New England Biolabs) and cloned into *Bgl* II digested pBUNA2pro (step 2), yielding plasmid pBUNA2pro-GLOX (step 3). pBUNA2pro-GLOX was sequenced to verify the absence of PCR errors.

Fig. 1

UV-64 protoplasts were prepared by a standard technique using cellullases and then co-transformed with p*PsURA5* and pBUNA2pro-GLOX using the PEG method.[18] The co-transformed clones (G strains) were screened by PCR, as described previously,[19] with the following modifications: primers PcGLOX F2 and PsGPD R1 were designed to amplify the *glx* gene fused with the *bee2* promoter.

**Enzyme assay**

*P. sordida* YK-624, G strains, and U strains (UV-64 protoplasts were singly-transformed with p*PsURA5*, yielding transformants designated as U strains) were incubated on PDA plates at 30 °C for 3 days, and 10-mm diameter disks then were punched out from the growing edge of the mycelia using a sterile cork borer. For determining the activities in the extracellular culture fluids, two mycelial disks from each source plate were placed into a 100-mL Erlenmeyer flask containing 10 mL nitrogen-limited Kirk medium,[20] which then was statically incubated at 30 °C for 2, 4, 6, and 8 days. Meanwhile, for detecting the activities in fungal-treated beech wood meals, these strains were cultured in 50-mL Erlenmeyer flasks containing 0.5 g of extractive-free beech wood meal (80-100 mesh) and 1.25 ml of distilled water, and incubated at 30 °C for 5, 10, 15, and 20 days. Following the culture period, the method described by Hirai *et al*. (1994) was modified for enzyme extraction. Briefly, fungal-treated wood meal was homogenized with 25 mL of 50 mM malonate (pH 4.5) or phosphate (pH 6.0) buffer containing 0.05% Tween 20 (Wako) using a Polytron PT1200E (Kinematica, Canada) homogenizer for a total of 5 min (20 s blending with 10-min intervals) at 4 °C.

MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol

105 to coerulignone (extinction coefficient at 470 nm (ε470) = 49.6 $mM^{-1}$ $cm^{-1}$).[21] The
106 reaction mixture (1 mL) contained 2,6-dimethoxyphenol (1 mM), $MnSO_4$ (1 mM), and
107 $H_2O_2$ (0.2 mM) in 50 mM malonate buffer (pH 4.5). GLOX activity was measured by
108 monitoring the oxidation of guaiacol to the tetramer (ε470 = 26.6 $mM^{-1}$ $cm^{-1}$). The
109 reaction mixture (1 mL) contained guaiacol (1 mM), 0.08 nkat of horseradish
110 peroxidase, and 2-methylglyoxal (1 mM) in 50 mM phosphate buffer (pH 6.0). A
111 standard curve was constructed for horseradish peroxidase (Y axis: production of the
112 tetramer; X axis: $H_2O_2$ concentration) and used to calculate the production of $H_2O_2$ in
113 the reaction system. One katal (kat) was defined as the amount of enzyme producing 1
114 mol of product per second.
115
116 **Determination of ligninolytic properties**
117 G strains, U strains, and *P. sordida* YK-624 were cultured in 50-mL Erlenmeyer
118 flasks containing 0.5 g of extractive-free beech wood meal (80-100 mesh) and 1.25 ml
119 of distilled water, which then were incubated at 30 °C for 28 days. After incubation,
120 weight loss, Klason lignin content, and acid-soluble lignin content of the fungal-treated
121 wood meal were determined, as previously described.[12] The selection factor (SF),
122 which is an indicator of ligninolytic selectivity, was calculated as follows: SF = lignin

loss/holocellulose loss. Holocellulose loss was calculated as follows: total weight loss – lignin loss.

**Transcriptional analysis of strain G-22**

Strain G-22 was inoculated in liquid medium as described above and then statically incubated at 30 °C for 2, 4, 6, and 8 days. The mycelia were then collected and stored at -80 °C pending transcriptional analysis. One hundred milligrams of mycelial mat was ground to a fine powder using a mortar and pestle under liquid nitrogen, and then total RNA was isolated from the powder using an RNeasy Plant Mini kit (Invitrogen). RT-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit (TaKaRa Bio, Otsu) and the gene-specific primer sets PcGLOX F3-PsGPD R1 (to amplify recombinant *glx*) and ActinF-ActinR (to amplify the actin-encoding gene, *act*). PCR was performed for 28 cycles of template denaturation at 95 °C for 30 s, primer annealing at 56 °C for 1 min, and DNA extension at 72 °C for 1 min using Ex Taq DNA Polymerase (TaKaRa Bio).

**Nucleotide sequence accession number**

The nucleotide sequences of *glx* derived from *P. chrysosporium* have been

deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/) under accession no. AB910536.

## Results and Discussion

### Isolation of recombinant *glx*-expressing transformants

*P. chrysosporium* was cultured in Kirk liquid medium for 5 days, and then genomic DNA was extracted from the mycelia. Based on the genome database of *P. chrysosporium*, two primers were designed to amplify the genomic DNA encoding GLOX. Genomic DNA of *glx* obtained by genome PCR was inserted between the *bee2* promoter and *gpd* terminator by restriction enzyme digestion and ligation (Fig. 1). This expression plasmid, pBUNA2pro-GLOX, was introduced into strain UV-64 together with p*PsURA5* as a marker plasmid. The presence of the *bee2* promoter-*glx* fusion gene in each uracil prototrophic clone was confirmed by PCR using genomic DNA as the template (data not shown). We obtained 16 of these “G” strains that were co-transformed with pBUNA2pro-GLOX and p*PsURA5*.

### GLOX production and ligninolytic properties of recombinant *glx*-expressing

**transformants**

In our previous reports, *P. sordida* YK-624 produces only LiP and MnP as ligninolytic enzymes,[12-15] and LiP activity has been hardly detected in the culture containing Mn ion.[12] Therefore, we focused MnP among ligninolytic enzymes, and the production of GLOX and MnP by G strains was compared with those by singly-transformed (p*PsURA5* alone) "U" strains. The mean GLOX activity in the culture medium inoculated with G strains for 5 days was 0.66 nkat/flask, whereas that in the culture medium of U strains was 0.19 nkat/flask (Fig. 2a). The mean MnP activity in the culture medium inoculated with G strains was 8.51 nkat/flask, whereas that in the culture media of U strains was 7.78 nkat/flask (Fig. 2b). These results indicate that G strains showed higher GLOX activities than U strains, although MnP activities of G strains did not apparently differ from those of U strains.

Fig. 2

The effect of recombinant *glx* expression on the ligninolytic properties of G strains was investigated. G strains showed 1.08-fold higher ligninolytic activity (Fig. 3a) and 1.18-fold higher SF values than U strains (Fig. 3b). In particular, strain G-22 showed 1.22-fold higher ligninolytic activity and 1.25-fold higher SF values than U strains (data not shown). These results indicated that the ligninolytic properties were improved by the recombinant expression of *glx* using the *bee2* promoter, with strain G-22 exhibiting

Fig. 3

177 the highest lignin degradation activity among the 16 screened co-transformants. In our
178 previous study, the promoter region of *bee2* was demonstrated to be a useful regulator
179 for the high expression of genes under ligninolytic conditions.[16] The results of the
180 present study are consistent with that previous work. The proposed role of GLOX in
181 lignin biodegradation is to generate $H_2O_2$ for LiP- and MnP-mediated reactions.[22]
182 Moreover, the identification of *glx* transcripts in decayed wood is consistent with a
183 close physiological connection between extracellular peroxidases and GLOX.[23] These
184 reports, together with our present results, suggest that GLOX plays an important role in
185 lignin degradation by white-rot fungi.

186 To confirm whether the improvement of the ligninolytic properties resulted from
187 an increase in GLOX production, GLOX and MnP activities produced in beech wood
188 meals inoculated with G-22 and wild type (WT) were determined. The MnP activity of
189 G-22 was similar to that of WT, and no drastic fluctuations were observed (Fig. 4b). In
190 contrast, higher GLOX activities were detected at 10 and 15 days in G-22, although
191 similar GLOX activities for each strain were detected at 5 days (Fig. 4a). These results
192 indicate that the improvement of the ligninolytic properties correlates with the higher
193 production of GLOX.

Fig. 4

194

**GLOX production and transcriptional analysis of G-22**

Transcriptional analysis was performed by RT-PCR to confirm whether the increment of GLOX activity reflected increased accumulation of *glx* transcript. Specifically, we analyzed a time course of GLOX activity in G-22 and WT. GLOX activity in G-22 increased gradually after 4 days, remaining relatively stable through 8 days (Fig. 5a). GLOX activity in G-22 was higher than that in WT during the incubation. The highest GLOX activity in G-22 was observed at 8 days, when activity was 7.2-fold higher than that in WT.

Fig. 5

We next analyzed the transcription of recombinant *glx* in G-22 by RT-PCR (Fig. 5b). The analysis indicated that high levels of recombinant *glx* transcript were detected after 4 to 8 days. These results suggest that the increase of GLOX activity in G-22 was caused by *bee2*-driven expression of the recombinant *glx*.

To conclude, we found that the recombinant expression of *glx* under the control of the *bee2* promoter is effective for the improvement of the ligninolytic properties in white-rot fungi. We also provide the evidence that physiologic productivity of GLOX in white-rot fungi is not enough under ligninolytic condition. In our next study, we propose to investigate the molecular breeding of superior lignin-degrading fungi by the simultaneous expression of *glx* and a MnP-encoding gene (*mnp4*). To our knowledge,

213 this is the first report to demonstrate that GLOX is involved in lignin degradation.

214

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216

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220

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Fig. 1 Plasmid map of the *glx* expression plasmid pBUNA2pro-GLOX.

Fig. 2 GLOX (a) and MnP (b) activities in the extracellular culture fluids inoculated with U strains and G strains for 5 days.

Fig. 3 Ligninolytic properties of U strains and G strains. Lignin degradation (a) and selection factor (b) by each strain for 28 days.

Fig. 4 GLOX (a) and MnP (b) activities detected in fungal-treated beech wood meals. G-22 (triangles) and WT (squares).

Fig. 5 Production of GLOX and expression of *glx* by strain G-22. (a) Time course of GLOX activity in strain G-22 (triangles) and WT (squares). (b) RT-PCR analysis of *glx* in strain G-22. The expression of actin was used as an internal control.

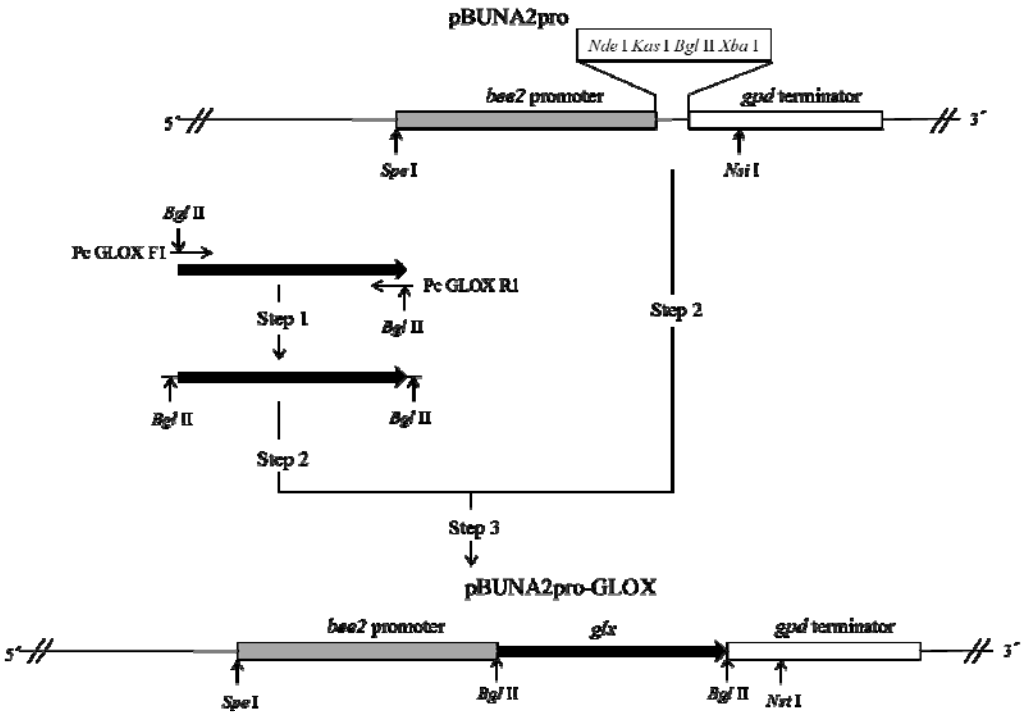

pBUNA2pro
Nde I Kas I Bgl II Xba I
bae2 promoter
gpd terminator
5'
3'
Spe I
Not I
Bgl II
Pc GLOX F1
Pc GLOX R1
Step 1
Step 2
Step 3
pBUNA2pro-GLOX
glx
Nrt I

Fig. 1

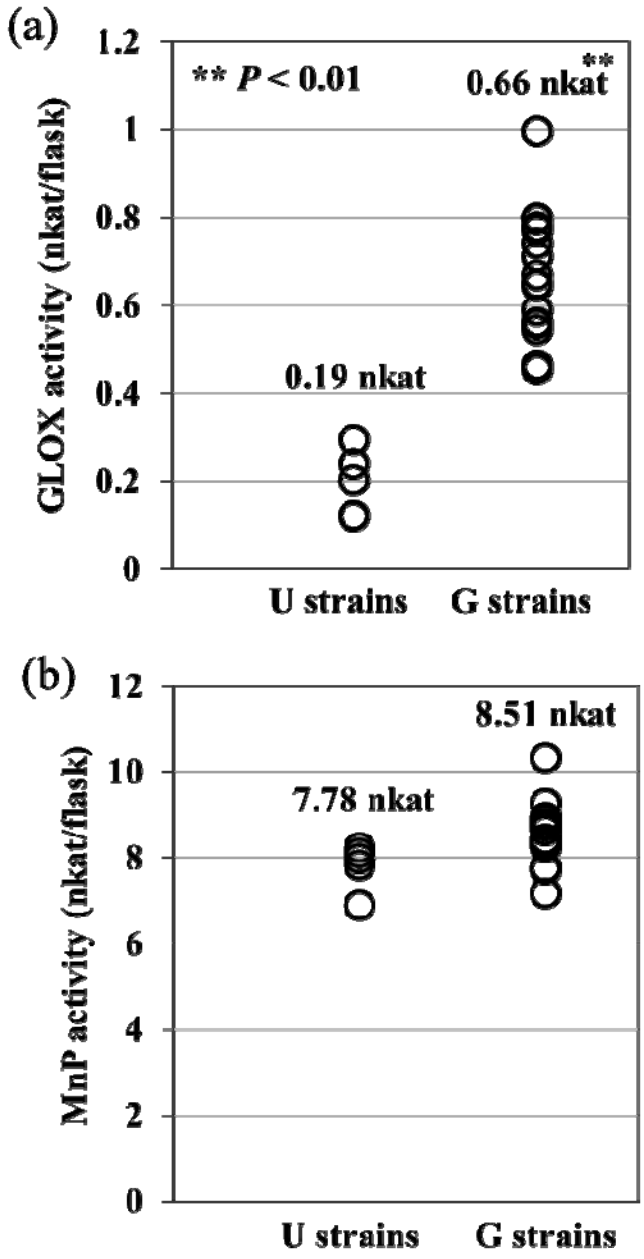

(a)
1.2
1
0.8
0.6
0.4
0.2
0
GLOX activity (nkat/flask)
** P < 0.01
0.66 nkat
**
0.19 nkat
U strains
G strains
(b)
12
10
8
6
4
2
0
MnP activity (nkat/flask)
8.51 nkat
7.78 nkat
U strains
G strains

Fig. 2

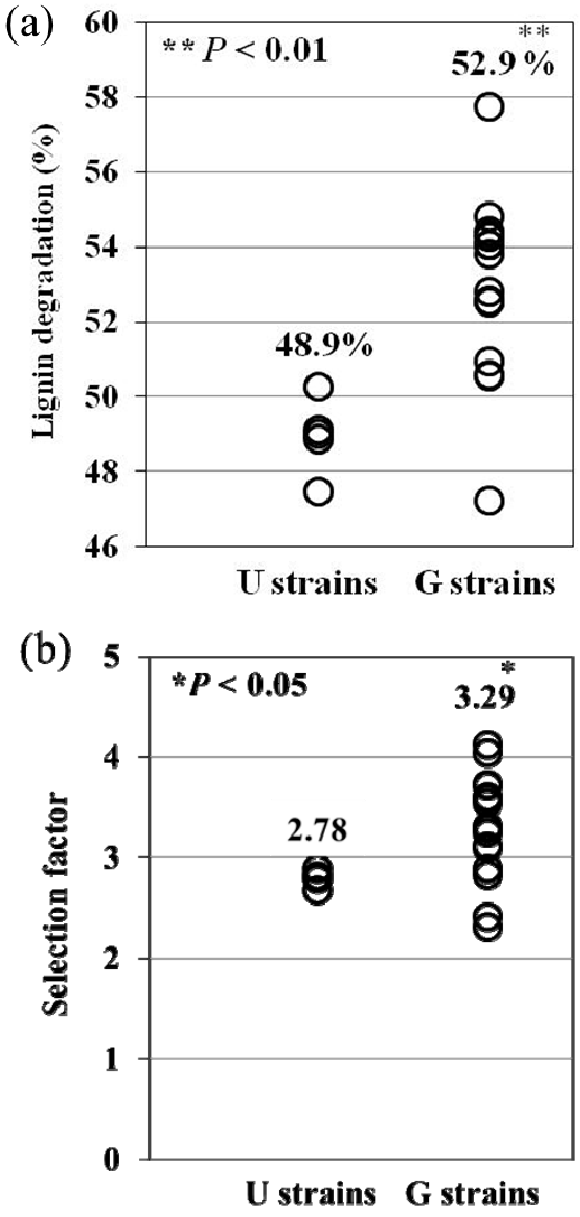
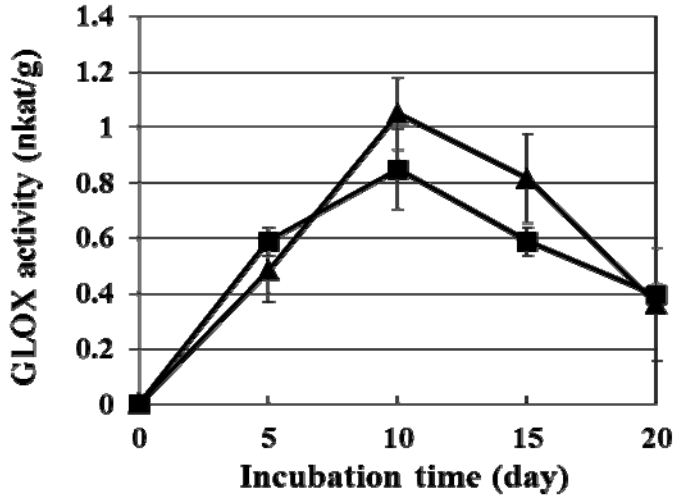
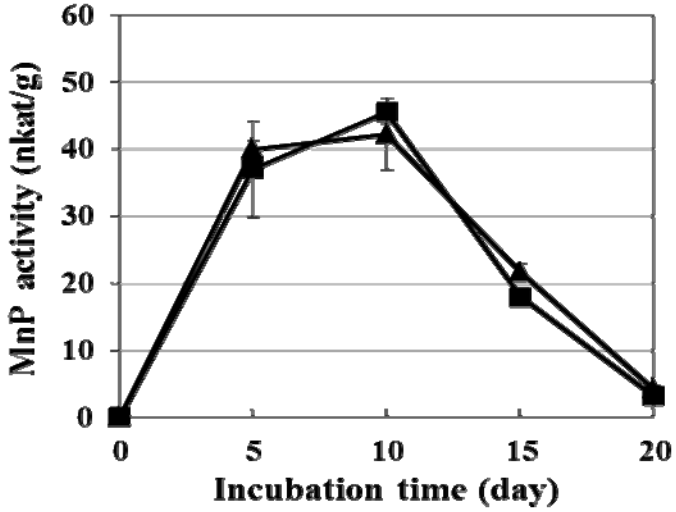

(a)
** P < 0.01
**
52.9 %
48.9%
Lignin degradation (%)
60
58
56
54
52
50
48
46
U strains
G strains
(b)
*P < 0.05
*
3.29
2.78
Selection factor
5
4
3
2
1
0
U strains
G strains

Fig. 3

(a)


GLOX activity (nkat/g)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
Incubation time (day)

(b)


MnP activity (nkat/g)
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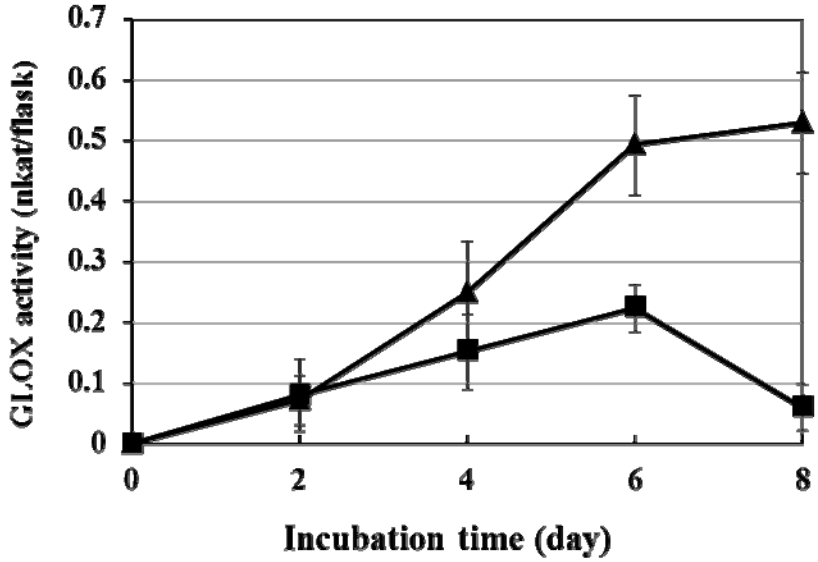
50
40
30
20
10
0
0
5
10
15
20
Incubation time (day)

Fig. 4

(a)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GLOX activity (nkat/flask)
0
2
4
6
8
Incubation time (day)

(b)

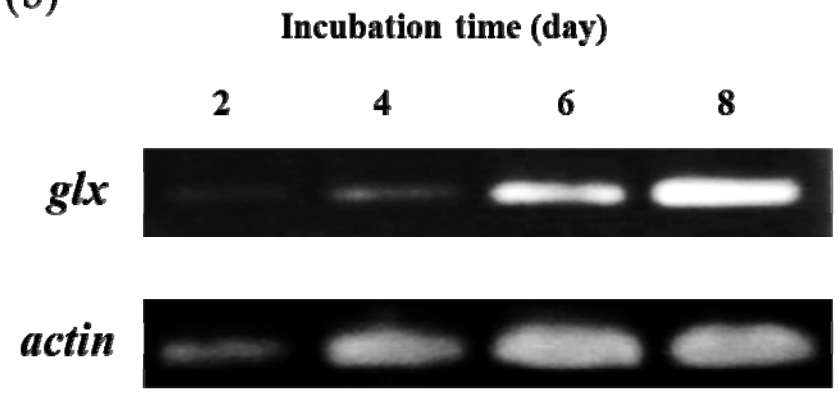

Incubation time (day)
2
4
6
8
glx
actin

Fig. 5