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Improved β -Glucan Yield Using an *Aureobasidium pullulans* M-2 Mutant Strain in a 200-L Pilot Scale Fermentor Targeting Industrial Mass Production

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Abstract β -(1 $\rightarrow$ 3)-D-glucans with β -(1 $\rightarrow$ 6)-glycosidic linked branches are known to be immune activation agents and are incorporated in anti-cancer drugs and health-promoting supplements. β -Glucan concentration was 9.2 g/L in a 200-L pilot scale fermentor using mutant strain *Aureobasidium pullulans* M-2, which was from an imperfect fungal strain belonging to *A. pullulans* M-1. The culture broth of *A. pullulans* M-2 had a faint yellow color, whereas that of the wild-type had an intense dark green color caused by the accumulation of melanin-like pigments. β -Glucan produced by *A. pullulans* M-2 was identified as a polysaccharide of D-glucose monomers linked by β -(1 $\rightarrow$ 3, 1 $\rightarrow$ 6)-glycosidic bonds through GC/MS and NMR analysis. When a conventional medium was used in the culture of *A. pullulans* M-2 in a 3-L jar fermentor, β -glucan concentration was 1.4-fold that produced by the wild-type. However, when medium optimized by statistical experimental design was used with dissolved oxygen at 10%, the β -glucan concentration was 9.9 g/L with yield of 0.52 (g β -glucan/g consumed sucrose), which was 2.9-fold that of the wild-type. This level of productivity was reproduced when the fermentation was scaled up 200-L. The industrial production of high β -glucan without melanin-like pigments is highly expected as a health-promoting supplement or functional food.

Keywords: *Aureobasidium pullulans*; β -glucan; black yeast; functional food; mutant

1. Introduction

β -Glucans are polysaccharides of D-glucose monomers linked by β -glycosidic bonds. Various types of β -glucan occur in wild organisms as storage molecules of saccharides that are an energy source and as structural molecules in cell walls. The β -glucan contained in mushrooms such as *Agaricus bisporus* [1] and *Pleurotaceae citrinopileatus*, which is a Chinese mushroom called Yuhuangmo [2], is also found in barley, oats and maize [3–5], and the cell walls of brewery yeast [6]. These β -glucans are considered non-digestible carbohydrates and classified as pathogen-associated molecular patterns, which can be recognized by pattern recognition receptors (PRRs). The effects of β -glucans on humans are considered to be mediated via PRRs such as dectin-1 and Toll-like receptors (TLRs) that are located at the surface of microbes and trigger various kinds of immune responses [7]. When PRRs are stimulated the expression of a cytokine gene is upregulated, which causes subsequent stimulation of cell-mediated immunity [8, 9]. This means that β -glucan increases phagocytosis activity and nitric oxide production and inflammatory responses in vivo and in vitro [10]. The production of inflammatory cytokines enhances various physiological activities such as immunopotentialiation, anti-tumor activity, and cancer cell proliferation suppression that maintain or promote good health [11–14]. Thus, applications of the β -glucans, not only as food additives and functional foods, but also as medical, pharmaceutical, and cosmetic raw materials are increasing year by year.

However, the supply of β -glucan cannot currently meet the demand because of low

productivity and high cost [15, 16]. Commercial β -glucan is extracted from the yeast cell wall and purified, which is associated with a high manufacturing cost. Moreover, some allergens originating from the yeast may contaminate the β -glucan product. It has been found that β -glucan is produced by a microorganism belonging to *Aureobasidium* sp. (commonly known as “black yeast”), which is an imperfect fungus widespread in nature, that exhibits a function equivalent, or superior, to that of the β -glucan derived from mushrooms [14, 16].

The present study aims to improve the production of β -glucan using strain improvement by UV mutation of the wild-type *A. pullulans* M-1. The culture medium of the mutant strain was optimized to improve β -glucan productivity and scaled up to 200-L to target industrial mass production. The chemical structure of the β -glucan produced by this method was characterized by chemical analysis.

2. Materials and Methods

2.1. Microorganism and mutation

The parent strain, *Aureobasidium pullulans* M-1 (Accession No. FERM BP-08615 deposited in the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tokyo, Japan), was cultured on a PDA (potato dextrose agar, Difco Laboratories Inc., Franklin lakes, NJ, USA) slant for 7 d. PDA is composed (per liter) of 4 g of potato extract, 20 g of dextrose, and 15 g of agar (pH 5.6). Colonies were suspended in 10 mL of phosphate buffered saline (PBS) and adjusted to a cell concentration of $\sim 10^6$ colony forming units (CFU)/mL. Aliquots (200 μ L) of this cell suspension were applied to a PDA

plate and the microbial cells were exposed to a 20-W UV lamp at a distance of 60 cm for 10 min. Under these conditions the cell survival rate was 0.1%. After UV exposure, the plates were subjected to static culture at 25°C for 4 d. The colonies that formed on the plates were cultured at 4°C for 3 d to induce chlamydospore formation.

2.2. Selection of a high β -glucan producer

The first selection was done by visual observation and comparatively large colonies were selected. The second selection was carried in flask culture. Production medium (40 mL), composed (per liter) of 12 g of sucrose, 2 g rice of bran, 0.83 g of sodium ascorbate, and 0.23 g ascorbic acid, was placed in a 300-mL Erlenmeyer flask and sterilized at 121°C for 20 min. The first selected colony was inoculated and cultured at 24.5°C in a rotary shaker at 150 rpm. The productivity of β -glucan of the mutant strain was confirmed using a 3-L fermentor (Bioneer-N300-3L, Marubishi Co. Ltd., Tokyo, Japan) with an aeration rate and agitation rate of 0.5 vvm and 200 rpm, respectively.

2.3. Gene sequence analysis

The ITS-5.8S and 28S rRNA-D1/D2 regions coding rRNA of the high β -glucan producer were investigated to confirm the strain species and its morphological and physiological properties according to previous reports [17, 18].

2.4. GC and EI-GC/MASS analysis

The sugar linkages of the β -glucan were analyzed according to the method described by

110 Anumura *et al.* [18]. Briefly, 100 g of sample were used for β -glucan extraction. Extracted
111 β -glucan (9 mg) was completely methylated by 100 mg of powdered NaOH and 0.6 mL of
112 methyl iodide followed by hydrolysis to form methylated monosaccharide. The methylated
113 monosaccharide was hydrogenated and acetylated to partially methylated alditol acetate. The
114 resulting alditol acetate was dissolved in chloroform and its chemical structure was analyzed
115 by GC (HP5890 series II, Hewlett-Packard, Palo Alto, CA, USA) and EI-GC/MASS (JMS
116 DX-303, JEOL, Tokyo, Japan) [19].

117 **2.5. NMR spectroscopy**

118 Purified sample was dissolved in dimethyl Sulfoxide-d₆ (DMSO-d₆) and then heated at 80°C
119 for 30 min, followed by H-D exchange by adding deuterium water. ¹³C NMR spectra were
120 recorded in DMSO-d₆ at room temperature using a Varian INOVA 500 NMR
121 spectrometer (Varian, Palo Alto, CA, USA) operating at 125.7 MHz. Purified sample
122 was dissolved in DMSO-d₆ and then heated at 100°C for 1 h, and ¹H NMR spectra
123 were recorded using an INOVA 600 NMR spectrometer at 599.5 MHz [20].

124 **2.6. Enzymatic analysis**

125 Purified sample (14.5 mg) was digested by 7.82 mg of cellulase I containing endo
126 β -(1→3)-glucanase (Wako Pure Chem., Osaka, Japan) or 5.34 mg of cellulase II containing
127 β -(1→3)-glucanase (Seikagaku Co., Tokyo, Japan) at 40°C for 16–24 h in acetate buffer (pH
128 4.5). The degradation product was analyzed by HPLC (LC-20A, Shimadzu, Kyoto, Japan)
129 with a TSKgel Sugar AXG column (15 cm × 4.6 mm I.D.) (Tosoh Bioscience LLC, Tokyo,

Japan). The eluent was 0.5 M potassium borate buffer (pH 8.7) at 70°C and the eluent flow rate was 0.4 mL/min. Excitation and emission wavelengths were 320 and 430 nm, respectively.

2.7. Optimization of β -glucan production in flask culture and confirmation of productivity in the 3- and 200-L fermentors

Box Behnken experimental design [21–23] was used to optimize the sucrose, rice bran, and sodium ascorbate concentrations in flask cultures. Statistical analysis was performed using STATISTICA (StatSoft Inc., OK, USA), and the regression of the parameters was solved by Mathcad 2001 Professional (Mathsoft Engineering and Education Inc., Cambridge, MA, USA). To confirm the possibility of industrial scale production of β -glucan by the mutant strain, 3-L fermentor (MDL300, Marubishi Co. Ltd., Tokyo, Japan) and 200-L stirred-tank reactors (Komatsugawa Chem. Eng. Co. Ltd., Tokyo, Japan) with working volumes of 2 L and 120 L, respectively, were used for β -glucan production. An optimized medium was used and the dissolved oxygen concentration (DO) was controlled at approximately 0, 10, and 30% by a DO controller with an agitation rate in the region of 100 to 150 rpm under a constant aeration rate of 0.5 vvm. Culture temperature was controlled at 24.5°C. During cultivation, the cell morphology was observed using an optical microscope (BX51, Olympus, Tokyo, Japan).

2.8. Measurement of β -glucan

A portion (100 g) of the culture broth was centrifuged at $190 \times g$, and the resulting

150 supernatant was precipitated by adding 70% ethanol. The ethanol precipitate that contained
151 high-molecular-weight substances was separated by centrifugation and digested with
152 α -amylase, protease, and amyloglucosidase. The enzyme-treated solution was diluted with a
153 four-fold volume of 95% ethanol to allow precipitation of β -glucan. The resultant 95%
154 ethanol precipitate moiety was subjected to air-drying overnight and further air-drying at
155 105°C for 3 h before being weighed. This gave the weight of the β -glucan content in the
156 culture broth.

157 **3. Results and Discussion**

158 **3.1. Screening of the high β -glucan producer**

159 *A. pullulans* M-1 cells were UV-irradiated at 0.1% of survival rate and 400 colonies of
160 comparatively large sizes compared to the wild-type were selected; this was followed by a
161 second selection. The colonies selected in the second screening described above were
162 inoculated into 300-mL Erlenmeyer flasks containing 40 mL of the production medium and
163 cultured for 7 d. Among the 400 colonies, a colony producing 5 g/L of β -glucan was selected
164 and designated as *A. pullulans* M-2 (Accession No. FERM BP-08615 deposited in the
165 International Patent Organism Depositary, National Institute of Advanced Industrial Science
166 and Technology).

167 **3.2. Identification of *A. pullulans* M-1 and M-2**

168 Molecular phylogenetic trees for the base sequence of the ribosomal RNA gene regions,

ITS-5.8S and 28SrRNA-D1/D2, were created by the neighbor-joining method using the top 20 sequences among the analogous sequences detected in an international DNA database. Comparison of ITS-5.8S and 28SrR sequences shows that the mutant strain classically defined as *A. pullulans* (Fig. 1) and belonging to an imperfect fungus *A. pullulans* is the same species as the parent strain *A. pullulans* M-1.

3.3 Comparison of the morphology of the parent strain with that of *A. pullulans* M-2 and their β -glucan productivities

When *A. pullulans* M-2 was cultured on PDA plate medium for 1 week, the shape of the vegetative hypha and the pattern of conidium formation were similar to those of the parent strain (Fig. 1A-1 and B-1) although the colony colors differed. The culture broth of *A. pullulans* M-1 had a dark green color, which is typical for *A. pullulans* (Fig. 1A-2), whereas *A. pullulans* M-2 exhibited a faint yellow color (Fig. 1B-2), suggesting that the M-2 strain does not accumulate any melanin-like pigment. The color of this product is very important to its commodity value because consumers prefer a transparent product rather than a dark one. This indicates that the β -glucan produced by *A. pullulans* M-2 is suitable for commercial product.

The β -glucan productivity of *A. pullulans* M2 was compared to that of the parent strain using a 3-L jar fermentor. The β -glucan concentration of *A. pullulans* M2 was 5.0 g/L at a culture time of 144 h, while that of the parent strain was 3.5 g/L (Fig. 2C).

3.4. Chemical structure of the β -glucan produced by *A. pullulans* M-2

The ^1H and ^{13}C NMR spectra of the β -glucan produced by *A. pullulans* M-2 are shown in Fig. 3. The ^1H signals at 4.5 ppm were assigned to the β -(1 $\rightarrow$ 3)-linkage (Fig. 3A). The ^{13}C signals at 103, 86, 68, and 61 ppm were assigned to the C-1 carbons of the β -(1 $\rightarrow$ 3; 1 $\rightarrow$ 6)-linkages, C-3 carbons of the β -(1 $\rightarrow$ 3)-linkages, branched C-6 carbons ($\text{CH}_2\text{-O-R}$), and unbranched C-6 carbons ($\text{CH}_2\text{-OH}$), respectively (Fig. 3B).

The β -glucans produced by *A. pullulans* M-1 were detected by GC and EI-GC/MASS after three consecutive methylations and their ratios are shown in Table 1. The ratio of branched to total glucose residues was higher than 60%, indicating that the β -glucans produced by *A. pullulans* M-2 were highly branched polysaccharides. Furthermore, from the HPLC analysis, the enzymatic digestion products were glucose and gentibiose (data not shown), suggesting the glucose in the glucan molecule could be assumed to be the β -anomer. From these chemical and enzymatic analyses, a possible chemical structure of the β -glucan produced by *A. pullulans* M-2 was derived and is given in Fig. 4.

3.5. Improvement of the β -glucan productivity

Independent variables corresponding to the sucrose (x_1), rice bran (x_2), and sodium ascorbate (x_3) concentrations were varied according to the Box-Behnken design, and the β -glucan concentrations at 120 and 144 h were recorded as responses and the statistical analyses are shown in Tables 2 and 3, respectively. The effect of the sodium ascorbate concentration (x_3) was negligible in this model, because its p-value was higher than 0.05. According to the analysis of variance, the responses were generated by a non-linear quadratic model described

208 as follows,

209
$$Z = -1.77 \times 10^{-1}x_1 + 1.19 \times 10^{-2}x_1^2 + 3.60 \times 10^{-1}x_2 + 3.60 \times 10^{-1}x_2^2 + 3.79 \times 10^{-2}$$

210 The correlation coefficient of this model is 0.89. Optimized sucrose and rice bran
211 concentrations were calculated to be 20 and 4.5 g/L, respectively. Using this optimized
212 medium, a β -glucan production level was expected to be 10.1 g/L.

213 **3.6. Scale-up of β -glucan production in a 200-L fermentor**

214 To confirm the possibility of industrial scale production of β -glucan by *A. pullulans* M-2,
215 β -glucan production using the optimized medium was carried out in a 3-L jar fermentor with a
216 controlled dissolved oxygen level (DO) during the production phase. When the DO level was
217 controlled at approximately 0% (Fig. 5A-1), the β -glucan concentration was 3.5 g/L (Fig.
218 5A-2). However, when the DO level was controlled at 10 or 30%, the β -glucan concentrations
219 were 9.9 and 9.0 g/L, respectively. The β -glucan yields, based on consumed sucrose at DO
220 levels of 0, 10, and 30%, were 0.28, 0.52, and 0.49 (g β -glucan/g consumed sucrose),
221 respectively. This indicates that the 10% DO level in the production phase was optimal for
222 β -glucan production. This result was scaled-up to the 200-L fermentor with the optimized
223 medium. The DO level was decreased gradually and controlled at 10% from the culture time
224 of 50 h. The reducing sugar concentration decreased gradually and was 2 g/L at the culture
225 time of 144 h. The β -glucan concentration increased with decreasing reducing sugar
226 concentration and reached 9.2 g/L with a β -glucan yield of 0.51 (g β -glucan/g sucrose) at 144
227 h (Fig. 5B), which was similar to that of the 3-L jar fermentor, suggesting that β -glucan

production in a 200-L stirred tank reactor was reproducible.

β -Glucan production levels observed in this present study were compared to production levels reported in related studies (Table 4). Sucrose was used as a carbon source. As a nitrogen source, NaNO_3 and $(\text{NH}_4)_2$ were used as inorganic nitrogen sources; otherwise yeast extract or rice bran were used as organic nitrogen sources. Typical *A. pullulans* strains produced 4.0 g/L of β -glucan at minimum yield, regardless of whether inorganic or organic nitrogen sources were used. The productivity of β -glucan using the mutant strain *A. pullulans* M-2 was 1.4-fold higher than that of *A. pullulans* M-1. Moreover, when the optimized medium was used, the β -glucan yield and productivity were 2.9 times higher than that of the wild-type. Based on the scale up criteria, such as agitation power per volume in the 200-L stirred tank reactor, the β -glucan yield and productivity were maintained in a 10-kL stirred tank reactor (data not shown) targeting for commercial production.

Recently, this β -glucan has become commercially valuable as a supplement for boosting immunity against the common cold, flu, allergies, high cholesterol, diabetes, and cancer. In addition, β -glucan is applied to the skin for dermatitis, eczema, wrinkles, bedsores, wounds, burns, diabetic ulcers, and radiation burns. This β -glucan produced by the black yeast mutant is colorless and does not contain melanin-like pigments, and is very suitable for industrial level production.

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323

Figure Legends

Fig. 1. Phylogenetic tree of *A. pullulans* M-1 and M-2. ITS-5.8S (A) and 28SrRNA-D1/D2 (B) regions coding rRNA gene from genomic DNA of *A. pullulans* M-1 and M-2 were analyzed, respectively.

Fig. 2. Morphological properties and β -glucan production in flask culture. The shape of the colony on PDA plate: *A. pullulans* M-1 (A-1) and M-2 (B-1). Bars in A-1 and B-1 denote 1 cm. Appearance of culture broth of flask culture: *A. pullulans* M-1 (A-2) and M-2 (B-2). Mycelial morphology of β -glucan producers: *A. pullulans* M-1 (A-3) and M-2 (B-3). Bars in A-3 and B-3 denote 20 μ m. Time courses of β -glucan production of *A. pullulans* M-1 and M-2 in 3-L jar fermentor (C). Open and closed circles in C denote *A. pullulans* M-1 and M-2, respectively.

Fig. 3. ^1H NMR (A) and ^{13}C NMR (B) spectra of β -(1 $\rightarrow$ 3,1 $\rightarrow$ 6)-glucan produced by *A. pullulans* M-2.

Fig. 4. Estimated chemical structure of β -(1 $\rightarrow$ 3,1 $\rightarrow$ 6)-glucan produced by *A. pullulans* M-2.

Fig. 5. Optimal DO level in a 3-L jar fermentor (A) and scale up of β -glucan production to a 200-L pilot scale fermentor with a working volume of 120 L (B) in the culture of *A. pullulans* M-2. Optimized medium, composed of 20 g/L of sucrose, 4.5 g/L of rice bran, and 0.5 g/L of sodium ascorbate, was used. Dotted, solid, and thick lines in A-1 denote DO level controlled at approximately 0, 10, and 30%, respectively. Triangles, circles, and squares in A-1 denote DO level controlled at approximately 0, 10, and 30%, respectively. Closed and open symbols

344 in A-2 indicate β -glucan and residual sugar concentration, respectively. Dotted line and open
345 and closed circles in B denote DO level (%), residual sugar, and β -glucan concentrations,
346 respectively.

Table 1. GC and EI-GC/MASS analysis of methylated β -glucan

Glycoside linkage	Ratio of linkage type (%)
Non-reducing end of glucose	39.1
$\rightarrow 3$ glucose $1 \rightarrow$	24.6
$\rightarrow 3, 6$ glucose $1 \rightarrow$	36.3

Table 2. Box-Behnken medium design and runs

Medium composition (g/L)			β -glucan concentration (g/L)	
Sucrose	Rice bran	Sodium ascorbate	At 120 h	At 144 h
10	1	1.5	3.63	3.65
20	1	1.5	4.33	3.71
10	1	1.5	4.73	4.62
20	5	1.5	8.87	8.44
10	3	0.5	5.50	4.96
20	3	0.5	8.89	9.17
10	3	2.5	4.92	4.52
20	3	2.5	8.36	7.41
15	1	0.5	2.97	4.30
15	5	0.5	6.94	6.46
15	1	2.5	2.72	2.85
15	5	2.5	6.43	5.72
15	3	1.5	6.99	6.14
15	3	1.5	6.82	6.44
15	3	1.5	7.02	6.32
15	2	0.8	6.85	6.52

Table 3. Statistical analysis of the flask cultures

Variable	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Sucrose (%)	15.226	2	7.633	11.103	0.004
Rice bran (%)	22.001	2	11.001	16.000	0.002
Sodium ascorbate (%)	2.417	2	1.209	1.758	0.233
Error	5.500	8	0.688		

Table 4. Comparison of β -glucan productivities in various *Aureobasidium* strains

Strain	Culture	Medium	Yield (g/L)	Time (h)	Productivity (g/L/h)	Reference
<i>A. pullulans</i>	Flask	Sucrose NaNO ₃	2.8	96	2.9×10^{-2}	15
<i>A. pullulans</i>	Flask	Sucrose (NH ₄) ₂	4.0	72	5.6×10^{-2}	14
<i>A. pullulans</i> MS-822 (KCTC1179BP)	50-L fermentor	Sucrose Yeast extract	2.5	168	1.5×10^{-2}	23
<i>A. pullulans</i> M-1	3-L fermentor	Sucrose Rice bran	3.5	144	2.4×10^{-2}	This work
<i>A. pullulans</i> M-2	3-L fermentor	Sucrose Rice bran	5.0	144	3.5×10^{-2}	This work
<i>A. pullulans</i> M-2	3-L fermentor	Optimized medium	9.9	144	6.9×10^{-2}	This work
<i>A. pullulans</i> M-2	200-L fermentor	Optimized medium	9.2	144	6.4×10^{-2}	This work