

Isolation of a *Peniophora* Strain Capable of Producing Ethanol from Starch and Kitchen Waste

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Abstract

A white rot basidiomycete identified as *Peniophora* sp. produced ethanol from glucose, mannose, cellobiose, and maltose with yields ranging from 0.37 to 0.41 g ethanol g consumed sugar⁻¹. *Peniophora* sp. was also capable of directly fermenting various types of starches, including soluble, corn, potato, and wheat starches, at a yield of 0.39 to 0.41 g ethanol g added starch⁻¹. The fungus also directly converted untreated kitchen waste to ethanol at 73% of the theoretical yield in the absence of externally added enzymes or nitrogen. Our findings indicate that *Peniophora* sp. efficiently hydrolyzes biomass to fermentable sugars, which are then directly converted to ethanol. The metabolic properties of this fungus may permit cost-effective and environmental friendly bioethanol production from various starting materials, including kitchen waste.

Keywords: Basidiomycete; *Peniophora*; Ethanol; Fermentation; Starch; Kitchen waste

Introduction

Worldwide greenhouse gas emissions are increasing and are thought to contribute to global warming, which has the potential to affect climate, ecosystems, and the spread of diseases. Among the various approaches to reduce greenhouse gas emission, the use of bioethanol has attracted international attention because it is a carbon-neutral fuel that is produced from naturally abundant biological materials. As bioethanol is predominantly derived from sugar or starch crops, it represents a sustainable, renewable, environmentally friendly transportation fuel that is a promising alternative to gasoline. Although bioethanol is increasingly being used as a fuel source around the world, the rapid increase in bioethanol use has also affected crop production patterns, resulting in increased food prices. Therefore, second-generation bioethanol production requires the development of economically feasible and sustainable processes utilizing renewable lignocellulosic materials that do not compete with food sources [1].

Lignocellulosic biomass is the most abundant and inexpensive material available for bioethanol production. Lignocellulose, which is composed of cellulose, hemicellulose and lignin, is hydrolytically stable and is not readily broken down into fermentable sugars. Cellulose and hemicellulose are polysaccharides that can be hydrolyzed to hexose and pentose sugars, whereas lignin is a phenolic compound that cannot be used for ethanol production. A major practical limitation in biomass-to-ethanol conversion is the cost associated with the enzymatic hydrolysis of lignocellulosic biomass, which requires enzymes such as cellulases. Although the enzymatic hydrolysis of lignocellulosic biomass has several advantages over acid hydrolysis, such as a lower environmental impact and reduced generation of by-products that negatively affect fermentation, the cost efficiency of this process requires improvement for commercial application.

Wood-decaying basidiomycetes play key roles in recycling forest resources through multi-enzyme systems that degrade lignocelluloses. For this reason, basidiomycetes may be useful for biorefinery processes that require the pretreatment of raw materials, including the production of biofuels and functional chemicals from lignocellulosic materials [2]. In our previous study, we isolated the white rot basidiomycete *Peniophora cinerea*, which is capable of producing ethanol from hexose sugars [3]. During subsequent screening for efficient ethanol-producing basidiomycetes, we found that a newly isolated strain, *Peniophora* sp. YM5314, produced ethanol from not only hexoses, but also from starchy materials, at higher efficiencies than the other evaluated species of *Peniophora*.

Household kitchen waste represents a valuable source of potential energy. Unlike lignocellulosic materials grown for fuel such as wood, agricultural and forest residues, which are restricted to environmentally suitable locations, kitchen waste is generated in large quantities wherever people live. As kitchen waste can be obtained at no cost and requires relatively low cost for transportation, it is expected to be an ecologically and economically sustainable raw material for bioethanol production in resource-poor countries, such as Japan. Several studies have examined bioethanol production from kitchen waste using an enzymatic saccharification step [4-11]; however, no studies have examined the use of a basidiomycete fungus for direct bioethanol production.

In the present study, the fermentability of starch and kitchen waste by *Peniophora* sp. YM5314 was examined to determine the possible application of this white rot fungus in ecological and economical bioethanol production.

Materials and Methods

Microorganism and culture conditions

Peniophora sp. YM5314 was maintained on 1.5% agar plates consisting of MYG medium (10 g malt extract l⁻¹, 4 g yeast extract l⁻¹,

and 4 g glucose l⁻¹). Fungal mycelia were grown on MYG agar plates for 5 days and three 0.5-cm² pieces of the mycelium mat were then inoculated into a 500-ml Erlenmeyer flask containing 50 ml MYG medium and further cultivated for 7 days at 28°C. Cells were harvested, washed once in sterile water, and transferred to a 500-ml Erlenmeyer flask containing 50 ml T medium (10 g yeast extract l⁻¹, 10 g KH₂PO₄ l⁻¹, 2 g (NH₄)₂SO₄ l⁻¹, and 0.5 g MgSO₄·7H₂O l⁻¹, pH 6.4) and 20 g l⁻¹ of the test carbon source were added. The cultures were incubated at 28°C without shaking under anaerobic conditions. Each experiment was performed in triplicate.

The kitchen waste used in this study was collected from the canteen of Tottori University, Japan and contained a moisture content of 77% ± 4%, with carbohydrates composing 8% ± 2% of the total solids. The culture medium for investigating ethanol production from kitchen waste was composed of 25 g kitchen waste and 40 ml water in a 500-ml Erlenmeyer flask, without pH adjustment. The initial pH of the prepared kitchen waste medium was 5.4 ± 0.2. As the composition of kitchen waste from the canteen exhibited daily variation, the experiments were repeated ten times using samples collected on different dates. The prepared media were sterilized in an autoclave at 121°C for 15 min before use.

Verification of strain identity

Identification of strain YM5314 was based on standard morphological and biochemical analyses and was carried out by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). The identity of this strain was determined by sequencing of the 28S rDNA D1/D2 and internal transcribed spacer (ITS) regions including 5.8S rDNA. DNA was extracted from mycelia using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and PCR was performed using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, South Plainfield, NJ, USA). Partial sequences were amplified with primer pairs targeting the D1/D2 (NL1 and NL4) [12] and ITS1-5.8S-ITS2 regions (ITS4 and ITS5) [13]. DNA sequencing was performed using an ABI PRISM 3130x1 Genetic Analyzer System (Applied Biosystems, Foster City, CA, USA). DNA sequences were compared with those in the GenBank database using BLAST [14], and multiple sequence alignments were conducted using ClustalW [15]. The sequences of both 28S rDNA (HM595611 or JF925333) and ITS1-5.8S-ITS2 rDNA (HM595565 or JF925333) from the *Peniophora* sp. strains available from GenBank/EMBL/DDBJ displayed 99% identity to the sequences of strain YM5314. According to the morphological, biochemical, and phylogenetic analyses, strain YM5314 was verified as *Peniophora* sp. The 28S rDNA and ITS1-5.8S-ITS2 rDNA sequence data from *Peniophora* sp. YM5314 have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers AB899814 and AB899815, respectively.

Analytical methods

Ethanol and residual sugar concentrations in culture filtrates were determined using a Prominence High-Performance Liquid Chromatography (HPLC) system (Shimadzu Co., Ltd., Kyoto, Japan) equipped with an RID-10A refractive index detector (Shimadzu) and a Shodex KS-801 column (8.0 mm × 300 mm; Showa Denko Co., Ltd., Tokyo, Japan) operating at 80°C with a distilled water mobile phase at a flow rate of 0.6 ml min⁻¹. The theoretical yield of ethanol was estimated to be 0.51 g ethanol g glucose⁻¹ (2 mol ethanol mol glucose⁻¹). When starch was used as the sole carbon source, the theoretical yield of ethanol was estimated to be 0.57 g ethanol g

starch⁻¹. The concentrations of starch and glucose released in the culture filtrates during fermentation on starch and kitchen waste were determined using an F-kit for starch (Roche Diagnostics, Mannheim, Germany) and a Glucose C2 test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The activities of α-amylase, α-glucosidase, and glucoamylase in culture filtrates during fermentation were assayed using commercial kits from Kikkoman Corp. (Chiba, Japan).

Results and Discussion

Production of ethanol from hexose sugars

The fermentation performance of the white rot fungus *Peniophora* sp. YM5314 was investigated in culture medium supplemented with the monosaccharides glucose and mannose as sole carbon sources. Both glucose and mannose were completely consumed within 48 h (Figure 1a and 1b). Following the depletion of each sugar, maximum ethanol concentrations of 8.2 and 7.3 g l⁻¹, which corresponded to ethanol yields of 0.41 and 0.37 g ethanol g consumed glucose and mannose⁻¹, respectively, were observed. When strain YM5314 was cultured on medium containing the disaccharides maltose and cellobiose, the carbon sources were gradually decomposed to glucose, with maximum ethanol concentrations of 8.1 and 7.9 g l⁻¹, respectively, reached after complete consumption of the sugars at 120 and 168 h, respectively (Figure 1c and 1d).

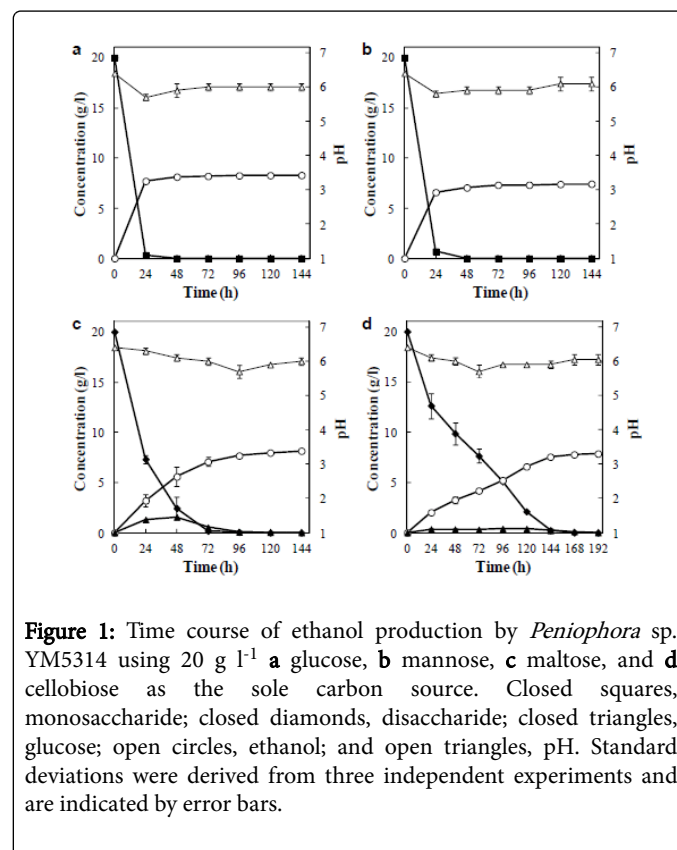


Figure 1: Time course of ethanol production by *Peniophora* sp. YM5314 using 20 g l⁻¹ a glucose, b mannose, c maltose, and d cellobiose as the sole carbon source. Closed squares, monosaccharide; closed diamonds, disaccharide; closed triangles, glucose; open circles, ethanol; and open triangles, pH. Standard deviations were derived from three independent experiments and are indicated by error bars.

The observed ethanol yields of 0.41 g ethanol g maltose⁻¹ and 0.40 g ethanol g cellobiose⁻¹ demonstrate that *Peniophora* sp. YM5314 effectively converts both of these sugars to ethanol. Thus, *Peniophora* sp. YM5314 has better ability than wild-type yeast *Saccharomyces*

cerevisiae for the fermentation of sugars in lignocellulose, as *S. cerevisiae* cannot assimilate cellobiose.

Direct ethanol production from starch

Peniophora sp. YM5314 was cultured for 168 h in medium supplemented with starch from soluble, corn, potato, and wheat, yielding maximum ethanol concentrations of 7.8–8.2 g l⁻¹, which corresponded to yields of 0.39–0.41 g ethanol g added starch⁻¹ (Figure 2a-2d).

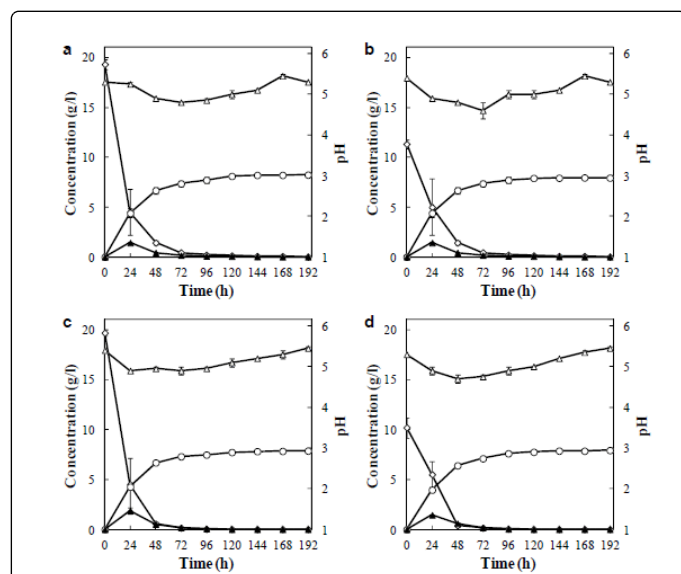


Figure 2: Time course of ethanol production by *Peniophora* sp. YM5314 using 20 g l⁻¹ **a** soluble starch, **b** corn starch, **c** potato starch, and **d** wheat starch as the sole carbon source. Open diamonds, starch; closed triangles, glucose; open circles, ethanol; and open triangles, pH. Standard deviations were derived from three independent experiments and are indicated by error bars.

Although differences in the solubility of the four examined types of starch were observed, with corn and wheat starch dissolving poorly in the medium after autoclaving, these solubility differences did not appear to influence ethanol production. During the first 24 h of each fermentation, a small amount of glucose from starch accumulated in the culture medium, followed by a decrease in glucose and a rapid increase in ethanol, indicating that this fungus maintained a balance between starch decomposition and sugar conversion to ethanol. Based on these findings, it was determined that strain YM5314 is able to convert various starches directly into ethanol at approximately 70% of the theoretical yield.

A recombinant *S. cerevisiae* strain with integrated α -amylase and glucoamylase genes was previously reported to produce 39 g l⁻¹ ethanol from 100 g l⁻¹ corn starch, corresponding to a maximum yield of 0.39 g ethanol g added starch⁻¹ (68% of the theoretical yield) [16]. The conversion performance observed here with a naturally occurring basidiomycete was comparable to that of recombinant *S. cerevisiae*, suggesting that *Peniophora* sp. YM5314 produces amylolytic enzymes that are able to directly convert starch, regardless of the type, to ethanol at relatively high yield.

Direct ethanol production from kitchen waste

As described above, *Peniophora* sp. YM5314 efficiently converted various types of starches to ethanol. To further characterize the fermentation properties of this fungus with respect to starchy materials, strain YM5314 was cultured using kitchen waste as a carbon source. Using this medium, a maximum ethanol concentration of 11.4 \pm 2.8 g l⁻¹ was obtained after 192 h of fermentation (Figure 3a). The levels of α -amylase and α -glucosidase increased in the culture medium during fermentation, whereas no significant secretion of glucoamylase was detected (Figure 3b).

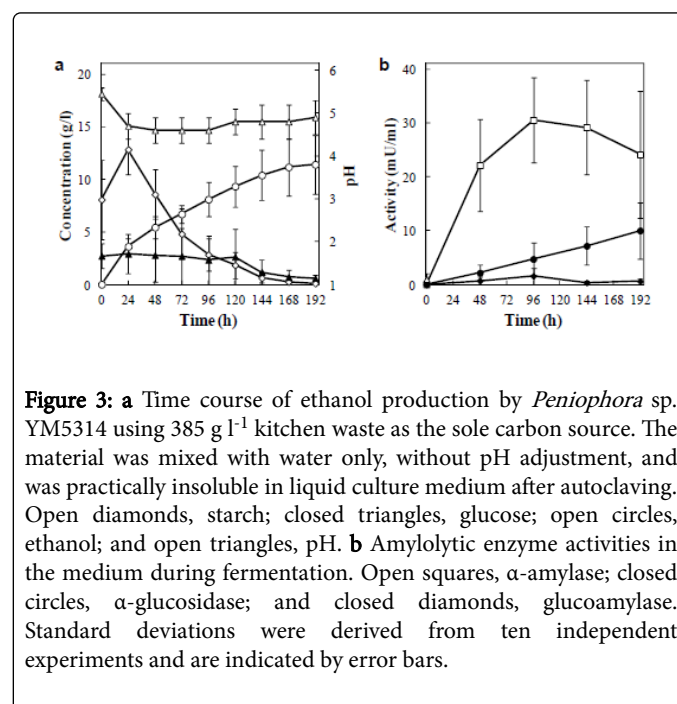


Figure 3: **a** Time course of ethanol production by *Peniophora* sp. YM5314 using 385 g l⁻¹ kitchen waste as the sole carbon source. The material was mixed with water only, without pH adjustment, and was practically insoluble in liquid culture medium after autoclaving. Open diamonds, starch; closed triangles, glucose; open circles, ethanol; and open triangles, pH. **b** Amylolytic enzyme activities in the medium during fermentation. Open squares, α -amylase; closed circles, α -glucosidase; and closed diamonds, glucoamylase. Standard deviations were derived from ten independent experiments and are indicated by error bars.

Therefore, it is possible that the utilization of starch present in the kitchen waste by strain YM5314 was due to the activity of both α -amylase and α -glucosidase. Although the activities of these two enzymes did not seem particularly high during fermentation, these enzymes may be produced at sufficient levels to facilitate direct ethanol fermentation.

Most starch in the kitchen waste was nearly entirely consumed after approximately 192 h, implying that strain YM5314 was able to recover and convert the available sugar to ethanol. The kitchen waste used in these experiments was randomly collected from the canteen on our campus and the composition of the waste therefore differed between collection days. Although ethanol production by this fungus was dependent on the carbohydrate content of the kitchen waste, excessive contamination of meat or fish tended to negatively influence the fermentation efficiency.

Based on the fermentation profiles presented here, strain YM5314 is able to directly produce ethanol from kitchen waste by simultaneous saccharification and fermentation without any pretreatment steps or additional enzymes. In a similar study, *S. cerevisiae* produced 30.9 g ethanol from 1 kg kitchen waste (sugar content, 11.8%), which corresponded to a theoretical yield of 60% based on the amount of glucose, through a saccharification process involving two kinds of glucoamylases, Glucochimu #20000 and Nagase N-40 [5]. Compared to this two-step saccharification and fermentation process, the single-

step fermentation process described here, which does not require exogenous enzymes for saccharification, had a higher yield of ethanol from kitchen waste (0.030 ± 0.007 g ethanol g kitchen waste⁻¹; 73% of the theoretical yield based on the amount of glucose).

Conclusion

In this study, the white rot basidiomycete *Peniophora* sp. YM5314 was capable of assimilating a broad spectrum of carbon sources and fermenting ethanol from starch and kitchen waste. Our findings demonstrate that the utilization of kitchen waste as a raw material for ethanol fermentation by this starch-degrading fungus may be possible for low-cost ethanol production and contribute to the effective recycling and reduction of kitchen waste and the associated CO₂ emissions resulting from incineration treatment. To our knowledge, this is the first report to describe the efficient fermentation of kitchen waste by a basidiomycete.

Peniophora sp. YM5314 appears to be a promising microorganism for the production of bioethanol from renewable resources, although further studies are required to improve the ethanol yield and productivity of this fungus. The direct production of ethanol from renewable resources by a naturally occurring basidiomycete may allow for sustainable bioethanol production by consolidated bioprocessing, as it is expected to reduce overall costs and the environmental footprint related to production.

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