Oxygen radical based on non-thermal atmospheric pressure plasma alleviates lignin-derived phenolic toxicity in yeast

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Abstract

Background: Vanillin is the main byproduct of alkaline-pretreated lignocellulosic biomass during the process of fermentable-sugar production and a potent inhibitor of ethanol production by yeast. Yeast cells are usually exposed to vanillin during the industrial production of bioethanol from lignocellulosic biomass. Therefore, vanillin toxicity represents a major barrier to reducing the cost of bioethanol production.

Results: In this study, we analysed the effects of oxygen-radical treatment on vanillin molecules. Our results showed that vanillin was converted to vanillic acid, protocatechuic aldehyde, protocatechuic acid, methoxyhydroquinone, 3,4-dihydroxy-5-methoxybenzaldehyde, trihydroxy-5-methoxybenzene, and their respective ring-cleaved products, which displayed decreased toxicity relative to vanillin and resulted in reduced vanillin-specific toxicity to yeast during ethanol fermentation. Additionally, after a 16-h incubation, the ethanol concentration in oxygen-radical-treated vanillin solution was 7.0-fold greater than that from non-treated solution, with similar results observed using alkaline-pretreated rice straw slurry with oxygen-radical treatment.

Conclusions: This study analysed the effects of oxygen-radical treatment on vanillin molecules in the alkaline-pretreated rice straw slurry, thereby finding that this treatment converted vanillin to its derivatives, resulting in reduced vanillin toxicity to yeast during ethanol fermentation. These findings suggest that a combination of chemical and oxygen-radical treatment improved ethanol production using yeast cells, and that oxygen-radical treatment of plant biomass offers great promise for further improvements in bioethanol-production processes.

Keywords: Atmospheric pressure plasma, Oxygen-radical treatment, Biorefinery, Bioethanol, Plant biomass, Vanillin

Background

Biorefinement of lignocellulosic biomass to liquid fuels or other chemicals is beneficial to sustainable energy and the environment [1]. Lignocellulose mainly comprises cellulose, hemicellulose, and lignin, and cellulose and hemicellulose are capable of converting fermentable sugars by enzymatic hydrolysis, whereas lignin plays a negative role in saccharification of the lignocellulosic biomass [2]. Lignin is an aromatic polymer comprising three primary units [hydroxyphenyl (H), guaiacyl (G), and syringyl (S)] that are randomly linked with aryl ether, ester, or carbon bonds [3, 4].

Bioethanol production from lignocellulose generally involves three steps: (1) pretreatment to break down complex lignocellulose structures, (2) enzymatic hydrolysis of polysaccharides (i.e., cellulose and hemicellulose) into fermentable sugars, and (3) fermentation to convert sugars into ethanol [5]. Pretreatment is required to alter the biomass by changing its chemical...
or physical properties and to allow increased enzyme accessibility to cellulose [6, 7], with various biological, chemical, and physical pretreatment methods having been developed [8–12]. Vanillin is generally generated as a byproduct during the process of fermentable-sugar production from lignocellulosic biomass, regardless of being herbage, softwood, or hardwood [13, 14]. The vanillin concentration in the lignocellulosic hydrolysate can vary depending on the types of biomass materials and treatment methods, with a wide range of vanillin concentrations (1–26 mM) reported in previous studies [15, 16]. Because vanillin is a potent inhibitor of yeast-specific ethanol fermentation via dose-dependent blockage of yeast growth and subsequent fermentation, vanillin toxicity represents a major barrier to reducing the cost of bioethanol production [17–20]. Several methods, including overliming, anion-exchange resin treatment, activated carbon treatment, sulphate treatment, and treatment with laccase, have been proposed to alleviate the negative effects of lignin-derived phenolics on biomass hydrolysates [21–25]; however, these methods require long processing times and are detrimental to the environment based on the release of organic waste [21, 23]. Additionally, utilization of these methods requires alkaline- or acid-resistant equipment, a neutralization step, chemical recovery, and waste treatment [21–25]. Therefore, the development of an environmentally friendly vanillin-removal process is an important prerequisite for the efficient production of bioethanol from lignocellulosic biomass.

In our previous work, we developed radical generators based on non-thermal atmospheric pressure plasma (NTAP) technology using an available radical generator with an oxygen–argon gas mixture to generate oxygen radicals [26, 27]. The radical generator provides high electron density, and we reported large amounts of atomic –O (3P) at an absolute density on the order of between 10^{13} cm^{-3} and 10^{14} cm^{-3} (equivalent to 1–10 ppm) [28]. Use of the NTAP-based radical generator has several advantages: (1) on-site generation, which avoids problems associated with chemical supply and storage; (2) reaction at ambient temperatures and pressures; (3) achievement of a rapid reaction with a high density of atomic oxygen radicals; and (4) a low cost relative to conventional low-pressure plasmas due to the absence of vacuum devices [29]. Moreover, pretreatment of plant biomass using a radical generator is more environmentally friendly than chemical methods, given that no chemical waste is produced. In our recent work, oxygen-radical pretreatment of cellulose and wheat straw enhanced cellulose degradation by cellobiohydrolases (CBHs) from the white-rot fungus Phanerochaete chrysosporium [30]. These findings indicated that the NTAP-based radical generator offers great promise for use in biorefining processes.

In this study, we analysed the effects of oxygen-radical irradiation against vanillin molecules, potent inhibitors of ethanol production by yeast. We also determined the effects of oxygen-radical treatment on lignin-derived phenolics generated by alkaline-pretreated rice straw.

Results and discussion

Oxygen-radical irradiation of vanillin

The effects of oxygen-radical irradiation of vanillin were examined using high-performance liquid chromatography (HPLC) and GC–MS (Fig. 1a and Additional file 1: Figure S1). Time-course analysis of vanillin conversion by oxygen-radical treatment using HPLC showed that the vanillin concentration in oxygen-radical-treated solutions decreased with increasing treatment time (Additional file 1: Figure S1). Vanillin (5.0 mM) decreased to 0.96 mM and was converted to vanillic acid (0.20 mM), protocatechuic aldehyde (0.14 mM), protocatechuic acid (0.01 mM), methoxyhydroquinone (0.03 mM), 3,4-dihydroxy-5-methoxybenzaldehyde (0.14 mM), and trihydroxy-5-methoxybenzene by oxygen-radical irradiation for 20 min using the radical generator (Fig. 1 and Additional file 1: Figure S2; Table 1). Additionally, we detected aromatic-ring-cleaved products, including methyl-2,5-dihydroxy-6-oxohexa-2,4-dienoate, 4-hydroxy-6-methoxy-6-oxohexa-2,4-dienoic acid, 4-formyl-6-methoxy-6-oxohexa-2,4-dienoic acid, 4-(2-methoxy-2-oxoethylidene)pent-2-enedioic acid, oxalic acid (3.03 mM), and methoxy oxalic acid, indicating that the benzene-ring of vanillin and its derivatives were cleaved by oxygen-radical irradiation. Moreover, we detected an unidentified but putative aromatic dimer compound (Fig. 1 and Additional file 1: Figure S2; Table 1). These results suggested that oxygen-radical irradiation promoted vanillin oxidation, monooxygenation, demethoxylation, decarbonylation, dimerization, and aromatic-ring fission (Additional file 1: Figure S3).

Previous studies indicated that the molecular weights of amino acids, such as Tyr, Phe, Trp, Cys, Met, Pro, His, Lys, Arg, Glu, Glu, Val, Leu, and Ile, change due to oxidation and hydroxylation by active species generated by NTAP irradiation [31–33]. Specifically, electron-rich groups, such as nitrogen- and sulphur-containing and aromatic compounds, were preferentially modified by the various active species [31–33]. Additionally, the aromatic rings of Tyr, Phe, Trp, and His are reportedly hydroxylated by NTAP irradiation [32]. Using Fourier transform and {^1}H nuclear magnetic resonance analysis, Asandulesa et al. [34] showed that the aromatic rings of benzyl alcohol, benzaldehyde, and benzyl chloride were cleaved and converted to aliphatic groups by NTAP irradiation.
Fig. 1 Conversion of vanillin by oxygen-radical treatment. a GC–MS chromatogram of vanillin solution (5.0 mM) irradiated with oxygen-radical treatment for 0 min and 20 min. Reaction products were trimethylsilylated and analysed by GC–MS. Identified reaction products are marked by arrows with numbers and shown in Table 1. b–e Treatment-time-dependent conversion of vanillin and the production of reactants. Error bars represent the mean±standard error of the mean of three independent experiments.
Moreover, similar results were observed using pyrolytic lignin and phenolic model compounds by ozonolysis [35–37]. Although the exact mechanism of vanillin conversion and aromatic-ring cleavage by oxygen-radical, plasma, or ozone treatment is not fully elucidated, oxygen-radical treatment would likely generate radicals in the gas phase that would react with lignin-derived phenolics to form radicals that promote ring cleavage. These findings indicated that vanillin oxidation, monooxygenation, demethoxylation, decarbonylation, dimerization, and aromatic-ring fission were generated by oxygen-radical treatment (Additional file 1: Figure S3).

**Effects of oxygen-radical treatment on yeast growth and ethanol production**

To examine the effects of oxygen-radical treatment of vanillin solution on yeast growth, we cultivated *Saccharomyces cerevisiae* S288c in YPD medium containing up to 5 mM vanillin irradiated with or without oxygen-radical. Figure 2 shows the yeast-growth curves associated with various vanillin concentrations. Compared with the absence of vanillin, yeast growth was inhibited by 8%, 35%, and 80% in the presence of 1.0 mM, 2.5 mM, and 5.0 mM vanillin, respectively, whereas the growth rates were 105%, 104%, and 83% in the presence of vanillin irradiated with oxygen-radical, respectively (Fig. 2a–d). The effect of several vanillin degradation products, such as vanillic acid, protocatechuic aldehyde, protocatechuic acid, methoxyhydroquinone, 3,4-dihydroxy-5-methoxybenzaldehyde, and oxalic acid on yeast growth was also determined (Additional file 1: Figure S4). Yeast growth with 2.5 mM vanillin was inhibited the most compared with that with the same concentration of its degradation products. These results indicate that vanillin degradation products generated by oxygen-radical treatment have lower toxicity against *S. cerevisiae* cells. The concentrations of vanillin degradation products except oxalic acid were lower than that of vanillin (Fig. 1 and Additional file 1: Figure S2; Table 1). Yeast growth was inhibited by 15% in the presence of 2.5 mM oxalic acid (Additional file 1: Figure S4). Compared with the absence of vanillin, yeast growth was inhibited by 8% in the presence of 1.0 mM vanillin, whereas the growth rate was 83% in the presence of 5.0 mM vanillin irradiated with oxygen-radical for 20 min, respectively (Fig. 2b, d). These results suggest that yeast growth in the presence of 5.0 mM vanillin irradiated with oxygen-radical may be inhibited by 20% by residual vanillin (0.96 mM) and oxalic acid (3.03 mM) generated from vanillin by oxygen-radical treatment (Fig. 2d). Moreover, ethanol concentration in culture supernatant after 16-h incubation in the absence of vanillin was 10.4 g/L (Fig. 3), whereas inclusion of vanillin inhibited ethanol production by 20%, 66%, and 88% at 1.0 mM, 2.5 mM, and 5.0 mM vanillin, respectively. Compared with the 16 h incubation in the absence of vanillin, ethanol production was 100%, 92%, and 83% in the presence of 1.0 mM, 2.5 mM, and 5.0 mM vanillin irradiated with oxygen radical, respectively (Fig. 3). The ethanol concentration in the oxygen-radical-treated vanillin solution at 5.0 mM was 7.0-fold greater than that from

**Table 1** Detected vanillin-specific compounds derived from oxygen-radical treatment

| No. | Identified compounds                                      | Concentration (mM) |
|-----|----------------------------------------------------------|--------------------|
| 1   | Methoxy oxalic acid                                       | –                  |
| 2   | Oxalic acid                                               | 3.031 ± 0.618      |
| 3   | Methyl-2,5-dihydroxy-6-oxohexa-2,4-dienoate               | –                  |
| 4   | Vanillin                                                  | 0.957 ± 0.133      |
| 5   | Methoxyhydroquinone                                       | 0.032 ± 0.008      |
| 6   | Protocatechuic aldehyde                                   | 0.143 ± 0.043      |
| 7   | 4-Hydroxy-6-methoxy-6-oxohexa-2,4-dienoic acid            | –                  |
| 8   | 4-Formyl-6-methoxy-6-oxohexa-2,4-dienoic acid             | –                  |
| 9   | Trihydroxy-5-methoxybenzene                              | –                  |
| 10  | 3,4-Dihydroxy-5-methoxybenzaldehyde                       | 0.139 ± 0.037      |
| 11  | 4-(2-Methoxy-2-oxoethylidene)pent-2-enedioic acid         | –                  |
| 12  | Vanillic acid                                             | 0.198 ± 0.050      |
| 13  | Unidentified compound that is contaminant in original reagent | –               |
| 14  | Protocatechuic acid                                       | 0.011 ± 0.003      |
| 15  | Unidentified compound that is putative aromatic dimer      | –                  |

Initial concentration of vanillin was 5.0 mM. The concentrations of vanillin and the reactants irradiated with oxygen-radical treatment for 20 min were quantified by GC–MS. Reaction products were trimethylsilylated and analysed by GC–MS. Numbers indicate the GC peaks shown in Fig. 1a. Mass spectra obtained from each GC peak are shown in Additional file 1: Figure S2.

* These compounds were not quantified because these reagents were not commercially available.
non-treated solution (Fig. 3). These results suggested that irradiation with oxygen radical alleviated vanillin toxicity against *S. cerevisiae* and helped to restore 80% of the ethanol yield as compared with no vanillin present.

Vanillin acts as a potent fermentation inhibitor that represses yeast growth and fermentative abilities [20, 38]. A recent study showed that vanillin suppressed translation initiation by affecting the ribosome-assembly process, thereby causing accumulation of cytoplasmic messenger ribonucleoprotein granules and processing bodies [39]. Furthermore, vanillin induces the accumulation of reactive oxygen species and mitochondrial fragmentation in *S. cerevisiae* and limits mRNA translation to reduce overall protein-synthesis levels, leading to vanillin-specific inhibition of yeast cell growth and ethanol fermentation [40, 41]. *S. cerevisiae* is a traditionally competitive cell factory used for bioethanol production due to its superior tolerance to ethanol and low pH, as well as its ease of genetic manipulation [42]. To overcome vanillin toxicity as a barrier to reduced bioethanol-production costs, vanillin-tolerant strains have been screened and engineered [38, 43–45]; however, these strains have not fully resolved the problems of toxicity associated with lignin-derived phenolics, which have been documented in other fermentable microorganisms (i.e., ethanol fermentation by *Thermoanaerobacter mathranii*, butanol fermentation by *Clostridium beijerinckii* and *Clostridium acetobutylicum*, butyric acid fermentation by *Clostridium tyrobutyricum*, hydrogen fermentation by *Thermoanaerobacter thermosaccharolyticum*, bacterial nanocellulose
production by *Gluconacetobacter xylinus*, and xylitol fermentation of *Candida tropicalis* [46–52]. Therefore, the presence of lignin-derived phenolics remains a problem in biorefining processes using lignocellulosic biomass. Our results suggest that oxygen-radical treatment as a potentially effective means of addressing vanillin toxicity to microorganisms during biorefining processes.

**Effects of oxygen-radical treatment on lignin-derived phenolics generated by alkaline pretreatment of plant biomass**

We examined the effects of oxygen-radical treatment of alkaline-pretreated rice straw slurry on yeast growth and ethanol production. The composition of cellulose, hemicellulose, lignin, ash, and total solids in non-pretreated rice straw and alkaline-pretreated rice straw with or without oxygen-radical treatment was determined (Table 2). After alkaline pretreatment, the biomass loss of native rice straw was 31.1% (Table 2). The remaining solid of alkaline-pretreated rice straw without oxygen-radical treatment was 68.9%, including 65.4% cellulose, 18.2% hemicellulose, 5.5% lignin, and 5.1% ash (Table 2). Oxygen-radical treatment did not affect the composition of alkaline-pretreated rice straw (Table 2).

We then performed vanillin conversion in the alkaline-pretreated rice straw slurry following oxygen-radical treatment for 20 min using HPLC (Fig. 4a) and GC–MS. Analysis of the soluble products from alkaline-treated rice straw revealed vanillin (3.32 mM), vanillic acid (0.13 mM), *p*-coumaric acid (2.11 mM), *t*-ferulic acid (0.69 mM), oxalic acid (0.50 mM), furfural (0.02 mM), and HMF (0.01 mM) (Table 3). These results indicated that lignin in native rice straw was converted to vanillin (7.5%), vanillic acid (0.3%), *p*-coumaric acid (5.2%), and *t*-ferulic acid (2.0%) in the alkaline-pretreated rice straw slurry without oxygen-radical treatment (Tables 2 and 3). However, vanillin concentration in the oxygen-radical-treated slurry decreased to 0.69 mM (Fig. 4a; Table 3). Additionally, *p*-coumaric acid, a potent inhibitor of yeast growth [53], was decreased to 0.31 mM in the oxygen-radical treated slurry (Table 3). Although yeast growths with 2.5 mM *p*-coumaric acid, oxalic acid, lactic acid, and furfural were 1.59-, 1.61-, 1.62-, and 1.60-fold, higher, respectively, than that with 2.5 mM vanillin, the compounds inhibited yeast growth (Additional file 1: Figures S4 and S5). These results implied that vanillin conversion by the oxygen-radical treatment of alkaline-pretreated rice straw enhanced yeast growth and ethanol production.

We then performed cellulase, from *Aspergillus niger*, hydrolysis of alkaline-pretreated rice straw slurry with or without oxygen-radical treatment to produce fermentable sugars to promote ethanol production by yeast. Following enzymatic hydrolysis, we analysed the soluble products in the alkaline-treated rice straw suspensions with or without oxygen-radical treatment.

**Table 2 The content of cellulose, hemicellulose, lignin, and ash in native, alkaline-pretreated and alkaline-pretreated with oxygen-radical-treated rice straw**

| Treatment | Total solids % | Composition (%) | Remaining solid (%) |
|-----------|----------------|-----------------|---------------------|
|           |                | Cellulose | Hemicellulose | Lignin | Ash |                |               |
| Alkaline  | Oxygen-radical | 96.1       | 34.4       | 29.5   | 13.4 | 13.1   | –               |
| +         | –              | 98.1       | 65.4       | 18.2   | 5.5  | 5.1    | 68.9            |
| +         | +              | 97.7       | 63.9       | 18.5   | 5.9  | 5.4    | 67.8            |

The contents of cellulose, hemicellulose, lignin, and ash in native and alkaline-pretreated rice straw with or without oxygen-radical treatment were determined according to previous methods. Data are presented as mean values of three independent experiments. The standard errors were <22%.

* Based on dry matter.
by reducing-sugar HPLC, finding that the contents of reducing sugars, such as glucose, cellobiose, cellotriose, and xylose were similar regardless of oxygen-radical treatment (Additional file 1: Figure S6). Cellulose to glucose conversion rates in the alkaline-treated rice straw slurry with or without oxygen-radical treatment were 31.0% and 32.7%, respectively. Commercially available cellulase from *A. niger* used in this study was not inhibited by up to 10 mM vanillin (data not shown).

We also determined the effect of irradiation of glucose (Additional file 1: Figure S7a). Glucose solutions (10, 25, 50 mM) were prepared, and the oxygen-radical was irradiated in these solutions. Glucose was not converted by the oxygen-radical treatment (Additional file 1: Figure S7a). We then determined yeast growth in 50 mM glucose solution with or without oxygen-radical treatment.
for 20 min (Additional file 1: Figure S7b). Compared with the glucose solution without oxygen-radical treatment, yeast growth was similar in oxygen-radical-treated solution (Additional file 1: Figure S7b). These results indicate that the irradiation of glucose is not affected on yeast growth. Our previous study reported that cleavage of the β-1,4-glycoside linkages in the cellulose backbone into smaller chains by oxygen-radical treatment promotes cellulose hydrolysis by allowing CBHs [30]. Because <i>A. niger</i> mainly secretes endo-β-1,4-glucanase and β-1,4-glucosidase and displays low levels of CBH production [54, 55], oxygen-radical treatment did not affect reducing-sugar production.

We then determined yeast growth in suspensions treated with oxygen radical for 20 min (Fig. 4b). After a 48-h incubation, yeast growth in oxygen-radical-treated suspensions was 5.8-fold higher than that of untreated suspensions (Fig. 4b). Furthermore, ethanol production from oxygen-radical-treated suspensions showed a 5.2-fold increase relative to that from untreated suspensions (Fig. 4c).

To elucidate the inhibitory effect of vanillin and p-coumaric acid in alkaline-pretreated rice straw suspensions, vanillin and p-coumaric acid were added to the oxygen-radical-treated suspension at final concentrations of 3.3 mM and 2.1 mM, respectively, followed by the determination of yeast growth and ethanol production, which revealed similar results to those obtained using alkaline-pretreated rice straw suspensions without oxygen-radical treatment (Fig. 4b, c). Compared with the addition of vanillin and p-coumaric acid, yeast growth rates in the suspensions were 1.8- or 4.6-fold in the presence of vanillin or p-coumaric acid at final concentrations of 3.3 mM or 2.1 mM, respectively (Fig. 4b). These results suggest that vanillin and p-coumaric acid conversions by oxygen-radical treatment of alkaline-pretreated plant biomass promote yeast ethanol production.

Because lignin-degradation products, such as vanillin, inhibit the cellulase activity of CBHs, oxygen-radical treatment of alkaline-pretreated rice straw represents an effective method for biorefining processes using cellulosic enzymes [56, 57]. These findings indicated that oxygen-radical treatment not only promoted cellulose degradation by CBHs, but also improved yeast ethanol production.

Various biological, chemical, and physical pretreatment methods have been developed [8–12]. For economic reasons, alkaline hydrolysis is commonly used to prepare lignocelluloses for enzymatic saccharification and fermentation [58]; however, vanillin is generated as a toxic byproduct during this process [13, 14]. Yeast cells are usually exposed simultaneously to vanillin during the industrial production of bioethanol from lignocellulosic biomass. According to our findings, a combination of chemical and oxygen-radical treatment methods would improve ethanol production using yeast cells (Fig. 5). Plasma discharge generated electrically might represent

### Table 3 Detected compounds in alkaline-pretreated rice straw slurry with or without oxygen-radical treatment

| No. | Identified compounds                        | Concentration (mM) | Without oxygen-radical | With oxygen-radical |
|-----|--------------------------------------------|--------------------|------------------------|---------------------|
| 1   | Methoxy oxalic acid                        | ND                 | –                      |                     |
| 2   | Oxalic acid                                | 1.130 ± 0.232      | 2.311 ± 0.329          |                     |
| 3   | Vanillin                                   | 3.320 ± 0.541      | 0.691 ± 0.144          |                     |
| 4   | Methoxyhydroquinone                        | ND                 | 0.082 ± 0.029          |                     |
| 5   | Protocatechuic aldehyde                    | ND                 | 0.077 ± 0.023          |                     |
| 6   | 4-Hydroxy-6-methoxy-6-oxoheaxa-2-dienoic acid | ND                  | –                      |                     |
| 7   | 3,4-Dihydroxy-5-methoxybenzaldehyde        | ND                 | 0.096 ± 0.030          |                     |
| 8   | 4-(2-Methoxy-2-oxoethylidene)pent-2-enedioic acid | ND                  | –                      |                     |
| 9   | Vanillic acid                              | 0.134 ± 0.052      | 0.055 ± 0.020          |                     |
| 10  | Lactic acid                                | 0.502 ± 0.132      | 0.991 ± 0.203          |                     |
| 11  | Furfural                                   | 0.021 ± 0.003      | 0.011 ± 0.003          |                     |
| 12  | HMF                                        | 0.010 ± 0.003      | ND                     |                     |
| 13  | p-Coumaric acid                            | 2.105 ± 0.478      | 0.314 ± 0.090          |                     |
| 14  | t-Ferulic acid                             | 0.687 ± 0.233      | 0.223 ± 0.044          |                     |

The concentrations of several compounds detected in alkaline-pretreated rice straw slurry with or without oxygen-radical treatment were quantified by GC–MS. These were trimethylsilylated and analysed by GC–MS.

ND not detected

* These compounds were not quantified because these reagents were not commercially available.
an attractive treatment process for the conversion of plant biomass to ethanol.

Conclusions
This study analysed the effects of oxygen-radical treatment on vanillin molecules, finding that this treatment converted vanillin to its derivatives, resulting in reduced vanillin toxicity to yeast during ethanol fermentation. Our results show that the oxygen-radical treatment of alkaline-pretreated lignocellulosic biomass reduces the yeast-inhibitory effects of vanillin by decreasing vanillin content while increasing the levels of various vanillin-derived molecules, thereby attenuating the inhibition of yeast growth and promoting ~fivefold higher levels of ethanol production relative to alkaline-pretreated lignocellulosic biomass without oxygen-radical treatment. These findings suggest that the oxygen-radical treatment of plant biomass offers great promise for further improvements in bioethanol-production processes.

Methods
Chemicals and materials
Vanillin, vanillic acid, 3,4-dihydroxy-5-methoxybenzaldehyde (Wako Pure Chemical Industries, Osaka, Japan), 2-methoxyhydroquinone (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), protocatechuic aldehyde (Sigma-Aldrich, St. Louis, MO, USA), and protocatechuic aldehyde (Nacalai Tesque, Kyoto, Japan) were purchased and used as inhibitors of yeast growth in cultures. Cellulase (mainly containing endo-β-1,4-glucanase and β-1,4-glucosidase) from A. niger [54, 55] was obtained from Tokyo Chemical Industry Co., Ltd., and its activity was 29,500 unit/g. Rice straw was grown and harvested on the farm at Meijo University (Aichi, Japan). The straw was cut, dried at 45 °C for 3 h, and milled to a particle size of 1 mm, followed by washing at a weight ratio of 1:20 of rice straw to distilled deionized water. The washed straw was dried at 45 °C for 24 h and used for subsequent experiments.

Oxygen-radical treatment
The oxygen-radical generator used in this study was based on an atmospheric pressure-discharge plasma generated with a gas mixture containing a small amount of O₂ (30 sccm) in argon (4.97 slm). The use of large amounts of argon provides a high electron density on the order of 10¹⁶ cm⁻³ [27]. Additionally, we expected that the use of argon as a buffer would decrease the three-body collision between oxygen species resulting in O₂ and O₃ molecules, thereby increasing atomic oxygen production in the atmosphere. The structure of the slit with a bent-flow channel downstream is capable of intercepting high-energy photons, and the electrically grounded potential on the flow channel terminates charged species.

A schematic illustration of the oxygen-radical generator is shown in Additional file 1: Figure S8a. Vanillin (1.0 mM, 2.5 mM, and 5.0 mM) dissolved in 0.25% acetonitrile solution (3.0 mL) was irradiated with oxygen radical using the oxygen-radical generator. A fixed distance of 1 cm was used between the slit exit of the radical generator and the surface of the liquid suspension. The suspension samples in Petri dishes (30-mm diameter) were placed on an automated stage for uniform treatment of the solution due to the shape of the
radical exit (0.5 × 16 mm). The speed of the automated stage was set at 4 mm/s, and a plastic chamber was covered to avoid mixing with ambient air.

Yeast strain, growth, and ethanol production
S. cerevisiae S288c was obtained from NITE Biological Resource Center (Tokyo, Japan) and cultured in liquid yeast-extract–peptone–dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) containing 1.0 mM, 2.5 mM, and 5.0 mM vanillin with shaking at 100 rpm at 28 °C for up to 16 h. Cell growth in the presence of vanillin with or without oxygen-radical irradiation was monitored by measuring the optical density at 600 nm. Ethanol in the culture supernatant was measured using an ethanol assay kit (Megazyme International, Bray, Ireland).

Alkaline pretreatment and oxygen-radical irradiation of rice straw
Prior to alkaline pretreatment, rice straw was milled to a particle size of 1 mm and then washed and dried at 45 °C for 24 h, after which the dried rice straw (20 g) was suspended in 400 mL of 1 N NaOH solution (at 5% (w/v) solid loading in 1 L Erlenmeyer flask), and two-step alkaline pretreatment was applied at 37 °C for 24 h with shaking at 100 rpm, followed by autoclaving at 120 °C for 60 min. The prepared alkaline-pretreated slurry was neutralized at pH 6 with 6 N HCl. Glycine (at a final concentration of 50 μM), which is not affected by oxygen-radical treatment [31], was added to the neutralized alkaline-pretreated slurry as an internal standard for quantitative analysis using GC–MS. Oxygen radical was then used to sequentially irradiate the slurry, as described.

Chemical composition analysis
The cellulose, hemicellulose, and lignin compositions of native rice straw, and the remaining solids filtered from the alkaline-pretreated rice straw slurry with or without oxygen-radical treatment were analysed according to the National Renewable Energy Laboratory (NREL) protocol [59]. Samples (300 mg) were mixed into 3 mL of 72% (w/w) sulfuric acid at 30 °C for 60 min. Then, the sulfuric acid was diluted to 4.0% by adding 84 mL deionized water. The mixture was incubated at 121 °C for 60 min. Then the mixture was cooled to room temperature, and the residue was removed by filtration and the supernatant was collected and determined by a Prominence reducing-sugar high-performance liquid chromatography (HPLC) analytical system (Shimadzu, Kyoto, Japan) to measure the monomeric sugar content including glucose, xylose, arabinose, galactose, and mannose. The concentration of cellulose and hemicellulose was calculated according to the monomeric sugar content.

Moreover, the acid-soluble lignin (ASL) content in the liquid was detected using a UV–visible spectrophotometer. The residue was used to determine the acid-insoluble lignin (AIL) content with a muffle furnace at 575 ± 25 °C for 24 h. Ash and total solids were also determined using the muffle furnace and a hot-air oven, respectively [60, 61].

Saccharification of alkaline-pretreated rice straw
Alkaline-pretreated rice straw slurry (with or without oxygen-radical treatment) was hydrolysed by cellulase from A. niger (Tokyo Chemical Industry Co., Ltd.) with enzyme loading at 6.0 mg of protein per gramme of cellulose. Saccharification proceeded at 37 °C for 48 h with shaking at 120 rpm. The hydrolysate was separated by filtration, and the filtrate was sterilized using a 0.22 μm polyethersulfone (PES) syringe filter and added to the yeast extract (at a final concentration of 1%) and peptone (at a final concentration of 2%) to culture yeast cells for 48 h. A schematic illustration of yeast growth and ethanol production using the alkaline-pretreated rice straw slurry with or without oxygen-radical and cellulase treatments following filter sterilization is shown in Additional file 1: Figure S8b.

Analytical methods
Vanillin solution (10 μL) treated with or without oxygen radical and the hydrolysate (10 μL) obtained from alkaline-pretreated rice straw with or without oxygen-radical and cellulase treatments following filter sterilization were analysed using an Acuity ultra-performance liquid chromatography (Waters, Milford, MA) equipped with an ADME-HR S5 column (150 × 4.6 mm i.d. × 5 μm pore size; Osaka Soda, Osaka, Japan). Vanillin solutions and the hydrolysates (500 μL) were lyophilized, trimethylsilylated using 50 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (Wako Pure Chemical Industries), and analysed using gas chromatography–mass spectrometry (GC–MS; GCMS-QP2010; Shimadzu, Kyoto, Japan) on a system equipped with a J&W DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm thickness; Agilent Technologies, Santa Clara, CA) [62]. Glycine (at a final concentration of 50 μM) was used as an internal standard for quantitative analysis using GC–MS. We determined the reducing sugar content in the hydrolysates obtained from alkaline-pretreated rice straw with or without oxygen-radical and cellulase treatments following filter sterilization. Reducing-sugars in the filtrates (10 μL) obtained from alkaline-pretreated rice straw with or without oxygen-radical and cellulase treatments following filter sterilization were also determined by monitoring
post-column derivatized reducing sugars that were sepa-
rated using a Prominance reducing-sugar HPLC analyti-
cal system equipped with a fluorescence detector. The
supernatant was separated on a Shim-pack 4.0 × 250-mm
ISA-07/S2504 column (Shimadzu) with a linear gradi-

tent of 0.1 M potassium borate buffer (pH 8.0) and 0.4 M
potassium borate buffer (pH 9.0) for 120 min at a flow
rate of 0.6 mL min⁻¹ [30, 63, 64].

Supplementary information
Supplementary information accompanies this paper at https://doi.
org/10.1186/s13068-020-1655-9.

Additional file 1: Figure S1. Treatment-time-dependent conversion of
vanillin (5.0 mM) and the production of reactants monitored by HPLC.
Identified reaction products are marked by arrows with numbers and
shown in Table 1. Figure S2. MS analysis of the trimethylsilyl (TMS)
derivatives among the reaction products generated from vanillin by
oxygen-radical treatment. Each number indicates the GC peaks shown in
Fig. 1a and Table 1. Figure S3. Vanillin oxidation, monoxygenation,
demethylation, decarboxylation, and aromatic-ring fission by oxygen-
radical irradiation. Each number indicates the GC peaks shown in Fig. 1b
and Table 1. Figure S4. Effects of vanillin degradation products on the
growth of S. cerevisiae. The yeast was grown in YPD medium supple-
mented with 2.5 mM vanillin degradation products, such as vanillic acid,
protocatechuic aldehyde, protocatechuate, methoxyhydroquinone,
3,4-dihydroxy-5-methoxybenzaldehyde, and oxalic acid. Yeast growth
was monitored by measuring optical density at 600 nm. Error bars
represent the mean ± standard error of the mean of three independent
experiments. Figure S5. Effects of several compounds generated from
alkaline-pretreated rice straw with or without oxygen-radical treatment
on the growth of S. cerevisiae. The yeast was grown in YPD medium
supplemented with 2.5 mM p-coumaric acid, t-ferulic acid, lactic acid,
and furfural. Yeast growth was monitored by measuring optical density
at 600 nm. Error bars represent the mean ± standard error of the mean
of three independent experiments. Figure S6. The content of glucose, cel-
lobiose, cellobiose, and xylene in alkaline-pretreated rice straw slurry with
or without oxygen-radical and cellulase treatments. Sugars released from
alkaline-pretreated rice straw after enzymatic hydrolysis using commer-
cially available cellulase from A. niger were quantified by reducing-sugar
HPLC. Data are presented as the mean ± standard deviation of three
experiments. Figure S7. Effects of oxygen-radical treatment of glucose on
the growth of S. cerevisiae. (a) TLC analysis of 10, 25, and 50 mM glucose
solutions irradiated with oxygen-radical treatment for 0 min (−) and 20
min (+). The procedure of TLC analysis was described previously [30]. (b)
The yeast was grown in 50 mM glucose medium containing yeast extract
(at a final concentration of 1%) and peptone (at a final concentration of
2%) with or without oxygen-radical treatment. Yeast growth was moni-
tored by measuring optical density at 600 nm. Error bars represent the
mean ± standard error of the mean of three independent experiments.

Figure S8. Schematic diagram of sample preparation for oxygen-radical

treatment. (a) Radical-treatment conditions were optimized to obtain
maximal atomic oxygen (O (3P j)) [1, 12]. All samples were suspended in 3-mL
solutions, and a fixed distance of 1 cm was used between the slit exit of
the radical generator and the surface of the liquid suspension. (b) Flow
chart of sample preparation used in this study for ethanol production
by S. cerevisiae using alkaline-pretreated rice straw slurry with or without
oxygen-radical and cellulase treatments.

Abbreviations
NTAP: non-thermal atmospheric pressure plasma; YPD: yeast-extract–pep-
tone–dextrose; GC–MS: gas chromatography–mass spectrometry; HPLC: high-
performance liquid chromatography; CBHs: cellobiohydrolases.

Acknowledgements
We would like to thank Editage (www.editage.jp) for English-language editing.

Authors’ contributions
SI, VG, KS, and MS performed the experiments; KS, MI, MK, and MS designed
the experiments; VG, MI, and MH optimized the oxygen-radical generator; and
SI, KS, MI, MK, and MS wrote the manuscript. All authors read and approved
the final manuscript.

Funding
This work was supported by a Grant-in-Aid for Scientific Research (19K05802
and partially supported by a grant (H30) from Toyoko Scholarship Founda-
tion, a research grant (H31) from The Yanmar Environmental
Sustainability Support Association and a MEXT-Supported Program for the
Strategic Research Foundation at Private Universities (S1511021).

Availability of data and materials
All data generated or analysed during this study are included in this published
article.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 29 October 2019   Accepted: 12 January 2020
Published online: 28 January 2020

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