

## A Microscale Protocol for Alkaline Nitrobenzene Oxidation of Lignins Using a Readily Available Reactor

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### Abstract

Nitrobenzene oxidation of lignin has long been one of the typical lignin chemical degradation methods. Previously, we reported a high-throughput and microscale protocol for the method. However, the reactor used in the protocol was available only in Japan and not supported outside Japan. In this study, we prepared an alternative protocol of alkaline nitrobenzene oxidation using an alternative reactor, which is available in many countries. Using the new protocol almost the same product yields as the previous protocol were obtained using a stable-isotope-dilution method. In addition, ethylvanillin, which is readily and commercially available, was also found to be usable as an alternative internal standard.

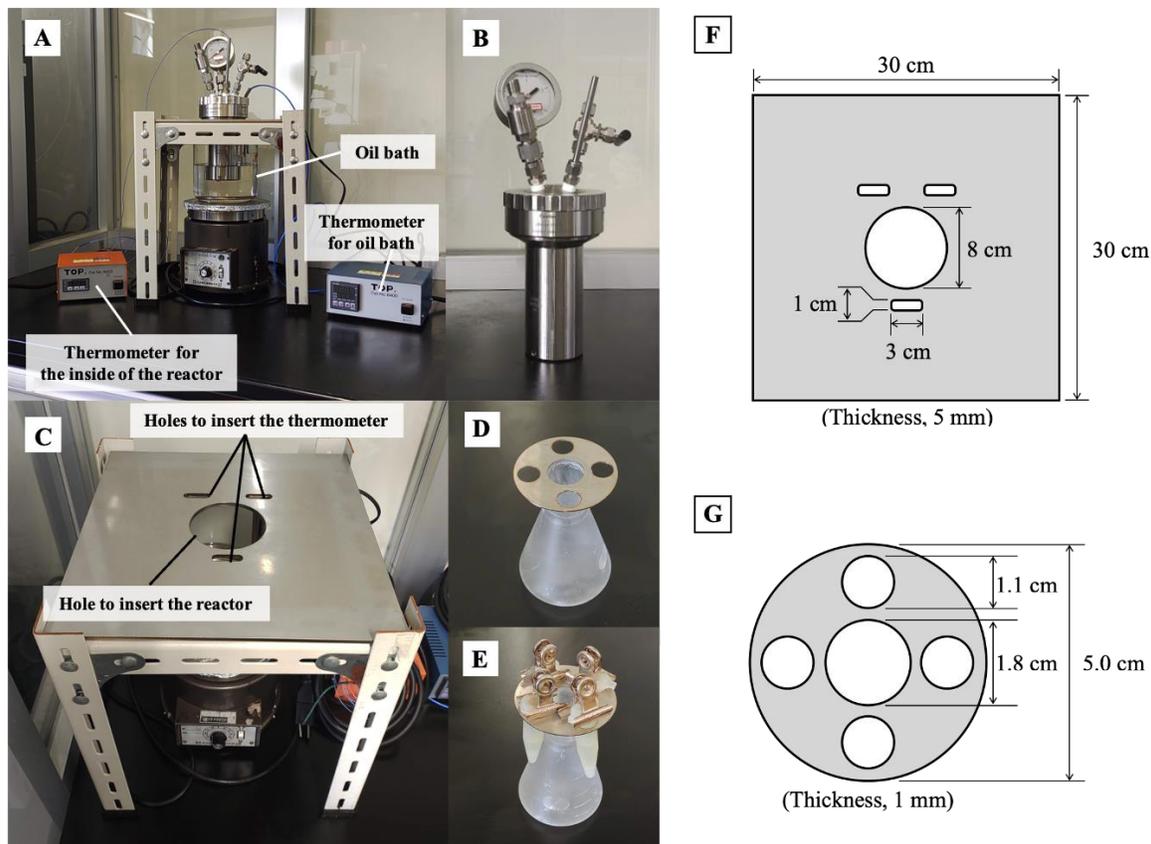
**Keywords:** Alkaline nitrobenzene oxidation; Lignin; Microscale protocol; Stable-isotope dilution

Recently, in the bioeconomy era, renewable resources have become increasingly important for establishing a sustainable society. Among the renewable resources, lignocellulosic resources are the most abundant, and their importance is therefore becoming increasing. As a basis for utilization of lignocellulose resources, characterization of lignin is important. During last decade, lignin analysis by multi-dimensional NMR (nuclear magnetic resonance spectrometry) technique has made great progress, while conventional chemical degradation analysis of lignin is still important for lignin structural characterization because it can provide information on lignin aromatic structures at low cost, quantitatively, and at high throughput.

Nitrobenzene oxidation of lignin has long been one of the basic lignin chemical degradation methods<sup>1,2</sup>. In the conventional method for nitrobenzene oxidation<sup>2</sup>, samples are reacted in stainless-steel vessels at 170°C in an oil bath. The vessels are sealed tightly with a screw cap fitted with a Teflon gasket. However, this process of sealing the vessel is problematic and often fails, leading to reactant leakage. To solve this problem, the conventional protocol<sup>2</sup> has been improved several times<sup>3-5</sup>. Katahira and Nakatubo employed an autoclave (Metal Reactor TEM-D 1000M; Taiatsu Techno Co., Ltd., Tokyo, Japan) as a reactor<sup>4</sup>. Later, we modified the method of Katahira and Nakatubo<sup>4</sup> and proposed a microscale and high-throughput protocol using stable-isotope internal standard<sup>3</sup>. However, this autoclave is not supported outside Japan and an instruction manual in English is not supplied. In this study, therefore, a new protocol using an alternative autoclave (Miniclave SUS 200 ml, Büchi-Glas-Uster, Uster, Switzerland; Fig. 1A and 1B), which is available in Japan and many countries in Europe, Africa, Asia, Oceania, and America, was developed.

Fig. 1A shows an overview of the system. The autoclave reactor (Fig. 1B) was inserted into a top plate (30 cm × 30 cm × 5 mm; diameter of the hole to insert the reactor,  $\phi$  8 cm; Fig. 1F), which was placed on a steel stand (Fig. 1C). The bottom of the reactor was deeply immersed in a silicone oil bath (Fig. 2B). A 50-ml Erlenmeyer flask with reaction tube folder (disk size,  $\phi$  5 cm; center hole,  $\phi$  1.8 cm; side hole,  $\phi$  1.1 cm; Fig. 1G) was placed in the miniclave SUS containing total 100 ml of distilled water (Fig. 1D, 1E and 2A). In this study, high-throughput microscale protocols using TEM-D

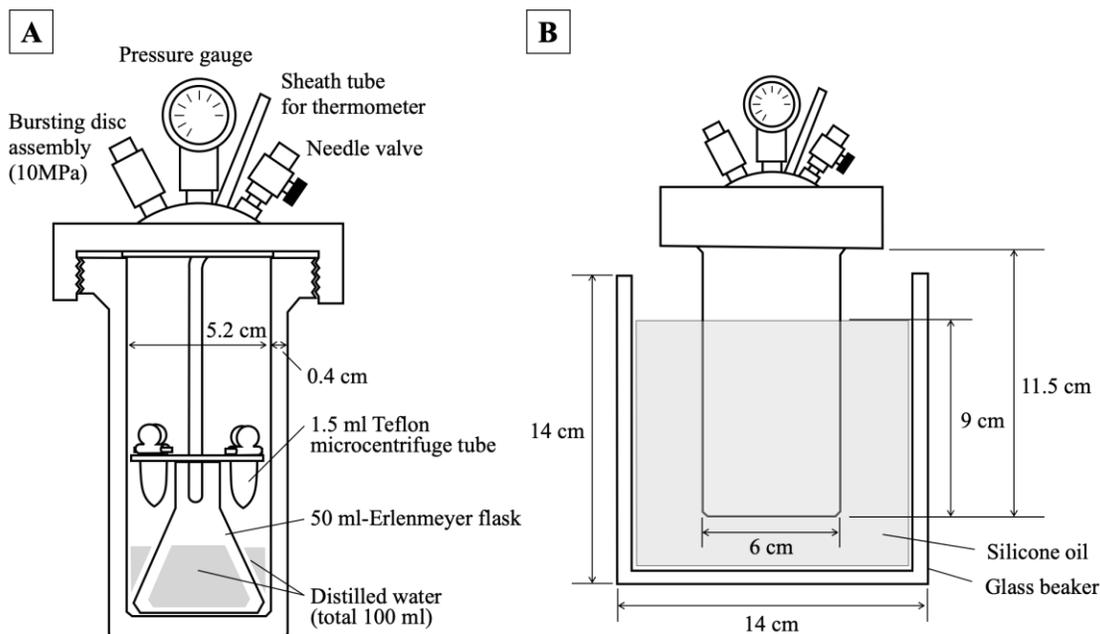
reactor<sup>3)</sup> and miniclave SUS (the present study) were referred to as proTEMd and proSUS, respectively. On the basis of the reaction condition with proTEMd (170°C, 0.7 MPa), the proSUS was optimized by measuring temperature and atmospheric pressure inside the miniclave SUS. By setting temperature of oil bath to 197°C, the environment inside the device reached 170°C (0.7 MPa) from room temperature (about 25°C) in about 1 hour after the start of heating (Fig. 3). If the amount of silicone oil (Shin-Etsu silicone, Cat.no. KF-96-100CS; Shin-Etsu Chemical Co., Ltd.) is low or the reactor is not deeply immersed (Fig. 2B), it will take more time to reach the set temperature.



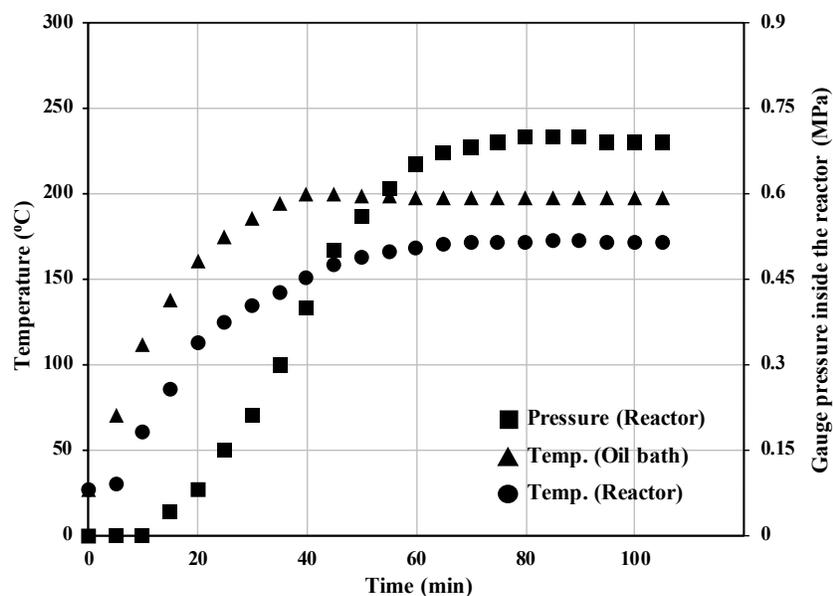
**Fig. 1.** Equipment for the new protocol of alkaline nitrobenzene oxidation.

(A) Overview of reaction equipment, (B) microclave SUS 200ml (Büchi Glas Uster), (C) steel stand and top plate for placing the reactor, (D) Tube holder on a 50-mL Erlenmeyer flask. (E) illustrates how the reaction tubes are placed in the holder. Design drawings of a top plate (F) and reaction tube holder (G).

To compare yields of products formed with proSUS under these conditions with those of proTEMd, extractive-free powders of acacia (sapwood) and sorghum (stem) were individually subjected to alkaline nitrobenzene oxidation with the protocols summarized in Table 1. A reaction and work-up process with proSUS was conducted exactly as the same manner as that of the proTEMd<sup>3)</sup>. Briefly, 10 mg of dried sample was placed into a 1.5 ml Teflon microcentrifuge tube (Flonchemical Co., Ltd.; 1.5-ml PFA sampling cup, Cat. no. 1322-02). NaOH (2M, 500  $\mu$ l) and nitrobenzene (30  $\mu$ l) were added to the tube and the resulting suspension was mixed by vortexing. The lid of the tube was clamped using a stainless-clip (KOKUYO Co., Ltd.; Medama Clip, Cat. no. Kuri-17B, Fig. 1E) to prevent the lid from opening. The reaction mixture in the tube was heated and pressurized at 170°C and 0.7 MPa for 1h in the autoclave reactor. After the reactor was cooled to about 100°C, the reaction tube was taken from the reactor and cooled on ice for 5 min. The internal standard was added to the reaction mixture and this solution was mixed by vortexing and centrifuged (2,000  $\times$  g). The supernatant of the total



**Fig. 2.** The autoclave reactor during reaction.  
 (A) Inside of miniclave SUS 200ml, (B) Position of miniclave SUS in oil bath.



**Fig. 3.** Time course of temperature and pressure during a reaction in proSUS.  
 Square, triangle, and circle symbols indicate gauge pressure of reactor inside, temperatures of the oil bath and reactor interior, respectively.

reaction mixture (250  $\mu$ l) was transferred to a 1.5-ml polypropylene microcentrifuge tube and extracted with ethyl acetate (200  $\mu$ l  $\times$  4). The aqueous layer was transferred to a new 1.5-ml polypropylene microcentrifuge tube, acidified to pH 2-3 by adding 60  $\mu$ l of 6 N HCl, and extracted with ethyl acetate (200  $\mu$ l  $\times$  3). The combined organic solutions were washed with a saturated aqueous NaCl solution, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and a portion (20  $\mu$ l) of this solution was evaporated under a high-vacuum.

The residue was dissolved in 8  $\mu$ l of *N,O*-bis(trimethylsilyl)acetamide (BSA) and left standing at 60°C for 45 min; then 0.8  $\mu$ l of BSA solution was subjected to gas chromatography-mass spectrometry (GC-MS) after trimethylsilylation. The yields of products (*p*-hydroxybenzaldehyde, vanillin, and syringaldehyde) were determined using a stable-isotope-dilution method by calculating the ratio between ion intensities corresponding to the MW of TMS ethers of each product (trimethylsilyl *p*-hydroxybenzaldehyde, MW 194.3; trimethylsilyl vanillin, MW 224.3; trimethylsilyl syringaldehyde, MW 254.4) and that of stable-isotope-labeled aldehydes (siAlds: trimethylsilyl [*ring*-<sup>13</sup>C<sub>6</sub>]-*p*-hydroxybenzaldehyde, MW 200.3; trimethylsilyl vanillin-*d*<sub>3</sub>, MW 227.4; trimethylsilyl syringaldehyde-*d*<sub>3</sub>, MW 257.4) as internal standards. Fig. 4 shows product yields formed with both protocols (proTEMd/siAlds and proTEMd/siAlds). The product yield obtained by the reaction with proSUS/siAlds was similar to that of proTEMd/siAlds in the reactions using acacia and sorghum. This result strongly suggests that the reaction with proTEMd can be reproduced by using proSUS.

**Table 1.** Comparison of alkaline nitrobenzene oxidation protocols.

	Katahira and Nakatsubo's method <sup>4,5)</sup>	High-throughput microscale protocol <sup>3)</sup> (proTEMd)	Microscale protocol (The present study; proSUS)
Oxidation reaction			
Vessel	18 ml Teflon tube	1.5 ml Teflon microcentrifuge tube	1.5 ml Teflon microcentrifuge tube
Amount of sample	50–100 mg	5–10 mg	5–10 mg
Reaction device	Metal Reactor TEM-D 1000M (Taiatsu Techno Co., Ltd.)	Metal Reactor TEM-D 1000M (Taiatsu Techno Co., Ltd.)	Miniclave SUS 200ml (Büchi Glas Uster)
Reaction conditions	170°C, 1h (Oil bath)	170°C, 1h (Band heater)	170°C, 1h (Oil bath)
Internal standard	5-Iodovanillin, acetovanillone	Stable isotope-labelled aldehydes (vanillin- <i>d</i> <sub>3</sub> and syringaldehyde- <i>d</i> <sub>6</sub> ), or acetovanillone	Stable isotope-labelled aldehydes ([ <i>ring</i> - <sup>13</sup> C <sub>6</sub> ]- <i>p</i> -hydroxybenzaldehyde, vanillin- <i>d</i> <sub>3</sub> and syringaldehyde- <i>d</i> <sub>6</sub> ), or ethylvanillin
Work up	Extraction with ethylacetate three times using a separatory funnel	Extraction with ethylacetate three times using 1.5 ml polypropylene microcentri- fuge tube, vortex and mini-centrifuge	Extraction with ethylacetate three times using 1.5 ml polypropylene microcentri- fuge tube, vortex and mini-centrifuge
	Solvent (about 30 ml) evaporation with a rotary evaporator	Solvent (about 0.5 ml) evaporation with a diaphragm pump or centrifugal concentrator	Solvent (about 0.5 ml) evaporation with a diaphragm pump or centrifugal concentrator
Throughput	4 samples / 8h	40–60 samples / 8h	4 samples / 8h

In general, stable-isotope-labeled compounds are not commercially available. Therefore, alternative internal standard compounds that are commercially available may be useful for determining the yield of a reaction product. Although we previously used acetovanillone as an internal standard <sup>3)</sup>, it has been reported that this compound was released as a product of alkaline nitrobenzene oxidation from some plants <sup>6,7)</sup>. Therefore, in this study, 300 nmol of ethylvanillin [3-ethoxy-4-hydroxybenzaldehyde, CAS no. 121-32-4; *m/z* 238.4 (TMS ether)] was used as an alternative internal standard, and yields of products were calculated from the ratio of ion intensity of *m/z* 238.4 (internal standard TMS ether) and *m/z* 194.3, 224.3, or 254.4 (product TMS ether). For preparation of calibration curve for each product, *p*-hydroxybenzaldehyde (0, 10, 25, 50, 100, or 200 nmol), vanillin (0, 75, 150, 300, 500, or 1000 nmol), or syringaldehyde (0, 75, 150, 300, 500, or 1000 nmol) was added to 500  $\mu$ l of 2M NaOH containing ethylvanillin (300 nmol), respectively. Moreover, the work-up and GC-MS analysis were performed in the same manner as proSUS and proTEMd. The calibration curve for each product is as follows:

$$Y_H = 0.348X_H \quad (0 \leq X_H \leq 1.71), \text{ for } p\text{-hydroxybenzaldehyde};$$

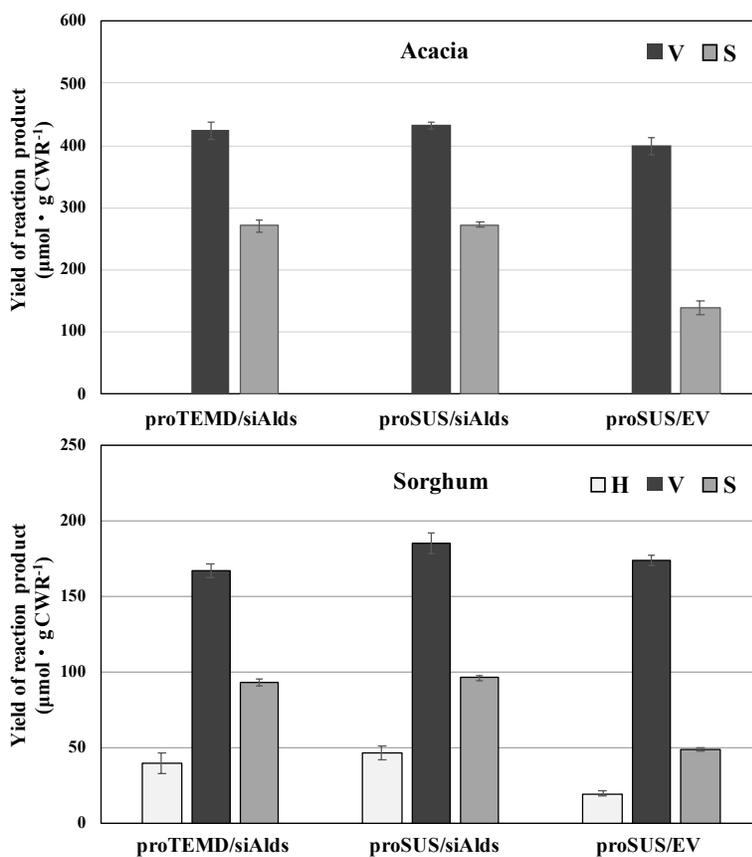
$$Y_V = 1.446X_V \quad (0 \leq X_V \leq 2.45), \text{ for vanillin};$$

$$Y_S = 0.408X_S \quad (0 \leq X_S \leq 8.58), \text{ for syringaldehyde};$$

$$Y = \text{molar ratio of each aldehyde to ethylvanillin};$$

$$X = \text{intensity ratio of ions corresponding to MW of each aldehyde TMS ether } (m/z \text{ 194.3, 224.3, or 254.4}) \text{ to that of ethylvanillin TMS ether } (m/z \text{ 238.4}).$$

The yield of *p*-hydroxybenzaldehyde and syringaldehyde in proSUS using ethylvanillin (proSUS/EV) were lower than those with proTEMd/siAlds, while that of vanillin was almost same in both protocols, proSUS/EV and proTEMd/siAlds (Fig. 4). It is considered that the product yields in proSUS/EV decreased because the extraction efficiency of the reaction product or absorption level onto extraction tools (*e.g.*, polypropylene tube) during work-up differs depending on the compound, clearly indicating the advantage of stable-isotope-dilution methods. The relative decrease of product yields was almost the same between the two plant materials; the reduction of *p*-hydroxybenzaldehyde and syringaldehyde was  $58.6 \pm 6.0\%$  and  $48.5 \pm 2.1\%$ , respectively. Therefore, the yields in proSUS/siAlds can be estimated by multiplying the yields in proSUS/EV using each factor for reaction product (2.42 for *p*-hydroxybenzaldehyde, 1.06 for vanillin, and 1.94 for syringaldehyde).



**Fig. 4.** Yields of reaction products obtained by alkaline nitrobenzene oxidation. H, *p*-hydroxybenzaldehyde; V, vanillin; S, syringaldehyde.

## CONCLUSIONS

1. A new microscale protocol (proSUS) for alkaline nitrobenzene oxidation using an autoclave that can be purchased in many countries was developed and showed the same reaction efficiency as the previous protocol (proTEMd).
2. Using the factors for each reaction product, the yield of the reaction product determined using stable-isotope-dilution method can be estimated even in the reaction using ethylvanillin as an internal standard.

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