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Increasing cellulosic ethanol production by enhancing phenolic tolerance of *Zymomonas mobilis* in adaptive evolution

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HIGHLIGHTS

- Cellulosic ethanol production of *Z. mobilis* was improved by adaptive evolution.
- *ZMO3_RS07160* played the key role in the improved phenolic aldehydes tolerance.
- Overexpression of *ZMO3_RS07160* enhanced cellulosic ethanol fermentability.

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ABSTRACT

Cellulosic ethanol fermentability of ethanologenic strain *Zymomonas mobilis* is severely inhibited by phenolic aldehydes generated from lignocellulose pretreatment. Here, a 198 days' laboratory adaptive evolution of *Z. mobilis* 8b in corn stover hydrolysate was conducted to increase its phenolic aldehydes tolerance and ethanol fermentability. The obtained *Z. mobilis* Z198 demonstrated a significantly improved conversion of the most toxic phenolic aldehyde (vanillin) by 6.3-fold and cellulosic ethanol production by 21.6%. The transcriptional analysis using qRT-PCR revealed that the gene *ZMO3_RS07160* encoding SDR family oxidoreductase in *Z. mobilis* Z198 was significantly up-regulated by 11.7-fold. The overexpression of *ZMO3_RS07160* in the parental *Z. mobilis* increased the ethanol fermentability to that of the adaptively evolved strain *Z. mobilis* Z198. This study provided a practical method to obtain a robust cellulosic ethanol fermenting strain, and a candidate gene for synthetic biology of biorefinery strains with strong phenolic aldehydes tolerance.

1. Introduction

Pretreatment is the inevitable step to overcome the biocalcitrance of lignocellulose, but various inhibitory compounds are also generated in this step (Yang and Wyman, 2008). Weak acids, furan aldehydes, and phenolics are derived from lignocellulose pretreatment as inhibitors to microbes (Ran et al., 2014). Among them, the phenolic aldehydes are the most stubborn inhibitors to be removed because of the wide derivations, low water solubility, and slow biodegradability (Qiu et al., 2020; Yan et al., 2019). Biodetoxification is a highly efficient and clean method on removing the lignocellulose derived inhibitors (Yi et al., 2019), but its degradation efficiency on phenolic aldehydes is relatively low and the prolonged biodetoxification leads to the increased loss of xylose sugar. Residual phenolic inhibitors after biodetoxification still show strong inhibition on ethanologenic strains such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Xie et al., 2016; Larsson et al., 2001).

Microbial robustness is a phenotype of microorganisms composed of various intracellular interactions and interlaced responses, making it difficult for rational design. Therefore, the rational design method on microorganisms may not be an effective way on changing this complicated property (Wang et al., 2016; Tan et al., 2015). On the other hand, laboratory adaptive evolution method may provide a practical option for improving microbial robustness to specific selection pressure, and also the genes responsible for laboratory adaptive evolution can be mined for further metabolic engineering (Kurosawa et al., 2015; Dunn and Rao, 2015; Jin et al., 2019). *Z. mobilis* is an anaerobic gram-negative ethanologenic strain for cellulosic ethanol fermentation with a lipopolysaccharide coating membrane and rich oxidoreductase enzymes (Zhang et al., 1995; Wang et al., 2017). Among phenolic inhibitors, *Z. mobilis* is highly susceptible to phenolic aldehydes, and less susceptible to the corresponding alcohols and acids derivatives (Franden et al., 2009). This unique property could be gradually upgrade inhibitor tolerance of

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Z. mobilis in adaptive evolution under the stress of phenolic aldehydes (Yi et al., 2015; Gu et al., 2015).

This study demonstrated a long-term adaptive evolution of *Z. mobilis* 8b in corn stover hydrolysate with the existence of phenolic aldehydes while furan aldehydes and weak acids were removed by biodetoxification. The finally obtained *Z. mobilis* strain was used for high corn stover solids loading simultaneous saccharification and co-fermentation, and both ethanol production and xylose utilization were significantly improved. Further analysis of qRT-PCR was used to mine the underlying mechanism of the improved fermentability after adaptive evolution. One key gene encoding SDR family oxidoreductase was identified by qRT-PCR essentially responsible for the enhanced phenolic tolerance and ethanol fermentability. This work provided not only a practical tool for improving fermentability of ethanologenic strains, but also an important gene for stronger robustness of general biorefinery fermentation strains.

2. Materials and methods

2.1. Strains and media

Z. mobilis 8b was obtained from ATCC (Manassas, VA, USA) and cultured in rich medium in rich medium (RM) (10 g/L yeast extract, 20 g/L glucose and 2 g/L KH_2PO_4) at 30 °C, without shaking. *Amorphotheca resinae* ZN1 was isolated and cultured on PDA slant and pretreated corn stover feedstock according to He et al. (2016) and Zhang et al. (2010b).

2.2. Reagents and enzymes

Cellic CTec 2.0 from Novozymes China (Beijing, China) was used for enzymatic hydrolysis with 205.6 FPU filter paper activity and 5,144 CBU per mL cellobiase activity (Ghose, 1987; Adney and Baker, 1996). The protein concentration (79.9 mg/mL) was measured according to Bradford (1976).

Phenolic aldehydes and yeast extract were purchased from local suppliers.

2.3. Raw material and its biorefinery processing

The composition of corn stover was measured with 35.6 ± 0.3% (w/w) of cellulose, 3.6 ± 0.4% (w/w) of hemicellulose, and 10 ± 0.1% (w/w) of ash by NREL LAP protocol (Sluiter et al., 2012). The pretreatment of corn stover was conducted according to Zhang et al. (2011). The inhibitors in the pretreated corn stover included 5.78 ± 0.01 mg/g dry matter (DM) of furfural, 3.52 ± 0.01 mg/g DM of 5-hydroxymethylfurfural (HMF), and 23.00 ± 1.01 mg/g DM of acetic acid. The biodetoxification by *A. resinae* ZN1 followed the protocol in He et al. (2016) and Zhang et al. (2010b). After biodetoxification, weak acid and furan aldehydes were totally removed; and partial phenolic aldehydes were removed with the residual content of 0.62 ± 0.12 mg vanillin, 0.12 ± 0.01 mg 4-hydroxybenzaldehyde, and 1.53 ± 0.13 mg syringaldehyde per gram of dry matter (DM).

The preparation of corn stover hydrolysate at 25% (w/w) solids loading was performed according to Wang et al. (2016). Briefly, the pretreated and biodetoxified corn stover was enzymatically hydrolyzed at 25% (w/w) solids loading with the enzyme dosage of 10 mg total cellulase protein per gram of cellulose. The corn stover hydrolysate was centrifuged at 10,000 rpm for 10 min to remove the insoluble solids and then used for adaptive evolution and flask fermentation.

2.4. Adaptive evolution and fermentation

Adaptive evolution of *Z. mobilis* 8b was conducted in 100 mL flask containing 20 mL corn stover hydrolysate by successive transfer every 24 h and repeatedly performed for 198 days. The inoculation ratio was 10% (v/v) for the initial 17 transfers and 5% (v/v) for the rest of the evolution culture. No shaking was done during the adaptive evolution. After 198 days' adaptive evolution, for the xylose utilization of the adaptive strain declined, a 21 days' adaptive evolution culture was conducted in the modified RM medium with 40 g/L xylose, and transferred every 3 days to enhance the xylose consumption capacity of the adaptive *Z. mobilis*.

Simultaneous saccharification and co-fermentation (SSCF) was conducted according to Zhang et al. (2010a). Briefly, SSCF was performed under 30% (w/w) solids loading using biodetoxified corn stover in 5L fermenter with enzyme dosage of 10 mg total cellulase protein per gram of cellulose. Pre-hydrolysis for 12 h at 50 °C and pH 4.8, and then SSCF at 30 °C and pH 5.8 by automatic feeding of 4 M KOH. 10 g/L yeast extract and 2 g/L KH_2PO_4 were added as the fermentation nutrients.

2.5. Analysis of glucose, xylose, ethanol and inhibitors

Glucose, xylose, ethanol, and inhibitors were analyzed according to Zhang et al. (2010a) and He et al. (2016).

2.6. Ethanol yield calculation

Xylose conversion ratio and ethanol yield (%) was calculated according to Zhang and Bao (2012).

2.7. qRT-PCR and DNA sequencing

The parental *Z. mobilis* 8b and the adaptive *Z. mobilis* Z198 strains were cultured and collected at 24 h at pH 5.5 and 30 °C in corn stover hydrolysate. RNA extraction and quantification, reverse transcription reactions, and qRT-PCR were carried out according to Yi et al. (2015).

Genomic DNA of *Z. mobilis* 8b and *Z. mobilis* Z198 was extracted according to maker's protocol. The gene fragment of *ZMO_RS07160* containing the total gene and its promotor was amplified from the genomic DNA, and then sequenced by Generay (Shanghai, China).

2.8. Construction of Z. mobilis recombinants

Shuttle vector pHW20a was used for the plasmid construction and transformation according to Dong et al. (2011) and Xia et al. (2018). The *Z. mobilis* recombinant was tested using corn stover hydrolysate and the biodetoxified corn stover by SSCF. The seed culture was conducted according to Yan et al. (2019). Tetracycline was added at 20 μg/mL.

3. Results and discussions

3.1. Adaptive evolution and fermentation evaluation of Z. mobilis 8b

The adaptive evolution of *Z. mobilis* 8b in corn stover hydrolysate was conducted by continuous transfer every 24 h. The corn stover hydrolysate still contained 0.38 g/L syringaldehyde, 0.03 g/L 4-hydroxybenzaldehyde, and 0.16 g/L vanillin after acetic acid and furan inhibitors were totally removed by biodetoxification. The cell growth, ethanol production, and glucose consumption steadily increased with the adaptive evolution for 198 days (approximately 9.6×10^3 cell divisions) until the fermentation performance was stable (Fig. 1) and the finally obtained adaptive strain was designated as *Z. mobilis* Z198.

The phenolic aldehydes conversion of the adaptive *Z. mobilis* Z198 was evaluated using the parental *Z. mobilis* 8b as control (Fig. 2). The vanillin conversion of the adaptive *Z. mobilis* Z198 was increased by approximately 6.3-fold comparing to the parental *Z. mobilis* 8b, while the conversion of 4-hydroxybenzaldehyde and syringaldehyde by the adaptive *Z. mobilis* Z198 were increased by 13% and 10%, respectively. Among the three representative phenolic aldehyde inhibitors, 4-hydroxybenzaldehyde was a strong inhibitor to *Z. mobilis* but its low content lessened its inhibition (Yi et al., 2015). Syringaldehyde was a weak inhibitor even a relatively high content (~1 g/L) to *Z. mobilis* (Gu et al.,

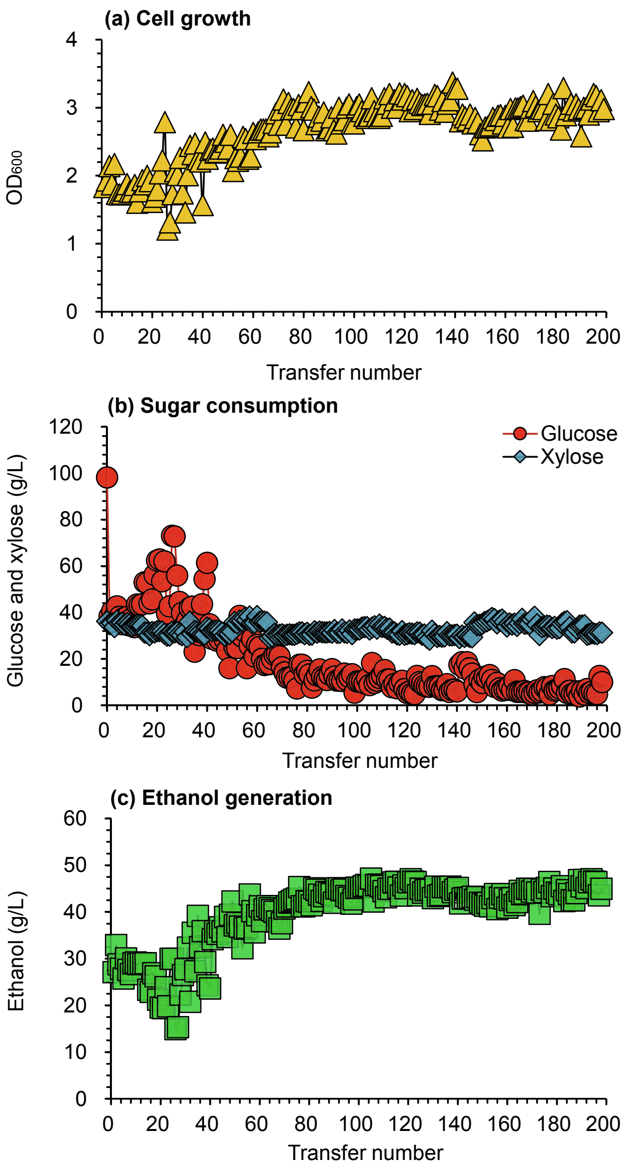

Fig. 1. Adaptive evolution of *Z. mobilis* 8b in corn stover hydrolysate. The transfer was conducted every 24 h at 30 °C without shaking. The inoculation ratio was 10% (*v/v*) for the initial 17 transfers and 5% (*v/v*) for the rest of the evolution culture.

2015). On the other hand, vanillin was a strong inhibitor not only because of its high toxic capacity to *Z. mobilis* but also for its relative high content in pretreated lignocellulose feedstock (Yi et al., 2015).

Cellulosic ethanol fermentability of *Z. mobilis* Z198 was evaluated by SSCF using biodetoxified corn stover under high solids loading (Fig. 3). The high solids loading correspondingly led to the high phenolic aldehydes content and the increased phenolic stress. The lag phase time of glucose consumption by the adaptive *Z. mobilis* Z198 was reduced to half of the parental *Z. mobilis* 8b. Ethanol production rate of *Z. mobilis* Z198 was 0.91 g/L/h, 21.3% higher than that of *Z. mobilis* 8b. Xylose utilization and ethanol titer of *Z. mobilis* Z198 were improved by 1.8-fold and 21.6%, respectively. Since phenolic aldehydes were the only inhibitors to *Z. mobilis* in the biodetoxified corn stover, the strengthened phenolic aldehydes conversion of *Z. mobilis* Z198 could be the major reason of the improved sugar consumption and ethanol fermentation.

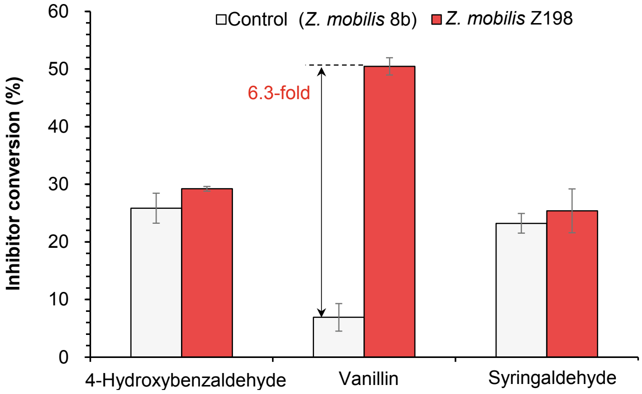

Fig. 2. Conversion of phenolic aldehyde inhibitors by the parental and adaptive *Z. mobilis*. The rich medium was separately amended with 0.3 g/L 4-hydroxybenzaldehyde, 1 g/L syringaldehyde, and 0.6 g/L vanillin. One colony of the strain was transferred into a 10 mL tube with 5 mL rich medium. The seed broth was transferred into a 100 mL flask with 20 mL rich medium for 12 h, and then used for inhibitors conversion fermentations with single inhibitor with initial 0.12 of cell density at the wavelength of 600 nm. The conversion ratio was calculated at 12 h after inoculation.

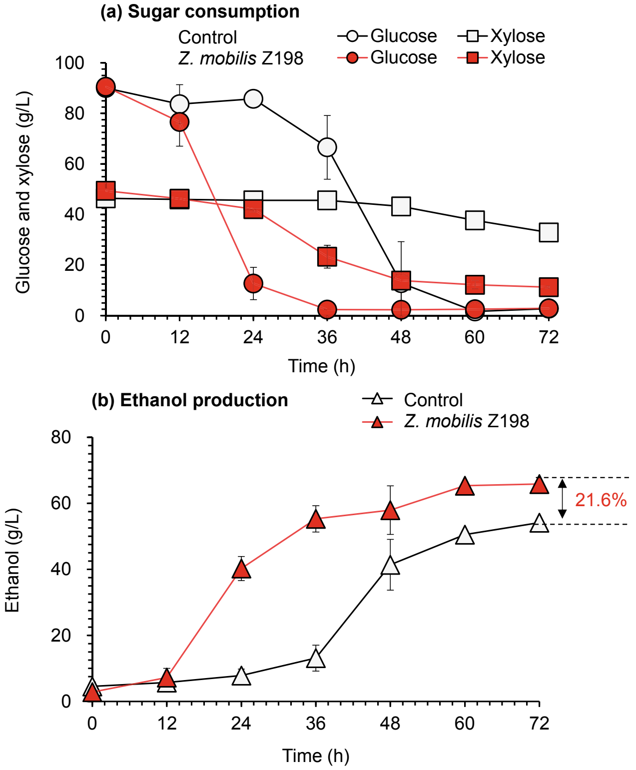

Fig. 3. Simultaneous saccharification and ethanol co-fermentation (SSCF) of corn stover by the adaptive *Z. mobilis* Z198. (a) Sugars consumption; (b) Ethanol production. The parental *Z. mobilis* 8b was used as the control of the adaptive *Z. mobilis* Z198. SSCF was performed using biodetoxified corn stover, in which the total cellulose content was 10.5% (w/w) and the total xylan content was 4.5% (w/w).

3.2. Transcriptional analysis and overexpression of the key gene

qRT-PCR were conducted on the genes related with the phenolic aldehydes conversion and ethanol production of the adaptive *Z. mobilis* Z198 and its parental *Z. mobilis* 8b cultured in corn stover hydrolysate (Fig. 4). The genes were selected based on the genome annotation (Gene

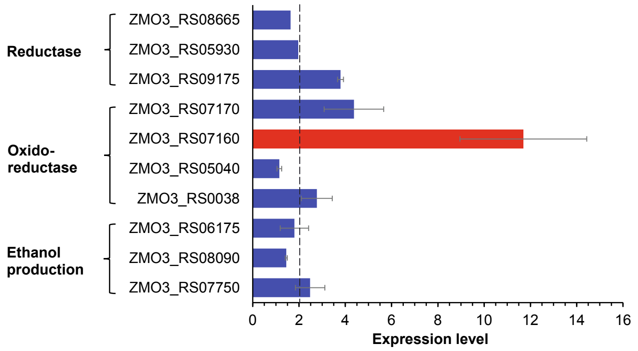

Fig. 4. Genes response to inhibitor tolerance and ethanol production in the adaptive *Z. mobilis* Z198 by qRT-PCR. The fold change of the regulation was defined as the mean expression level in the adaptive *Z. mobilis* Z198 and normalized to the parental *Z. mobilis* 8b. Cells were harvested at 24 h when culturing in corn stover hydrolysate. Three independent experiments were conducted and indicated with the error bars.

Bank accession number NZ_CP023682) and our previous study (Yi et al., 2015), including (i) three reductases *ZMO3_RS09175* (encoding aldo/keto reductase), *ZMO3_RS08665* (encoding alkene reductase), and *ZMO3_RS05930* (encoding pyrroline-5-carboxylate reductase); (ii) four oxidoreductases *ZMO3_RS0038* (encoding SDR family oxidoreductase), *ZMO3_RS05040* (encoding NAD(P)-dependent oxidoreductase), *ZMO3_RS07160* (encoding SDR family oxidoreductase), and *ZMO3_RS07170* (encoding SDR family oxidoreductase); (iii) three genes on ethanol synthesis pathway *ZMO3_RS07750* (encoding zinc-binding alcohol dehydrogenase), *ZMO3_RS08090* (encoding iron-containing alcohol dehydrogenase), and *ZMO3_RS06175* (encoding pyruvate decarboxylase), which were also responsible for phenolic aldehydes conversion (Wang et al., 2017).

The results show that one reductase *ZMO3_RS09175* encoding aldo/keto reductase demonstrated approximately 3-fold up-regulation, and two SDR family oxidoreductases *ZMO3_RS07160* and *ZMO3_RS07170* were up-regulated 11.7- and 3.4-fold, respectively. The *ZMO3_RS07160* gene with the maximum up-regulation among selected genes was further investigated for the improved phenolic aldehydes tolerance. The gene blasting result showed that the complete sequence of *ZMO3_RS07160* starting from the promoter region in the adaptive *Z. mobilis* Z198 and the parental 8b had no point mutation, thus the regulation change occurred only at the transcription level of the adaptive *Z. mobilis* Z198.

The function of this specific gene *ZMO3_RS07160* for phenolic aldehyde tolerance was further verified by expressing the gene in the parental *Z. mobilis* 8b using the pHW20a plasmid (Dong et al., 2011). The recombinant was fermented both in corn stover hydrolysate (CSH) and by SSCF using biodetoxified corn stover (Fig. 5). The expression of *ZMO3_RS07160* in *Z. mobilis* 8b significantly increased ethanol production by 23.6% in CSH and 21.8% in SSCF. Comparing to the parental *Z. mobilis* 8b, the increase of ethanol production by the *ZMO3_RS07160* overexpressed *Z. mobilis* recombinant, 21.8% in CSH and 23.6% in SSCF, was coincidentally the same with the increase of the adaptive strain *Z. mobilis* Z198, 21.6% (Fig. 4). The result indicated that the regulation of the single gene *ZMO3_RS07160* played an important role in the enhancement of cellulosic ethanol fermentability of *Z. mobilis* strain, and this gene was also pivotal for the improved phenolic aldehyde conversion, especially for the most toxic vanillin. This key gene could be used to improve phenolic aldehydes conversion (especially for vanillin) of other fermentation strains by metabolic engineering.

4. Conclusions

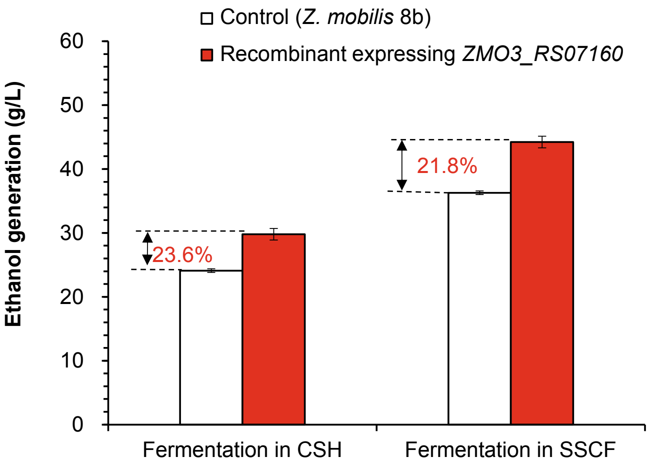

Fig. 5. Ethanol fermentation evaluation of the *Z. mobilis* recombinant expressing the gene *ZMO_RS07160*. *Z. mobilis* recombinant harboring the empty pHW20a was used as the control. The fermentation was conducted for 72 h. The separate hydrolysis and fermentation was conducted in corn stover hydrolysate (CSH) containing phenolic aldehydes. The simultaneous saccharification and co-fermentation (SSCF) was performed using biodetoxified corn stover, in which the total cellulose content was 8.5% (w/w) and the total xylan content was 2.8% (w/w).

The adaptive evolution of *Z. mobilis* strengthened phenolic aldehydes conversion (especially for the most toxic vanillin) by 6.3-fold and cellulosic ethanol production by 21.6% under high solids loading. The up-regulation of *ZMO3_RS07160* encoding SDR family oxidoreductase was considered to be the key factor for the enhanced phenolic aldehydes tolerance. Furthermore, the overexpression of *ZMO3_RS07160* in the parental *Z. mobilis* 8b also upgraded its phenolic inhibitors tolerance and increased the ethanol fermentability to the same level of the adaptive *Z. mobilis* Z198.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.124926.

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