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BIOCONVERSION OF LIGNOCELLULOSIC MATERIALS BY FUNGAL "ENZYME COCKTAIL" WITH THE CONTRIBUTION OF A GLYCOSIDE HYDROLASE FROM *XYLARIA POLYMORPHA* TO RELEASE CARBOHYDRATES AND BIOMETHANOL

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Abstract. A bifunctional glycoside hydrolase GH78 from the ascomycete *Xylaria polymorpha* (*Xpo*GH78) with catalytic versatility to both glycosides and esters, may be beneficial for the efficient degradation of plant cell-wall complexes containing both diverse sugar residues and esterified structures to apply in, for example, the production of biofuels from plant feedstock. Furthermore, the disintegrating effect of enzymatic lignocellulose treatment can be significantly improved by using different kinds of hydrolases and a phenol oxidase. Accordingly, the conversion of rape straw meal with *Xpo*GH78 used in this study was improved in the presence of accessory enzymes, i.e. cellulases, xylanases (Cell&Xyl) and/or laccase (*Xpo*Lac), without any chemical treatment. Synergistic conversion of rape straw resulted in a release of 17.3 mg of total carbohydrates (e.g., arabinose, galactose, glucose, mannose, xylose) per gram substrate after incubating for 72 hrs. Of which, the amount of glucose and galactose increased by 1.5 and 6 times, respectively, compared with commercial hydrolases used alone. Moreover, the treatment of rape straw with *Xpo*GH78 led to a marginal biomethanol release of approx. 17 µg g^{-1} and improved to 270 µg g^{-1} (increased by approx. 16 times) by the cooperation with above mentioned accessory enzymes.

*Keywords***:** Glycoside hydrolase; enzyme cocktail; ascomycetous fungus; *Xylaria polymorpha*; lignocelluloses.

Classification numbers: 1.3.2, 1.4.2.

1. INTRODUCTION

Lignocellulose-containing biomass, the largest renewable reservoir of potentially fermentable carbohydrates produced by plant photosynthesis, has a yearly supply of approximately 200 billion metric tons worldwide, of which lignocellulosic substances account for approximately 60 % of total plant biomass produced on earth [1, 2]. As a raw material for

biotechnological and industrial applications, this huge resource is becoming more and more economically important, not least against the background of intensified utilization of biomass in the sense of the biorefinery concept and the idea of sustainable development [3,4]. Lignocellulose is a major constituent of plant cell-wall polymers, providing rigidity and mechanical stability and protecting plants from microbial attack. The particular properties of lignocellulose are based on the structure of its major components - cellulose (backbone), hemicelluloses (xylan; covering material) and lignin (molecular glue) - which are strongly intermeshed and chemically bonded by non-covalent forces and covalent cross-linkages [5,6]. In addition, pectin is also a major matrix component within the lignocellulose of cell walls, especially in non-woody fibers. However, it is not usually found in woody tissues because secondary wall thickening replaces almost all pectin with lignin [7].

Due to the rigidity and mechanical stability of lignocellulose, it is important to successfully use this biomass to develop enzyme cocktails that will break down plant cell walls into small usable fractions [8]. In addition to well-described oxidative enzymes (e.g., laccase, lignin peroxidase, manganese peroxidase, etc.) which involved in lignocellulose degradation [9 - 11], a number of hydrolytic enzymes belonging to glycoside hydrolases and carbohydrate esterases are needed for a complete conversion [12, 13]. Glycoside hydrolases (GHs; or glycosidases, EC 3.2.1.x) catalyse the hydrolysis of a wide variety of glycosidic linkages between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Although hundreds of glycoside hydrolases from different microorganisms have been identified and biochemically characterized, the search for new glycoside hydrolases with better properties for industrial applications is continuing [8].

In a previous study, we focused on the isolation as well as the catalytic and molecular characterization of a novel GH78 glycoside hydrolase (belonging to the GH78 family by its sequence) from the wood rot ascomycete *X. polymorpha* (designated as *Xpo*GH78) [14]. Thus, *Xpo*GH78 is the first fungal enzyme of the GH family 78 that exhibits alkyl-aryl esterase activity on numerous natural and synthetic esters and enables the soft rot fungus to partially hydrolyze the lignocellulosic complex. Such catalytic versatility combined in one protein with activities towards both glycosides and esters could be beneficial for the efficient degradation of plant cell-wall complexes containing both diverse sugar residues and esterified structures. Based on this research, it would be worthwhile to use this multifunctional hydrolytic enzyme alone and in combination with accessory hydrolases and oxidase ("enzyme cocktail") for the efficient conversion of lignocellulosic materials to carbohydrates and biomethanol.

2. MATERIALS AND METHODS

2.1. Materials

Fungus and enzyme production

The wood decay ascomycete *Xylaria polymorpha* (Pers.) Grev.isolated in Viet Nam (Cuc Phuong National Park) was grown at 23 ^{o}C on the plates of malt extract agar (MA; 20 g malt extract per liter) and stored at 4 ^{o}C in the culture collection of INPC-VAST. The pre-culture was prepared by transferring an agar plug (1 cm) from stock culture onto a new MA plate, which was then incubated at 23 ^{o}C. The 3-week-old pre-cultures were readily served for the subsequent experiments.

For enzyme production on solid-state culture, approx. 2 kg wheat straw was pre-soaked with distilled water overnight and stored in a 10-liter autoclavable plastic bag (H+P Labortechnik, Oberschleiβheim, Germany). After sterilization (121 °C for 30 min), inoculation was performed by using 2 overgrown MA plates of *X. polymorpha* and incubated at 23 °C for 6 - 8 weeks.

Lignocellulosic sources such as birch wood, wheat bran, rape straw, birch wood xylan, beech wood xylan, oat spelt xylan and wheat arabinoxylan, etc. were provided by Sigma-Aldrich (Steinheim, Germany) and Megazyme (Bray, Ireland). The commercially available preparation Ecopulp® X-200 of AB Enzymes (Darmstadt, Germany) is a mixture of different recombinant hydrolases produced by *Trichoderma reesei* (carboxymethylcellulase 1.6 U mg^{-1} and glucuronoxylanase 1 U mg^{-1}; designated as Cell&Xyl). The purified laccase (128 U mg^{-1}) was provided by René Ullrich (TUD-IHIZ).

2.2. Liquid cultivation

First, a minimal medium containing the essentials for fungal growth, e.g., 0.5 g of $MgSO_4$, 1.5 g of KH_2PO_4, 2.0 g of yeast extracts per 1 litre was prepared. Thereafter, 100-ml Erlenmeyer flasks containing 50 ml of the above minimal medium (pH 6.0) each were supplemented with 2 % (w/v) of potential feruloyl esterase-stimulating substances, i.e. different ester- and lignocellulosic sources: birch wood, beech wood, wheat bran, wheat straw, rape straw, birch wood xylan, beech wood xylan, oat spelt xylan, and wheat arabinoxylan as well as triesters: triacetin and olive oil. Afterwards, 5 mL of the suspension was transferred into sterilized liquid medium (121 °C for 30 min) for the incubation at 23 ºC on a rotary shaker (200 rpm).

2.3. Enzyme assay

*Xpo*GH78 activity regarding its feruloyl esterase (FAE) aspect was determined by hydrolytic demethylation of 1 mM methyl ferulate to the ferulic acid in 3-(*N*-morpholino) propanesulfonic acid (100 mM MOPS) buffer at pH 6.0. The reaction was initiated by the incubation of reaction mixtures at 37 ºC for a suitable time depending on enzyme sample (10-30 min) and then terminated by an equal volume of acetic acid/acetonitrile (11.3 %; v/v) as stop solution [15]. After centrifugation, the released ferulic acid was analysed by HPLC as described below. FAE activity was expressed in international units (U) in which one unit was defined as the amount of enzyme required to catalyse the transformation of 1 micromole substrate methyl ferulate per minute (µmol min^{-1}) under above conditions.

2.4. Isolation and purification of *Xpo*GH78

The purification of *Xpo*GH78 basing on its FAE activity was performed by using an FPLC ÄKTA™ system (GE, Freiburg, Germany). The enzymatic extract from *X. polymorpha* culture was applied to different steps of anion-exchange and size-exclusion chromatography as described previously [14].

2.5. Hydrolysis of lignocellulosic materials by an enzyme cocktail containing *Xpo*GH78

Hydrolysis of fine milled rape straw (< 200 µm in size) was investigated by using *Xpo*GH78 and further hydrolases (Cell&Xyl) and oxidoreductases (*Xpo*Lac), which were commercially available (Ecopulp X-200) or previously purified, to improve the efficiency of the

conversion process. Rape straw material (3 g per reaction) was *in vitro* incubated with *Xpo*GH78 (0.4 U mg^{-1}) and selected enzymes in 100 mM sodium citrate buffer at pH 6.0.

The reactions (total 30 ml) containing the above components were incubated at 23 °C for 72 hrs. To follow the synergistic effects and the single effects of each enzyme used, enzymatic conversion was performed with each enzyme alone, and on the one hand, in combinations with all hydrolases (*Xpo*GH78 and Ecopulp X-200 preparation). On the other hand, it was performed with all tested enzymes (*Xpo*GH78, Ecopulp X-200 preparation, and laccase). Controls containing heat-denatured enzymes (95 ^{o}C for 15 min) were used.

2.6. High performance liquid chromatography (HPLC)

Reaction aliquots were shortly centrifuged (12,000 rpm) and then transferred into 1.5-mL HPLC vials. For monosaccharide analysis, substances were routinely eluted on a Rezex™ HPLC ion-exclusion column [RPM-Monosaccharide Pb^{+2} (8 %), 7.8 mm × 300 mm, Phenomenex®] using an Agilent HPLC system (1200 series) equipped with a refractive-index detector (RID) and reference monosaccharides.

2.7. Gas chromatography tandem mass spectrometry (GC-MS)

The GC-MS method was applied to estimate the enzymatic demethylation of rape straw by *Xpo*GH78 alone or by enzyme cocktails. After incubation of corresponding substrates under the conditions described above, the released biomethanol by enzymatic hydrolysis was analyzed using a GC-MS system (Hp 6890 series, Agilent, Germany) fitted with a Zebron column (ZB-WAXplus, 250 µm × 30 m, 0.25 µm film thickness, Phenomenex®, Germany). The data were acquired according to GC retention time and electron impact MS in the selected ion monitoring (SIM, 31 m/z) mode at 70 eV [18].

3. RESULTS AND DISCUSSION

3.1. Production of GH78 glycoside hydrolase on different lignocellulosic and ester substrates

This fungus *Xylaria polymorpha* was grown in liquid cultures supplemented with different lignocellulosic substances consisting of ester bonds and serving as the sole carbon source. As shown in Fig. 1, wheat straw, wheat arabinoxylan, wheat bran and oat spelt xylan remarkably stimulated the hydrolytic production in comparison to the control, which did not contain any inducer. At the applied concentrations of wheat straw and wheat arabinoxylan (2 %), the highest levels (~ 120 U l^{-1}) of FAE activities were obtained. Both substances seem to be the most suitable natural inducers for an improved enzyme production. At the same concentration, oat spelt xylan and wheat bran had also an enhancing effect that turned out to be approximately half of the maximal attained activities of wheat straw substrates (~ 60 U l^{-1}). Other tested carbon sources such as triacetin and olive oil did not effectively stimulate the respective enzyme production. For larger-scale enzyme production used for further enzymatic conversion of biomass, the straw-like substrate was chosen and a multifunctional GH78 glycoside hydrolase (*Xpo*GH78; molecular mass of 98 kDa and a weak acidic *p*I value of 3.7) was purified from *X. polymorpha*'s culture with regarding its feruloyl esterase activity as described previously by Nghi *et al.* [14].

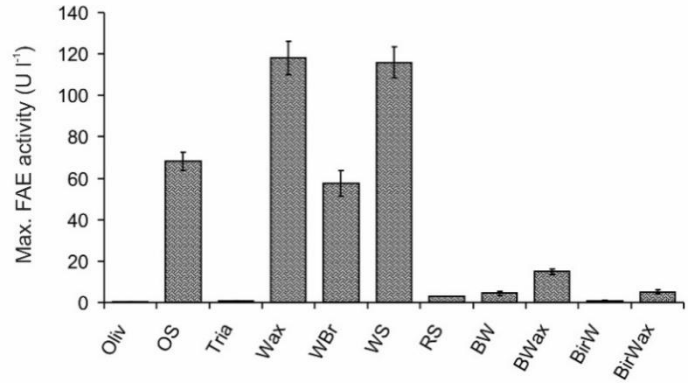

Figure 1. Overview of the maximum FAE activities of multifunctional GH78 glycoside hydrolase (*Xpo*GH78) from *X. polymorpha* in liquid cultures during 21-day incubation with potential inducing agents: olive oil (*Oliv*), oat spelt xylan (*OSXyl*), triacetin (*Tria*), wheat arabinoxylan (*Wax*), wheat bran (*WBr*), wheat straw (*WS*), rape straw (*RS*), beech wood (*BW*), beech wood xylan (*BXyl*), birch wood (*BirW*), and birchwood xylan (*BirXyl*).

3.2. Release of carbohydrates from rape straw

Although rape straw (RS) did not well induce the production of XpoGH78 in vivo by fungusX. polymorpha, a synergistic effect was observed for the release of sugars from rape straw meal caused by enzymatic treatment with single hydrolases or oxidases, or in a combination of these enzymes. It can be assumed that the multifunctional XpoGH78 hydrolyzes ester bonds in the peripheral area of the lignocellulose between the monolignols of lignin and the hemicellulose moieties. Moreover, the enzyme can facilitate the hydrolysis of polysaccharides to the corresponding mono- and dimers, and the XpoLac may oxidize the emerging mono- and polyphenols followed by subsequent polymerization reactions. The increase of reducing sugars is obviously due to a depolymerizing effect caused by cellulase and xylanase activity (Ecopulp X-200). Thus, the release of the five most abundant sugars (i.e., arabinose, galactose, glucose, mannose, xylose) contained in the cellulose and hemicellulose moieties of straw material were then detected and quantified by HPLC as shown in Table 1. Increasing amounts of glucose (4.7 to 5.9 mg g^{-1}) could be measured after treatment with the Ecopulp X-200 preparation, or subsequently supplemented with XpoGH78 and finally by using all enzymes together. Such a synergistic effect was also observed for galactose increased up to 6-fold (0.5 to 3.0 mg g^{-1}) compared with commercial hydrolases (Cell&Xyl) used alone.

The comparison of the sum of all five detected sugars released by each enzymatic reaction system is shown in Fig. 2. The effect caused by the multifunctional *Xpo*GH78 (total amount of released carbohydrates: ~ 6 mg g^{-1}) is a half of that observed for the Ecopulp X-200 preparation (Cell&Xyl; 12 mg g^{-1}). It is no surprise that the oxidase *Xpo*Lac alone did not affect the liberation of sugar from rape straw meal. Notably, the overall carbohydrate release was increased up to 16 mg g^{-1} by using a combination of *Xpo*GH78 and the crude Ecopulp X-200 preparation. The addition of *Xpo*Lac to this enzyme cocktail containing only hydrolases further increased the effect up to 17.3 mg g^{-1}. An equal amount of the reducing sugars contents [16.7 mg g^{-1} (33.3 µg ml^{-1}] was obtained during the enzymolysis of rape straw by a recombinant *Lentinula edodes*endoglucanase (LeCel12A). Current results also suggested that rape straw could be more efficiently hydrolyzed by this enzyme compared with other straws, i.e., wheat straw, corn straw, and rice straw [19]. Saha & Cotta [20] used an optimized treatment for the saccharification of wheat straw with different commercially available enzyme preparations and up to 300 and 150

mg g^{-1} of glucose and xylose, respectively, were obtained after 24 hrs of incubation. Nevertheless, a higher reaction temperature (45 ^{o}C) and chemical pretreatment were used in this study in contrast to the mild reaction conditions applied for the *Xpo*GH78-catalysed conversion. Additionally, Vancov & McIntosh [21] demonstrated the increase in sugar release from ~ 135 to 190 mg g^{-1} by a doubling of the reaction temperature from 60 to 121 °C. The efficiency of enzymatic hydrolysis of lignocellulosic material depends on the enzyme amount or substrate used. Therefore, reaction parameters for the straw conversion by *Xpo*GH78 with and without accessory enzymes can be further optimized to improve the release of economically important metabolites.

As a result, the synergetic action of tested enzymes was proved by the release of *C*-5 and *C*-6 carbohydrates (glucose, xylose, galactose) from rape straw meal, which was clearly enhanced by the use of either the combination of all hydrolytic enzymes (Cell&Xyl and *Xpo*GH78; glucose release of 5.5 mg g^{-1}) or the addition of *Xpo*Lac (glucose release of 5.9 mg g^{-1}).

Table 1. Release of sugars from rape straw by enzymatic treatment.

Reaction system	Designation	Release of carbohydrates (mg g^{-1})				
		Arabinose	Galactose	Glucose	Mannose	Xylose
Hydrolase Ecopulp X-200	*Xpo*GH78	1.8	0.2	1.4	0.9	1.4
	Cell&Xyl	1.3	0.5	4.7	2.3	3.4
Hydrolase & Ecopulp X-200	*Xpo*GH78 Cell&Xyl	2.5	1.5	5.5	3.0	3.5
Hydrolase & Ecopulp X-200, Laccase	*Xpo*GH78 Cell&Xyl *Xpo*Lac	2.3	3.0	5.9	2.6	3.5

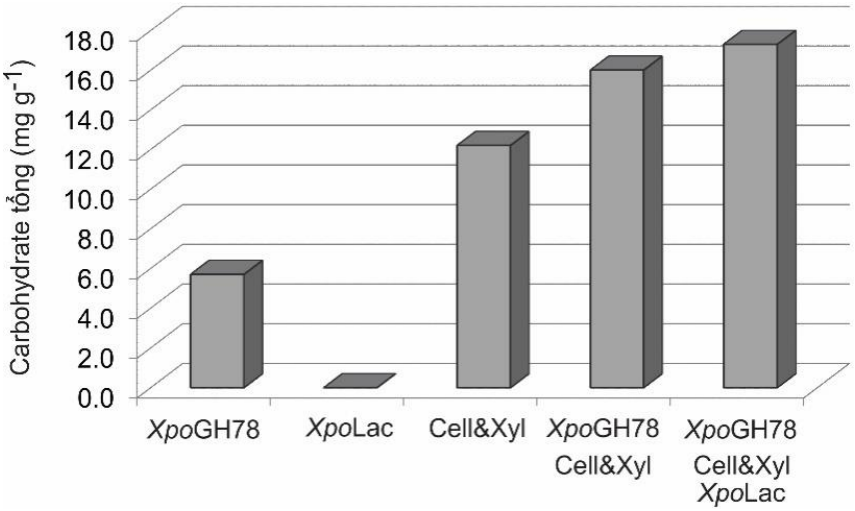

Figure 2. Sum of the released carbohydrates from milled rape straw after treatment with different enzymes (*Xpo*GH78, *Xpo*Lac, and Cell&Xyl) as single applications, and in two enzyme combinations [(i) *Xpo*GH78 and Cell&Xyl; (ii) *Xpo*GH78, Cell&Xyl, and *Xpo*Lac]. The values are corrected by the sugar amounts in the corresponding controls containing heat-denaturated enzymes.

3.3. Release of biomethanol from rape straw

Demethylation activity of XpoGH78 on the methyl-groups occurring in the pectin moieties of rape straw was demonstrated by GC-MS detection of biomethanol as the reaction product (Fig. 3). For that reason, the improvement of biomethanol release by the synergistic action of hydrolases and oxidases was investigated. The incubation of rape straw with XpoGH78 for 72 hrs at 23 °C led to a marginal biomethanol release of approx. 17 µg g^{-1} and with crude cellulose/xylanase to 94 µg g^{-1}. This effect could be considerably improved by the cooperation of both preparations together, which led to a biomethanol release of approx. 170 µg g^{-1}. The addition of XpoLac to this hydrolase cocktail finally increased the biomethanol release up to 270 µg g^{-1} (Fig. 4).

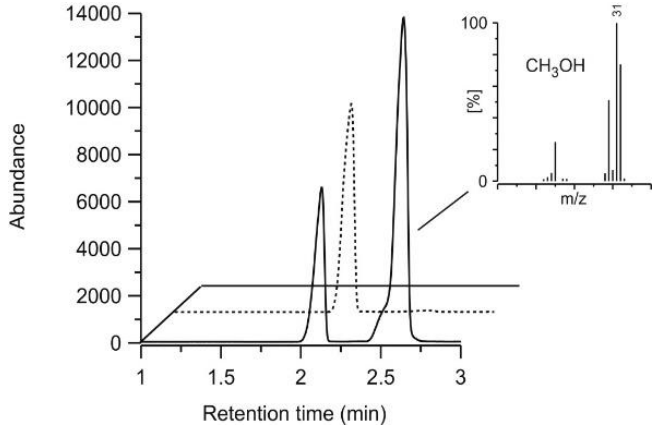

Figure 3. GC-MS elution profile of the enzymatic demethylation of rape straw (*solid line*) after 48 hrs of incubation. Control without enzyme (*dashed line*) insert shows the mass spectrum of biomethanol as the reaction product.

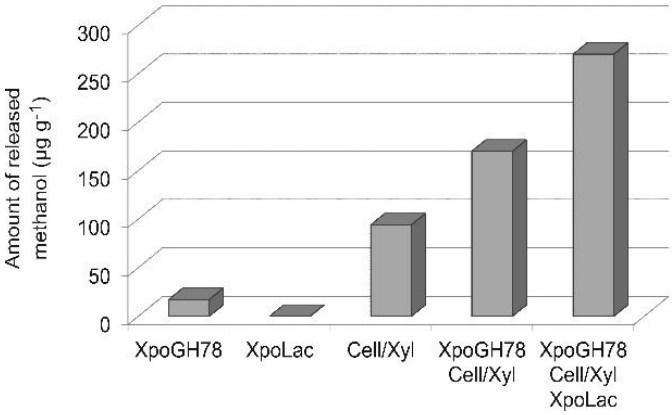

Figure 4. Release of biomethanol from milled rape straw after demethylation by different enzymes (*Xpo*GH78, *Xpo*Lac and Cell&Xyl) as single applications, and in two combinations of enzyme systems [(i) *Xpo*GH78 and Cell&Xyl; (ii) *Xpo*GH78,Cell&Xyl, and *Xpo*Lac)].

Demethylation or pectin methylesterase activity of *Xpo*GH78 was evidenced by the release of biomethanol from the cell wall heteropolymer pectin. The enzymatic hydrolysis of ester bonds in the carboxymethyl group of the galacturonic acids in pectin may explain the release of

biomethanol from rape straw. The highest amount of approx. 270 µg g^{-1} (≈ 27 %) biomethanol was obtained by using the enzyme cocktail containing *Xpo*GH78/*Xpo*Lac and Cell&Xyl. This finding may be of general biotechnological interest, since the production of the so-called biomethanol from agricultural wastes could be used for the sustained synthesis of fuel supplements.

The *Xpo*GH78 possesses the pectin methyl-esterase activity to hydrolyze methoxyl side chains of the pectic substrate, a cell wall component of rape straw. Indeed, esterase-catalyzed conversion of methoxylated substrates, e.g., phenylalkanoate methyl esters [22, 23], and complex lignocellulosic substrates has been recently reported [24,25]; the direct release of biomethanol from lignocellulosic materials by demethylating microbial hydrolases like *Xpo*GH78 is still quite scarce.

4. CONCLUSION

The ability of purified GH78 glycoside hydrolase (*Xpo*GH78) to hydrolyze lignocellulosic materials was tested for rape straw with and without the addition of accessory enzymes. As main metabolites, carbohydrates and biomethanol could be detected, which are all of biotechnological interest. Synergistic conversion of rape straw in a combination of all hydrolytic enzymes (*Xpo*GH78, Cell&Xyl, and *Xpo*Lac) resulted in the release of 17.3 mg g^{-1} of total carbohydrates (arabinose, galactose, glucose, mannose, xylose) after incubating at 23 °C for 72 hrs. Moreover, the demethylation activity of *Xpo*GH78 and other hydrolytic- and oxidative enzymes (Cell&Xyl, and *Xpo*Lac) on rape straw led to the generation of approx. 270 µg g^{-1} of biomethanol. Here, we have provided the investigation and demonstration of applicability of enzyme cocktail-based methods (by glycoside hydrolase and oxidase) without chemical pre-treatment, which could be useful in different applications, i.e., 2^{nd} -generation biofuels (advanced biofuels) manufactured from non-food biomass.

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CRediT authorship contribution statement. Author 1: Experimental performance, data analysis, and manuscript preparation. Author 2: Experimental performance, Formal analysis. Author 3: Methodology, Formal analysis. Author 4: Manuscript revision, supervision.

Declaration of competing interest. The authors declare that we 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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