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Combinatorial Enzyme Approach to Convert Wheat Insoluble Arabinoxylan to Bioactive Oligosaccharides

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Abstract

Combinatorial enzyme technology was applied for the conversion of wheat insoluble arabinoxylan to oligosaccharide structural variants. The digestive products were fractionated by Bio-Gel P4 column and screened for bioactivity. One fraction pool was observed to exhibit antimicrobial property resulting in the suppression of cell growth of the test organism ATCC 8739 *E. coli*. It has a MIC value of 1.5% (w/v, 35˚C, 20 hr) and could be useful as a new source of prebiotics or preservatives. The present results further confirm the science and useful application of combinatorial enzyme approach.

Keywords

Combinatorial Enzyme Approach, Wheat Insoluble Arabinoxylan, Bioactive Oligosaccharides

1. Introduction

Plant cell wall polysaccharides consist of polymeric backbones decorated with various types of substitutions [1]. For example, the hemicellulose polymer xylan contains a β-1,4-linked xylopyranosyl main chain decorated with at least five types of side groups: acetyl, phenolic (ferulic and coumaric) acid, glucuronyl, and arabinofuranosyl residues. Cleavage of these side groups requires acetylxylan esterase, feruloyl esterase, a-glucuronidase, and a-L-arabinofuranosidase, respectively [2]. The side group composition varies depending on the source of substrates used in the pretreatment and extraction of the xylan polymer [3]. Adding to the complexity is the fact that ferulic acid, which is an essential structural component in cell wall structure, can function in dimerization crossing xylan and other polysaccharides. Some ferulic acid esterases are also known to be

diferulic esterases [3].

The presence of these side groups as well as their positions, density, and types of linkages influences the pattern of enzymatic degradation of the main chain polymer and vice versa. These cooperative interactions determine the structural outcome of the oligosaccharide fragments produced. The enzymatic removal of the side groups individually and/or sequentially constitutes a combinatorial design for generating vast libraries of structurally diverse oligosaccharides that would translate into different and unique reactivity and functional properties. The diverse population can often be screened with high-throughput methods for candidates possessing the target biological and/or functional properties. The novel concept of this "combinatorial enzyme technology" is schematically represented in **Figure 1** and has been described in a recent review [4]. We applied the design to produce libraries of pectic oligosaccharides and feruloyl oligosaccharides. Repeated fractionation and screening resulted in the isolation of bioactive oligosaccharide species with antimicrobial activity [5] [6]. The present work describes the combinatorial digestion of pretreated wheat insoluble arabinoxylan using 5 sets of enzyme compositions to produce libraries of xylo-oligosaccharides followed by gel fractionation to isolate bioactive species.

2. Materials and Methods

2.1. Materials

Wheat insoluble arabinoxylan (WIA) was obtained from Megazyme (Wicklow,

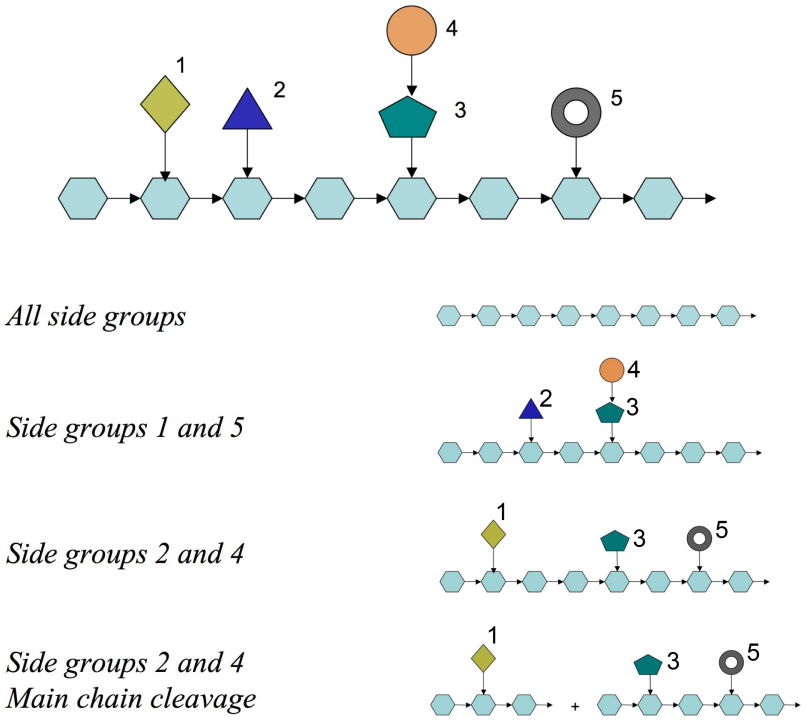

Figure 1. A schematic representation of combinatorial enzyme digestion to produce libraries of oligosaccharides of diverse structures [4].

Ireland). All carbohydrases: endo-1,4-β-D-xylanases from *Cellvibrio mixtus* (E-XYNBCM); α-L-arabinofuranosidase from *Bifidobacterium* sp. (E-AFAM2); feruloyl esterase from *Clostridium thermocellum* (E-FAEZCT) were obtained from Megazyme (Wicklow, Ireland). *E. coli* (ATCC 8739) was obtained from American Type Culture Collection (Manassas, VA). Culture media were purchased from Sigma (St. Louis, MO). Bio-Gel P-4 was obtained from BioRad (Richmond, CA). HPTLC plates were purchased from Analtech (Newark, DE). All chemicals and solvents were of analytical or HPLC grade.

2.2. Pretreatment of WIA

WIA (1.5 g) was soaked in 28.5 ml water overnight and then autoclaved for 20 min at 121˚C at 20 - 21 psi in a stainless steel reactor tube of 1" OD × 4.5" L × 0.65" thickness, with 1" stainless steel swagelock end fittings) [7]. The pretreated WIA was washed 4x with water before being subjected to enzyme digestion.

2.3. Enzymatic Hydrolysis of WIA

To prepare for each digestion mixture, 1 ml pretreated WIA (4% concentration stock in suspension) was centrifuged for 10 min at 5000 rpm, and 300 ml of the supernatant was carefully removed, leaving 700 ml WIA residues for digestion. A cocktail of three enzymes FAEZCT, AFAM2 and XYNBCM in various composition ratios (yielding 5 different combination sets, **Table 1**), plus 0.1 ml 0.5 M potassium phosphate pH 6.0, and H_2O to adjust a final reaction volume of 300 ml were added. A total of 20 reactions for each set were incubated at 37˚C in a water shaker bath at 225 rpm at specific time durations. The reaction mixtures were cooled to room temperature, centrifuged (10 min 5000 rpm), and the supernatants were combined and lyophilized for Bio-Gel column fractionation.

2.4. Gel Filtration Chromatography

The combined supernatants were centrifuged (20 min, 5000 rpm), filtered, and lyophilized. The dried product was redissolved in 9 ml of 0.2 M ammonium

Table 1. Enzymes formulated in various combination ratios.

Set	XYNBCM	FAEZCT	AFAM2	Buffer	Water
1	15 mg (48 ml)			100 ml	152 ml
2	15 mg (48 ml)	0.42 mg (30 ml)		100 ml	122 ml
3	15 mg (48 ml)		0.16 mg (83 ml)	100 ml	69 ml
4	15 mg (48 ml)	0.42 mg (30 ml)	0.16 mg (83 ml)	100 ml	39 ml
5		0.42 mg (30 ml)	0.16 mg (83 ml)	100 ml	87 ml

Buffer = 500 mM potassium phosphate, pH 6.0 (Final conc. in rxn = 50 mM). For each set, the final enzyme solution volume was adjusted to 300 ml with added water. This Table presents enzyme cocktails used per reaction tube. A total of 20 reactions (=20 ml enzyme cocktails plus autoclaved WIA) were combined for each of the combined for each set.

bicarbonate buffer, pH 6.0, filter-sterilized, and applied to a Bio-Gel P4 column (2.5 × 100 cm) equilibrated in 0.2 M ammonium bicarbonate buffer (degassed and filter sterilized). Elution flow rate was 7.5 ml/20 min/fraction. Fractions were monitored for unsaturation by A_{235} reading, for phenolic acids by A_{325}, and for total carbohydrates by the phenol-sulfuric acid method [8] [9].

2.5. Analysis of Hydrolysis Products

Enzymatic hydrolysis of WIA was measured by the formation of reducing ends of oligosaccharides produced by XYN action and by AFA action using the DNSA method [10]. Total carbohydrate was determined by the phenol-sulfuric acid method [8]. Ferulic acid was determined using Folin-Ciocalteu reagent according to Ainsworth *et al.* [11].

2.6. High-Performance Thin-Layer Chromatography

The oligo samples were developed by HPTLC (20 × 10 cm HPTLC silica gel F235 plate with EtOAc/HOAc/1-PrOH/HCOOH/H_2O (25:10:5:1:15). The unsaturation of oligo fragments was observed by spraying the developed plate with $KMnO_4$ reagent (3 g $KMnO_4$, 20 g K_2CO_3, 5 ml 5% aqueous NaOH, and 300 ml H_2O). The plate was next sprayed with 10% H_2SO_4 in methanol containing 1 mg/ml orcinol, followed by heating at 90˚C for visualization of oligosaccharides.

2.7. Growth Experiments and Culture Conditions

Freeze-dried powder of *E. coli* (ATCC 8739) was rehydrated with 1 ml sterile MH (Mueller Hinton) broth. Colonies grown on MH agar plate were transferred to grow in a 5 ml culture (35˚C, 220 rpm, 4 hr) and the absorbance at 600 nm was measured after 4 hr incubation. The culture was diluted with MH broth to a final concentration of 1 × 10^3 cfu/ml based on a standard curve. The standard curve was constructed by plotting the number of colonies (by plate count) vs OD600 (of liquid culture). Individual pooled fractions of WIA oligosaccharides were dissolved in MH broth at specific concentrations, filter-sterilized (2 mm HT Tuffryn membrane syringe filter, Pall Corporation), and added to the diluted *E. coli* 8739 culture. Cell growth was determined by measuring the absorbance at 600 nm of appropriate dilutions (~1:20 with MH) of the cell culture using a microplate reader (SpectraMax M2, Molecular Devices, CA). The oligo concentration was determined by the phenol-sulfuric acid method based on a xylose standard curve. The standard microdilution method was used to determine the minimum inhibitory concentration (MIC), which is defined as the lowest concentration of an antimicrobial that inhibits the visible growth of a test microorganism (such as ATCC 8739 used in this study) in overnight incubation [12].

3. Results and Discussion

Pretreatment is a critical step in cellulosic ethanol production in that the process helps to loosen the substrate structure to increase its accessibility to enzyme di-

gestion. Hot water pretreatment has been demonstrated to effect dissolution of carbohydrates without causing degradation of the products [13] In our previous studies, it has been shown that hot water pretreatment of corn fiber resulted in a 4-fold increase of ferulic acid hydrolysis using combined enzymes of FAE and XYN, compared to the untreated samples under the same reaction conditions [7].

The reaction mixture obtained after combinatorial enzyme digestion was fractionated by a Bio-Gel P4 column to yield four pooled fractions based on monitoring A_{235} reading (for unsaturation), Abs_{325} (for phenolic acids), and Abs_{590} (for total carbohydrates by the phenol-sulfuric acid method). The molar ratios used in the combinations (**Table 1**) were based on the results obtained from a series of preliminary testings of varying the concentrations of each of the three enzymes. The production of FA, diFA, and xylose equivalent were analyzed to yield an estimation of the optimal amount of enzymes used for hydrolysis (**Figure 2**).

The soluble supernatant from combinatorial enzyme digestion of pretreted WIA was fractionated by Bio-Gel P4 into 4 peaks (**Figure 3**). The fractions of each peak were pooled and lyophilized and analyzed for antimicrobial activities. For the testings, the initial inoculation of the test microorganism *E. coli* ATCC 8379 was carefully controlled to a starting titer of 1×10^3 cfu/ml so that all experiments were performed with the same initial conditions. The chromatogram shows that the four pooled fractions contained varying degrees of unsaturation, phenolic moiety, and reducing sugars. However, only pool fraction #2 (F2) possessed inhibitory effect on the test *E. coli* (**Figure 4**). **Figure 5** shows that the inhibitory effect increased with the concentration (0 to 2%), and a suppression of cell growth was achieved at ~1.5% w/v, which was the MIC (minimum inhibitory concentration) value. The inhibitory effect on cell growth was observed in extended time, amounting to 51%, 56%, and 32% for 16. 24 and 48 hr, respectively (**Figure 6**).

The mechanism of inhibition might be related to the unique structural properties of the olgosaccharide. F2 had an unsaturated structure as confirmed by the

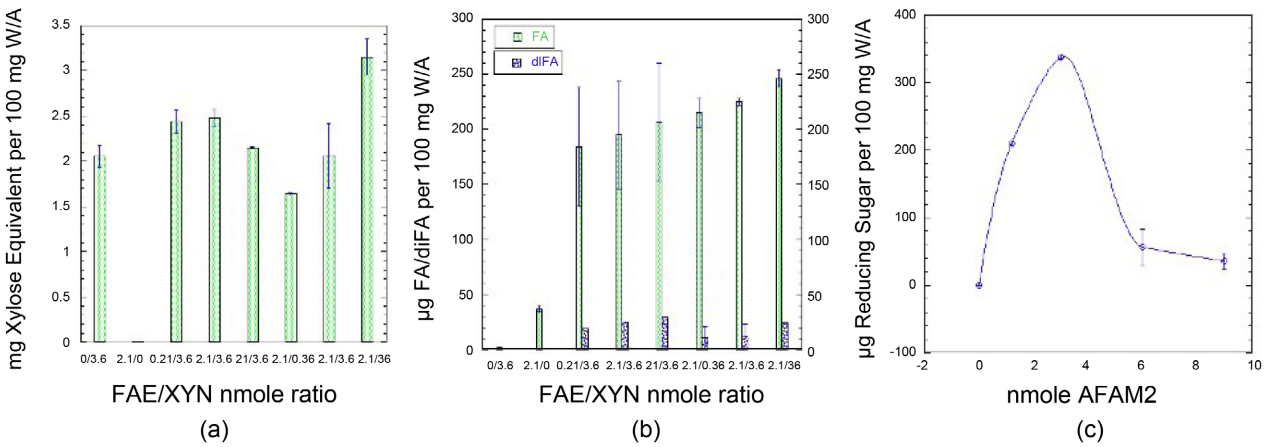

Figure 2. Preliminary analysis of varying concentrations of each of the three enzymes on the digestion of pretreated WIA.

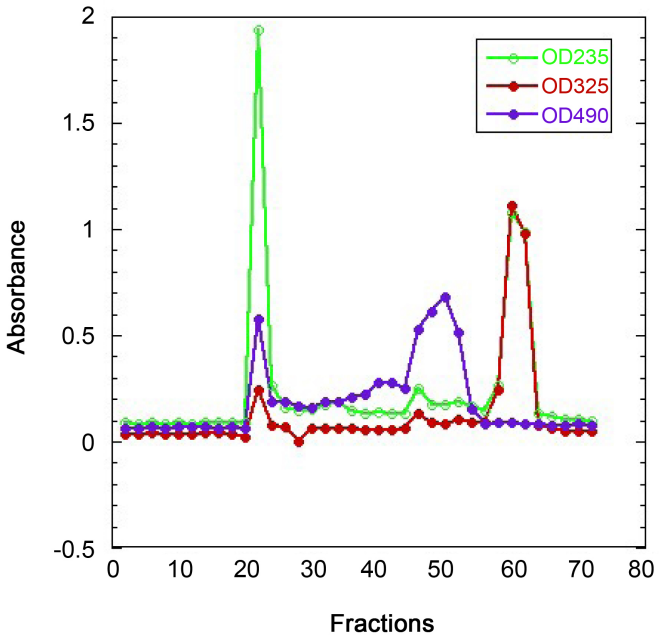

Figure 3. Bio-Gel P4 column chromatography of recombinatorial enzyme digest of pretreated WIA. Conditions: Bio-Gel P4 column - 2.5 × 100 cm, elution buffer - 0.2 M ammonium bicarbonate, flowrate - 7.5 ml/20min/tube. Fraction pools: F1 (20 - 24), F2 (36 - 44), F3 (45 - 55), F4 (58 - 64).

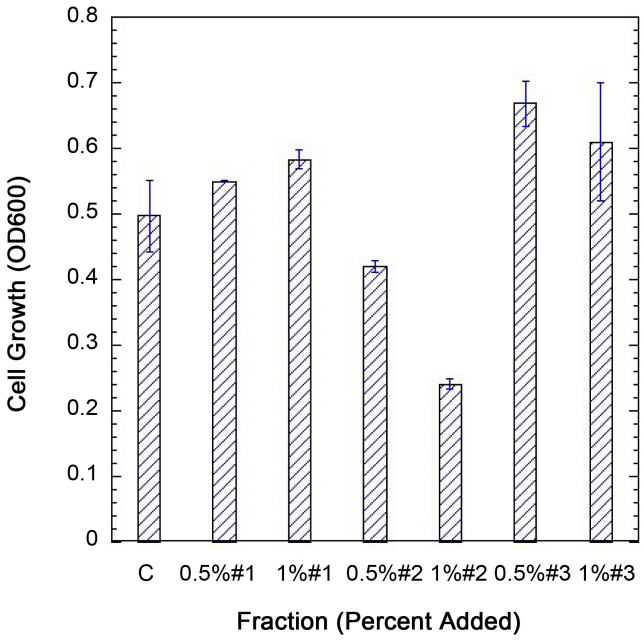

Figure 4. Comparison of effects of F1, F2, and F3 on cell growth (35˚C, 20 hr). An ON culture of *E. coli* ATCC 8739 in MH incubated at 35˚C was diluted to appropriate concentration. F1, F2, and F3 at 2 different concentrations (0.5 and 1%) were added. The incubation was continued for 20 hr. Cell growth was determined by measuring A_{600} of appropriate dilutions of the cell culture. The cfu/ml cell density was calculated based on the conversion curve.

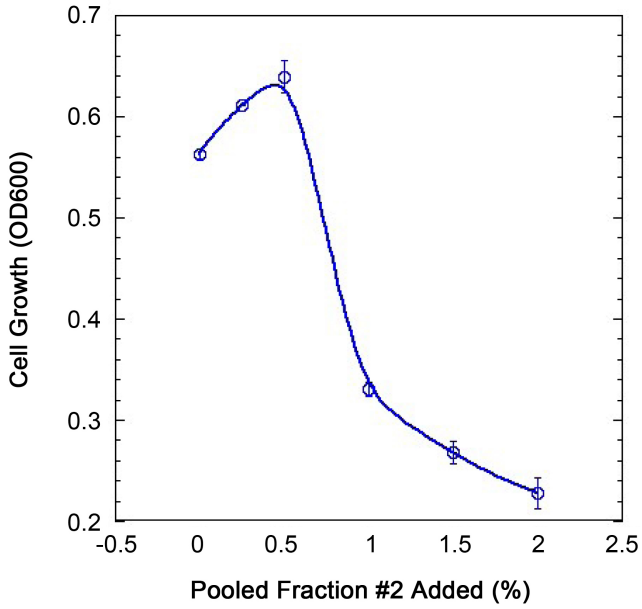

Figure 5. Increasing concentration effect of F2 on cell growth. An overnight culture of *E. coli* (ATCC 8739) in MH broth at 35˚C was diluted to appropriate titer concentration, and various concentration of the F2 (0 to 2%) were added. The incubation was continued for 20 hr. Cell growth was determined by measuring A_{600} of appropriate dilutions of the cell culture, and the cfu/ml cell density was calculated based on the conversion curve.

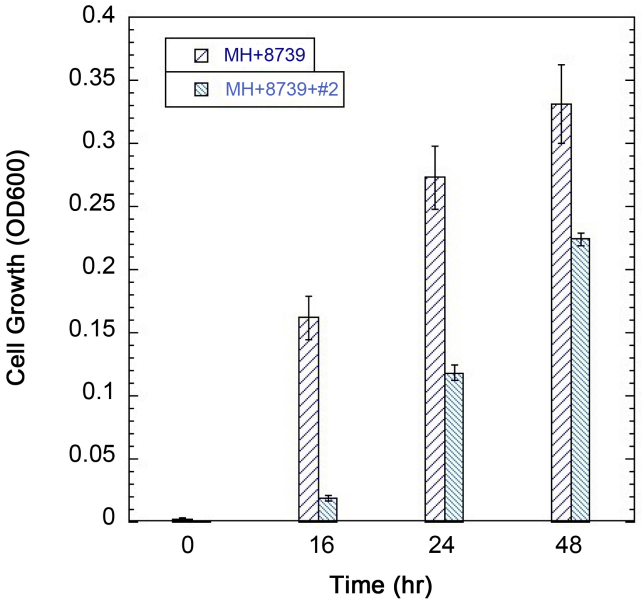

Figure 6. Time course of the effect of F2 on cell growth. An overnight culture of *E. coli (*ATCC 8739) in MH broth incubated at 35˚C was diluted to appropriate titer concentration, and the oligos F2 at 1.5% were added. The incubation was continued for 2 days with sampling at 16, 24 and 48 hr. Cell growth was determined by measuring A_{600} of appropriate dilutions of the cell culture, and the cfu/ml cell density was calculated based on the conversion curve.

absorbance measurement at 235 nm. The active oligo also had a size range estimated of 1.5 kDa, generally considered in the range of low molecular weight oligosaccharides. The active fraction F2 contained an average of 10 xylose units in length carrying 1 FA moity per 6.5 xylose units (**Table 2**).

The presence of reactive double bonds in the active oligo species may be a contributing factor to its inhibitory effect on cell growth. Double bonds are electrophilic and readily participate in a variety of reactions, resulting in crosslinking and inactivation of biomolecules. The presence of reactive double bonds of some classes of phenolic compound, has been associated with the ability to facilitate membrane permeability and attributed to antimicrobial activity [14]. Phenolic compounds found in hydrolysis of lignocellulosic materials have been shown to contain antimicrobial activities, comparable to the common preservative sodium benzoate [15].

The size range of the oligosaccharide may may play a key role in facilitating passage through the cell membrane. Many antimicrobial oligosaccharides reported in the literature are low molecular weight molecules [16] [17]. High molecular weight oligosaccharides are shown to prevent efficient utilization and expression of relevant functional and biological activities. The present results seem to support the suggestion that large molecular size would not allow for the molecule to penetrate the cell membrane, or have interactions with intracellular constituents and processes of the cell.

Food preservatives are generally applied in the range of 0.1%. The use of non-digestible oligosaccharides (NDO) has gained popularity as functional food ingredients. Oligosaccharides have also been promoted in recent years as alternatives for antibiotics as antimicrobial growth performance promoters (AGP) in animal production [18]. The health cause-effect of AGP and NDO is generally linked to the modification of microflora, and thus the physiological conditions of the intestinal system [19]. In both regards, oligosaccharides are commonly used in sub-minimum inhibitor concentrations acting to modulate the microbiota composition.

Table 2. Analysis of phenolic acid, total carbohydrate, reducing sugar in gel column fractions.

Gel column fraction	Phenolic acid[1] (nmole FA/mg)	Total Ccarbohydrate[2] (nmole xylose/mg)	Reducing Sugar[2] (nmole reducing end/mg)
Fraction 1	192.74 ± 43.29	1152.33 (0.95)	342.19 (0.93)
Fraction 2	141.99 ± 0.88	932.53 (1.0)	93.25 (0.98)
Fraction 3	248.87 ± 0.34	559.52 (0.99)	193.17 (0.99)
Fraction 4	194.96 ± 4.22	479.58 (1.0)	213.15 (1.0)

[1]Calculated from duplicate sample analyses. [2]Results from slope of plotting analyses of three weigh concentrations of the sample. R2 values in parenthesis.

4. Conclusion

This project has applied combinatorial enzyme technology to create oligosaccharides of diverse structures from pretreated WIA. A fractionated oligosaccharide species has been shown to suppress the growth of the test organism ATCC 8739, with a MIC value of 1.5%. The active oligosaccharides may be useful as a new source of high-value preservatives or as alternatives for antimicrobial growth promoters. The present study also demonstrates the theory, feasibility, and practical application of the combinatorial enzyme approach.

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Reference to a company and/or products is only for purposes of information and does not imply approval of recommendation of the product to the exclusion of others that may also be suitable. All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status, or handicap. The authors declare that there is no conflict of interest regarding the publication of this paper.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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