



Title	Nonreducing terminal chimeric isomaltomegalosaccharide and its integration with azoreductase for the remediation of soil-contaminated lipophilic azo dyes
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Citation	Carbohydrate Polymers, 305, 120565 https://doi.org/10.1016/j.carbpol.2023.120565
Issue Date	2023-04-01
Doc URL	http://hdl.handle.net/2115/91442
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Type	article (author version)
File Information	CarbohydratePolymers120565.pdf



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1 **Nonreducing terminal chimeric isomaltomegalosaccharide and its integration with**
2 **azoreductase for the remediation of soil-contaminated lipophilic azo dyes**

3
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20
21 **Abstract**

22 Lipophilic azo dyes are practically water-insoluble, and their dissolution by organic
23 solvents and surfactants is harmful to biological treatment with living cells and enzymes. This
24 study aimed to evaluate the feasibility of a newly synthesized nonreducing terminal chimeric
25 isomaltomegalosaccharide (N-IMS) as a nontoxic solubilizer of four simulated lipophilic azo dye
26 wastes for enzymatic degradation. N-IMS bearing a helical α -(1 \rightarrow 4)-glucosidic segment derived
27 from a donor substrate α -cyclodextrin was produced by a coupling reaction of cyclodextrin
28 glucanotransferase. Inclusion complexing by N-IMS overcame the solubility issue with equilibrium
29 constants of 1,786–242 M⁻¹ (methyl yellow > ethyl red > methyl red > azo violet). Circular
30 dichroism spectra revealed the axial alignment of the aromatic rings in the N-IMS cavity, while
31 UV–visible absorption quenching revealed that the azo bond of methyl yellow was particularly
32 induced. Desorption of the dyes from acidic and neutral soils was specific to aqueous organic over
33 alkali extraction. The dissolution kinetics of the incorporated dyes followed a sigmoid pattern
34 facilitating the subsequent decolorization process with azoreductase. It was demonstrated that after
35 soil extraction, the solid dyes dissolved with N-IMS assistance and spontaneously digested by

36 coupled azoreductase/glucose dehydrogenase (for a cofactor regeneration system) with the
37 liberation of the corresponding aromatic amine.

38

39 **Keywords:** azobenzene dye, biological treatment, chimeric structure, dye-contaminated soil
40 remediation, saccharide solubilizer, methyl yellow

41

42 **1. Introduction**

43 Lipophilic azo dyes are persistent and accumulate in the environment, especially in sludge
44 waste or soil near processing manufacturers, with a potential risk of carcinogenic effects and
45 genotoxicity (Zanoni et al., 2013). Remediation of highly polluted sites with aromatic pollutants is
46 complicated, as at least two essential series of soil washing and decomposition are mandatory
47 (Fabbri, Prevot, Zelano, Ginepro, & Pramauro, 2008). To date, the removal of hydrophobic dyes by
48 surfactants and organic solutions is common, followed by various recent technologies, such as
49 electrochemical photocatalysis, ozonation, and Fenton oxidation (Muthuraman & Teng, 2009;
50 Tehrani-Bagha & Holmberg, 2013; Hitam & Jalil, 2020). However, the latter drawbacks are the
51 limitation of light penetration in colored effluents and the separation of fine catalysts from the
52 treated medium (Fabbri et al., 2008). The biodegradation of water-soluble azo dyes under aerobic,
53 anaerobic, and combined anaerobic-aerobic systems has been eagerly studied (O'Neill et al., 2000).
54 Nevertheless, these approaches may face some limitations, e.g., larger land area and long operation
55 time. In contrast, *ex situ* biological treatment with a microbial enzymatic system is a great
56 advantage for highly specific dye degradation and elimination of deleterious byproducts regardless
57 of land size (Husain, 2006). Although the critical step is to find an appropriate lipophilic dye
58 solubilizer for exchanging organic solvents or surfactants after the soil extraction, this integration
59 process has not been revealed before.

60 Azo dyes are synthetic colorants where the structure carries one or more chromophore azo
61 groups ($-N=N-$) associated with benzene or naphthalene rings or sometimes with aromatic
62 heterocycles, forcing them to have a core hydrophobic nature. Only anionic azo dye-substituted
63 sulfonate ($-O-SO_3Na$) is highly water-soluble over the entire pH range, which has applicability in
64 textile dyeing. The major class of anionic lipophilic azo dyes widely used as acid-base indicators,
65 optical materials or chemosensors having carboxylate substituents, e.g., ethyl red (ER) and methyl
66 red (MR), or hydroxyl groups, e.g., azo violet (AV), are only water-soluble in alkaline pH
67 (Tehrani-Bagha & Holmberg, 2013). The opposite charge distribution in cationic azo dyes
68 presented in amino substituents of the azobenzene dyes, e.g., methyl yellow (MY), is a
69 consequence of alkali-insolubility (Matazo, Ando, Borin, Santos, 2008). As mentioned above, the
70 solubilization of lipophilic dyes mostly requires surfactant, organic excipients, and pH adjustment
71 with extreme acid or alkali (only for an ionizable dye) or an emulsion and a liposomal formulation,

72 most of which are highly susceptible to further enzymatic treatment, whereby lipophilic dye
73 digestion can be more bioavailable if the dye states are maintained in a soluble form. Therefore, the
74 remediation of lipophilic dyes by implementation with a mild solubilizer will be highly beneficial.
75 In this context, pure nonvolatile saccharide solubilizers have been proposed, e.g., cyclic α -(1 \rightarrow 4)-
76 linked oligosaccharides (cyclodextrin; Song et al., 2019) and linear isomaltomegalosaccharide (L-
77 IMS) with chimeric segments of short α -(1 \rightarrow 4)-linkages at a reducing terminal of the α -(1 \rightarrow 6)-
78 glucosidic main chain (Lang et al., 2014a). Regarding the latter approach, we recently established
79 the enzymatic synthesis approach of chimeric IMS bearing longer segments of α -(1 \rightarrow 4) than L-
80 IMS, and the physicochemical analysis emphasized the role of water solubility enhancement of
81 poorly soluble compounds mainly arising from the α -(1 \rightarrow 4)-segment (Lang et al., 2022a, b). Such
82 knowledge influenced us to synthesize a new IMS structure by extending the length of the α -
83 (1 \rightarrow 4)-segment at the "nonreducing terminal" of the single IMS (N-IMS, Fig. 1A). N-IMS is
84 hypothesized to incorporate a lipophilic molecule through host-guest interactions, and the complex
85 is soluble in a water phase, similar to the properties of cyclodextrin. Furthermore, the helical chain
86 of N-IMS is simplified to be digestible (with α -amylase) and expected to be safe for the biological
87 purposes. N-IMS thus becomes the eco-friendly carbohydrate material having chimeric
88 conformation with bifunctionality: the main chain of long α -(1 \rightarrow 6)-segment contributes to the
89 inherent high water solubility (Lang et al., 2022b), and the α -(1 \rightarrow 4)-segment with helical chain
90 supplies the specific inclusion ability. In addition, the oxygen-insensitive azoreductase (BrAzo)
91 gene from *Brevibacillus laterosporus* TISTR1911 was cloned and overexpressed (Lang et al.,
92 2013a). The enzyme appeared to have potential use as a long-term biocatalyst under the coupling of
93 BrAzo and glucose dehydrogenase (GDH) for the regeneration of the cofactor β -nicotinamide
94 adenine dinucleotide reduced form (NADH). This study aims to forward N-IMS as a lipophilic dye
95 solubilizer and establish biotreatment of dye-contaminated soil using BrAzo/GDH for a potentially
96 sustainable system.

97 In this study, we newly reported the synthesis of N-IMS in which the α -(1 \rightarrow 4)-segment
98 derived from α -cyclodextrin was introduced towards the α -(1 \rightarrow 6)-main chain of a commercially
99 available dextran T 10 by cyclodextrin glucanotransferase (CGTase; EC2.4.1.19) from *Bacillus*
100 *macerans*. The putative helical conformation of the N-IMS and its hydrophobic binding capacity
101 was evaluated by the formation of homogeneous complexes with iodine and fluorescence probe, 6-
102 (*p*-toluidino)-2-naphthalene-6-sulfonate (TNS) in water, respectively. The intermolecular
103 interaction was verified by the methods of continuous variation using Job's plots and two-
104 dimensional (2D) ^1H - ^1H nuclear overhauser enhancement spectroscopy (NOESY). The soil was
105 adsorbed with AV, ER, MR, and MY (dye structures, see Fig. 2A) as representative site-polluted
106 lipophilic dyes. We then investigated the basic principles of dye desorption from these soils with an
107 alkaline and aqueous organic solvent combination. The specific binding capacity of the dye and N-

108 IMS complexes as well as the bioavailability test with the BrAzo coupling system for the
109 decolorization of the incorporated dyes were discussed for the first time.

110

111 **2 Materials and methods**

112 **2.1 Azo dyes, chemicals, and enzymes.** Amylose (DP = 28, synthetic product; DP, average degree
113 of polymerization) was purchased from Glico Nutrition (Osaka, Japan). Deuterium oxide (D₂O)
114 was obtained from Kanto Chemical (Tokyo, Japan). TNS and deuterium chloride (35%, w/v, in
115 D₂O) were purchased from Sigma–Aldrich (St. Louis, MO, USA). MR [4-
116 (dimethylamino)azobenzene-2'-carboxylic acid], 4-dimethyl-*p*-phenylenediamine, and GDH
117 (EC1.1.1.47) from *Bacillus* sp. were obtained from Wako Pure Chemical Industries (Osaka, Japan).
118 ER [4-(diethylamino)azobenzene-2'-carboxylic acid], AV (synonym, magneson; 2,4-dihydroxyl-
119 4'-nitroazobenzene), and MY [4-(dimethylamino)azobenzene] were obtained from Tokyo
120 Chemical Industry (Tokyo, Japan). Dextran T 10 was purchased from Amersham Biosciences
121 (Uppsala, Sweden) and fractionated to have DP of 48 (average value). α -Cyclodextrin and
122 maltooctaose were a gift from Nihon Shokuhin Kako (Tokyo, Japan). CGTase (liquid form
123 containing 13.8 ± 2.5 U/mL) was donated from Amano (Nagoya, Japan). For CGTase assay,
124 enzyme diluted appropriately was incubated at 20 °C in 100 μ L with 20 mM maltose and 20 mM α -
125 cyclodextrin in 50 mM sodium acetate buffer, pH 5.5. The reaction mixture was withdrawn at 3, 10,
126 and 20 min and heated at 100 °C for 10 min for termination of reaction. Maltooctaose of product
127 was determined by high-performance anion-exchange chromatography with pulsed amperometric
128 detection (HPAEC-PAD, Osaka Soda, Osaka, Japan) with a CarboPac PA1 column (Dionex,
129 Sunnyvale, CA, USA) eluted with 640 mM NaOH solution supplemented with 10 mM sodium
130 acetate. One unit of CGTase was defined as the amount of enzyme to produce 1 μ mol maltooctaose
131 by coupling α -cyclodextrin to maltose per min. *Myo*-inositol and maltooctaose were utilized as
132 internal and external standards, respectively. Heterologous expression of dextran glucosidase from
133 *Streptococcus mutans* by *Escherichia coli*, in which the enzyme catalyzes the exo-wise hydrolysis
134 of dextran molecules at the terminus to release free glucose, was prepared in our laboratory (Saburi,
135 Mori, Saito, Okuyama, & Kimura, 2006). α -Amylase (type I-A, EC3.2.1.1) from porcine pancreas,
136 β -amylase (type II-B, EC3.2.1.2) from barley crude, and dextranase (EC3.2.1.11) from *Penicillium*
137 sp. were obtained from Sigma–Aldrich. β -Nicotinamide adenine dinucleotide oxidized form (NAD⁺)
138 and other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

139

140 **2.2 N-IMS synthesis.** N-IMS was prepared according to the method in Patent no. 6655246
141 (Kimura, Hara, & Lang, 2020) with slight modification; dextran T 10 (9.6 g) and α -cyclodextrin
142 (9.7 g) were dissolved by boiling in a microwave at the molar concentrations of 25 mM and 200
143 mM, respectively in 50 mL of 50 mM sodium acetate buffer, pH 6.0, and preincubation at 20 °C.

144 The reaction mixture was further incubated with CGTase (1 mL; 0.28 U/mL) for 3 h before boiling
145 for 10 min. The mixture was subsequently incubated with dextran glucosidase (1 U/mL) at 37 °C
146 for 24 h to digest the residual dextran T 10, boiled, and centrifuged at 11,300 ×g to obtain the
147 supernatant. N-IMS in the supernatant was purified by precipitation with precooled methanol (60%,
148 v/v), dialysis with a 3500 kDa Spectra/Por7 membrane (Rancho Dominguez, CA, USA) to remove
149 glucose and oligosaccharides with DPs ≤21, and passage through a manually packed ion exchanger
150 Amberlite MB-4 column (Organo, Tokyo, Japan) before freeze-drying.

151

152 **2.3 Characterization of N-IMS.** The linkage composition and DP were obtained by ¹H NMR and
153 gel filtration HPLC analyses, respectively, according to previous methods (Lang et al., 2014a). The
154 yield was determined by the amount of N-IMS divided by the amount of acceptor substrate
155 (dextran T 10). Structure analysis of N-IMS using α-amylase treatment and gel filtration HPLC
156 followed our previous methods of Lang et al. (2022a). Additionally, β-amylase and dextranase
157 digests were performed similarly at pH 4.8 and 5.2, respectively. For an iodine staining assay, N-
158 IMS (2 mg/mL in water, 125 μL) was mixed with iodine reagent (2.0 g iodine and 20.0 g potassium
159 iodide in 1 L with water; freshly prepared; 5 μL), then fulfilled with 370 μL water, and allowed to
160 stand for 5 min. The absorption spectra were monitored over the range of 350–800 nm without
161 dilution (U2900 spectrophotometer, Hitachi, Tokyo, Japan). Synthetic amylose (DP = 28) was used
162 as a reference. TNS is practically nonfluorescent in an aqueous solution but adapts a large
163 fluorescence intensity in more hydrophobic environments (Beyer, Craig, & Gibbons, 1973). To
164 determine the stability constant (K_c) of N-IMS, the complexes of 0.5 μM TNS with N-IMS (0, 1, 2,
165 4, 6, 8, and 10 mg/mL) in water were prepared by mixing them for 30 min in the dark at 25 °C and
166 then transferred into 96-well black plate. The emission spectra were recorded from 400–550 nm by
167 a fluorescence microplate reader (Tecan, Infinite M200-HAFH, Tokyo, Japan) at an excitation
168 wavelength of 360 nm. The K_c value was calculated using Equation 1 according to the methods of
169 Buranaboripan, Lang, Motomura, & Sakairi (2014) and Lang et al. (2022b):

$$170 \quad \frac{1}{I-I_0} = \frac{1}{I'-I_0} + \frac{1}{K_c(I'-I_0)[H]} \quad (\text{Equation 1})$$

171 In the equation above, I_0 , I , and I' are the initial fluorescence intensities of TNS without N-IMS,
172 with N-IMS of different concentrations ($[H]$), and with N-IMS at the maximum concentration,
173 respectively.

174

175 **2.4 Solubility test of lipophilic dyes with N-IMS and inclusion analysis.** Excess amounts of four
176 dyes (AV, ER, MR, and MY; 1.0 mg) were mixed vigorously with 100 μL N-IMS at 0–10 mM (0,
177 2, 4, 6, 8, and 10 mM) in water at 25 °C for 4 h. The supernatant was collected by centrifugation at
178 12,000 ×g for 10 min three times. This portion was then diluted with 1 M NaOH solution, and the
179 dye concentration was quantified by measuring the maximum absorptions (λ_{max}) at 556, 450, and

180 434 nm for AV, ER, and MR, respectively, using calibration curves constructed with the dye
181 standards dissolved in 1 M NaOH. In the case of the MY test, DMSO was utilized instead with a
182 λ_{\max} of 425 nm. The equilibrium constant (K_s) was determined using Equation 2:

$$183 \quad K_s = \frac{\text{Slope}}{S_0(1-\text{Slope})} \quad (\text{Equation 2})$$

184 where the slope was obtained from the initial straight line of the plots of lipophilic dye
185 concentrations against N-IMS concentrations (see Fig. 2B; open-circled plots), and S_0 is the
186 solubility of the dye in water in the absence of N-IMS by following previous methods (Higuchi &
187 Connors, 1965; Lang et al., 2014a). The same experiment was performed again with N-IMS at 0–
188 50 mM (0, 10, 20, 30, 40, and 50 mM) in 50 mM Britton-Robinson buffer, pH 6.0, for comparison.
189 Only for the MY test were the samples centrifuged before spectroscopic measurement to remove
190 precipitant due to salts in buffer precipitated in DMSO. The influence of medium pH on the water
191 solubility of MR was investigated in the pH range of 4.0–9.0 in 50 mM Britton-Robinson buffer
192 with and without 10 mM N-IMS. Inclusion ability was estimated by recording the UV–vis spectra
193 of N-IMS solution (0, 1, 5, 10, 15, and 20 mM) containing dyes as guest compounds (20, 40, and
194 35 μM for AV, ER, and MR, respectively, in 50 mM Britton-Robinson buffer, pH 6.0). The MY
195 test was carried out at 40 μM in the same buffer at pH 2.0, as the dye is acid-soluble. For the
196 conformational analysis, the dyes in excess were dissolved in 100 μL of 50 mM N-IMS dissolved
197 in 50 mM Britton-Robinson buffer (pH 6.0). The soluble complexes (50 μL) were then taken and
198 dried in vacuum. The dried complexes were dissolved in 0.5 mL water, and the circular dichroism
199 spectra were recorded from 200–550 nm by a Jasco Ps-450 spectropolarimeter (Tokyo, Japan).

200 Stoichiometry of MY and N-IMS in the complexes was estimated based on a Job's plot
201 method (Buranaboripan, Lang, Motomura, & Sakairi, 2014). The total concentration of N-IMS and
202 MY was maintained constantly at 100 μM (i.e., $[\text{MY}] + [\text{N-IMS}] = 100 \mu\text{M}$) in 0.1 M Britton-
203 Robinson buffer, pH 1.0. After standing for 15 min, the absorption spectra of a series of MY and
204 N-IMS mixtures were recorded from 200–650 nm. The ΔAbs value at 318 or 512 nm (λ_{\max} ; see Fig.
205 4A) was obtained from the absorbance difference of MY in the presence and absence of N-IMS. In
206 Job's plots, the x-axis value corresponds to the mol ratio of MY (P ; see Fig. 4B), where $P =$
207 $[\text{MY}]/([\text{MY}] + [\text{N-IMS}])$. The y-axis value becomes the ΔAbs multiplied by P . The MY
208 complexed with the short-amylose (DP = 28) and α -cyclodextrin was prepared for comparison.

209 The ^1H NMR and 2D NOESY analyses were recorded for pure MY and its inclusion
210 complex (formed from mixture of 20 mM MY and 20 mM N-IMS), respectively, at 298 K using a
211 Bruker 500 MHz spectrometer. MY is soluble in acidic solution, however, the N-IMS-associated
212 inclusion interaction might weaken the ionization of dye. Therefore, acidic condition was formed
213 by dropwise-addition of deuterium chloride (5 μL) to dissolve about 2.25 mg of MY in 0.5 mL D_2O
214 (20 mM MY) containing 20 mM N-IMS, and the solution pD measured afterward was 0.57. The

215 samples were kept overnight at 25 °C and centrifuged before analysis. For NOESY, the spectrum
216 acquired a mixing time of 100 ms and 16 scans.

217

218 **2.5 Preparation of artificial dye-contaminated soils (dye soil) and their desorption test.**

219 Moistureless garden soil was homogenized thoroughly in water, autoclaved at 121.5 °C for 15 min,
220 washed with water three times, and dried in a vacuum (the so-called control soil). To mimic the
221 soils contaminated with industrial discharge, two methods of soil treatment were prepared by
222 applying initialized fixing dyes to the soils at 1.0 mg/g as follows. (1) Dyes adsorbed to acidic soil:
223 the control soil (3 g) was suspended in 30 mL of 0.1 M sodium acetate buffer, pH 3.0, in three
224 Falcon tubes and then mixed with AV, ER, and MR (3 mg, predissolved in 1 mL of 1 M NaOH).
225 The tubes were mixed frequently by vortexing at 25 °C for 3 h. The dye soil was collected by
226 centrifugation, repeatedly washed with water twice and dried. At this time, MY adsorption was
227 skipped, as it is alkali-insoluble. (2) Dyes adsorbed to neutral soil: the above protocols were
228 prepared identically, but all four dyes were dissolved in ethanol (1 mL) before placing them into
229 the water-suspended control soils. To evaluate the efficiency of dye adsorption, the desorption of
230 dye from (1) and (2) was performed by two methods. (i) Extraction by KOH. Dye soil and control
231 soil (10 mg) were mixed with 1 mL of 0.1 M KOH in microtubes by vortexing for 4 h. The
232 extracted portion was 400 µL after centrifugation at 3000 ×g for 10 min and acidified with 100 µL
233 of 6 M hydrochloric acid. This allowed the dye to incorporate into the ethyl acetate layer (500 µL)
234 after mixing by vortexing and centrifuging. The upper organic layer (100 µL) was taken and
235 evaporated to dryness. The dye content in the dried portion was quantified following the methods
236 described in Section 2.4. (ii) Extraction by chloroform + water + methanol + acetone immiscible
237 solution (CWMA, 1:1:1:1, v/v) was carried out based on the best recovery data reported by Imran
238 et al. (2015). Briefly, dye soil and control soil (10 mg) were mixed with CWMA by an ordered
239 addition of 250 µL of chloroform, acetone, methanol, and water and mixed for 4 h. The lipophilic
240 dyes were separated into the bottom layer of chloroform/acetone after centrifugation (3,000 ×g, 10
241 min). This portion was withdrawn (100 µL) and dried by speed-vacuum. The dye content
242 determinations followed the methods described in Section 2.4, and DMSO was utilized for the MY
243 test.

244

245 **2.6 Azoreductase assays.** Dye stocks (AV, ER, MR, and MY, 500 µM) were prepared in ethanol,
246 and the solubility of dyes in enzymatic assays was maintained with 10% (v/v) ethanol in 50 mM
247 Britton-Robinson buffer, pH 6.0. The BrAzo activity assay (1 mL) contained 100 µL dye stock
248 (final dye concentration, 50 µM), 200 µM NADH, and BrAzo at 25 °C. One unit of BrAzo activity
249 was defined as the amount of enzyme catalyzing the reduction of 1 µmol of dye per min (Lang et
250 al., 2013a). BrAzo and GDH were coupled for the continuous generation of NADH. In this sharing

251 relationship, we measured the enzyme units of BrAzo and GDH by following the decrease and
 252 increase in the absorbance of NADH at 340 nm, respectively. The BrAzo activity assay contained
 253 50 μM MR sodium salt, 200 μM NADH, and BrAzo, whereas the GDH assay contained 50 mM
 254 glucose, 200 μM NAD^+ , and GDH. The activity units were defined as 1 μmol NADH oxidized or
 255 NAD^+ reduced per min, using an extinction coefficient of $6.3 \text{ mM}^{-1} \text{ cm}^{-1}$ for BrAzo and GDH,
 256 respectively. In the Ping-Pong Bi-Bi mechanism, 2 mols of NADH are used to reduce 1 mol of azo
 257 dye via the NADH pathway (Bin et al., 2004); therefore, the unit concentrations of BrAzo and
 258 GDH were initially maintained at 1 and 2 U/mL throughout the study, respectively, as GDH was
 259 required to supply 2 mols of NADH to the BrAzo reaction for 1 mol of azo dye reduction.

260

261 **2.7 Lipophilic dye decolorization in the coupling reaction of BrAzo/GDH/N-IMS**

262 **2.7.1 Decolorization of four dyes.** The batch reaction mixture of N-IMS for dye dissolution and
 263 decolorization was determined with a 96-well plate through a Multiskan Ascent microplate reader
 264 (Thermo Fisher Scientific, Shanghai, China). The 100 μL assay was fixed with dried portions of 2
 265 mM AV, ER, MR, and MY. To prepare this solid dye plate, 5 μL of 40 mM dye stock in ethanol
 266 solution was placed at one side of the well bottom, keeping the center clear for the possible well-
 267 read, and the plate was stored at 60 $^{\circ}\text{C}$ for 20 min to remove residual ethanol. Then, 100 μL of N-
 268 IMS solution at 0, 1, 5, 10, and 20 mM in 50 mM Britton-Robinson buffer, pH 6.0, was added to
 269 the well plate by a multichannel pipette. For the biodegradation test, the above N-IMS solutions
 270 were prepared identically but supplemented with glucose (50 mM) and transferred into the dried
 271 dye wells. Then, the enzymes (BrAzo 1 U/mL and GDH 2 U/mL) and NAD^+ (2 mM) were placed,
 272 and the plate was wrapped by a clear sheet. The shaking mode was started immediately at the
 273 beginning with 960 rpm for 10 s. The absorbance at 450 nm was autorecorded every 2 min for 120
 274 min. The concentrations of dissolved dyes were calculated with the calibration curves. Sigmoid
 275 model parameters were obtained by fitting (Equation 3) with the experimental data of dissolved dye
 276 concentration (y) as a function of time (x) using Solver-based Microsoft Excel programming:

$$277 \quad y = C_0 + \left(\frac{ax^b}{c^b + x^b} \right) \quad (\text{Equation 3})$$

278 where C_0 is the dye concentration. The a (μM) is $C_M - C_0$, where C_M (μM) represents the dye
 279 concentration at equilibrium. The b ($\mu\text{M}/\text{min}$) is the initial rate (slope) of solid dye transformation
 280 to a soluble form, and c (min) is the sigmoid midpoint or time point at which the sigmoidal curve
 281 reaches half of its maximum solubility. Consequently, the inclusion capacity of dyes in the N-IMS
 282 (q_N , mg/g) complex was calculated by Equation 4:

$$283 \quad q_N = \left(\frac{aMW_{\text{dye}}V}{1000M_1} \right) \quad (\text{Equation 4})$$

284

285 where a is obtained from the Equation (3). M_1 is the mass of N-IMS utilized (mg) and V is the
286 assay volume (1×10^{-4} L).

287

288 **2.7.2 Decolorization process of MY extract.** MY was scaled up for 1.6 g of neutral soil by
289 CWMA solution (160 mL) following the protocol described in Section 2.5, filtrated to remove the
290 residual soil by a GF/A glass microfiber filter (GE Healthcare, Buckinghamshire, UK), and dried.
291 The dried portion was redissolved in methanol to adjust to an initial concentration of 2 mM,
292 transferred to 1 mL into two microtubes and allowed to dry again by speed vacuum. Tube no. 1 was
293 replenished with BrAzo/GDH and additives (1 mL) with the same composition and concentration
294 of the assay in Section 2.7.1. Tube no. 2 was filled with BrAzo/GDH including additives and 20
295 mM N-IMS in 1 mL. The tubes were incubated at 25 °C with stirring by a ball-shaped magnetic bar.
296 Portions of the reaction mixture (100 μ L) were withdrawn (after 20 min and 1–3 days) and heated
297 at 100 °C for 10 min to terminate the enzyme reaction. The aromatic amines were extracted with
298 100 μ L of ethyl acetate, and the upper layer (50 μ L) was taken after centrifugation at 12,000 \times g (10
299 min, 25 °C), dried, and redissolved in 50 μ L of an HPLC mobile phase containing acetonitrile and
300 0.1% (v/v) phosphoric acid in the ratio of 3:7, v/v. The samples were subjected to HPLC analysis
301 using the same instruments of Lang et al. (2014b) with a Cosmosil Cholesterol column (4.6 \times 150
302 mm, Nacalai Tesque), and the isocratic eluent was run at 40 °C with a flow of 1 mL/min and a UV
303 detector at 245 nm. 4-Dimethyl-*p*-phenylenediamine was used as an external standard.

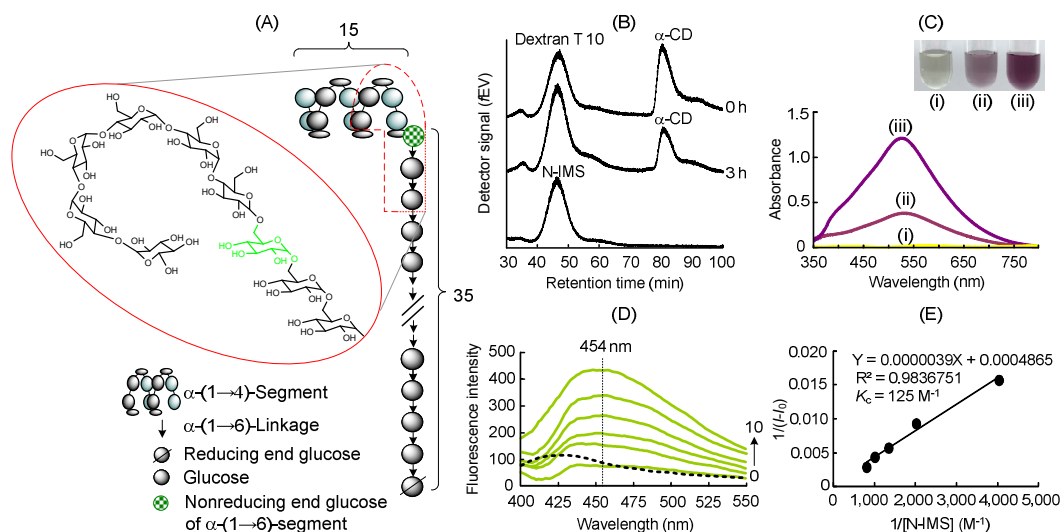
304

305 **3 Results and discussion**

306 **3.1 Synthesis and characterization of N-IMS.** CGTase is known to catalyze an opening of
307 cyclodextrin and subsequently transfer the linear dextrin towards the nonreducing end of the glucan
308 acceptor (known as the coupling reaction) (Yoon & Robyt, 2006). In this study, CGTase was
309 exploited to graft an α -(1 \rightarrow 4)-segment of α -cyclodextrin donor molecules to dextran T 10 (DP =
310 48). The hydrophobicity was expected to arise from a stereochemical constraint of the new segment
311 as the chain of sole α -(1 \rightarrow 6)-glucosyl units of dextran is only highly hydrophilic. The increase of
312 the peak area around the original dextran and decrease in about half of initial α -cyclodextrin in the
313 reaction mixture were detectable in the gel filtration HPLC at the end of the CGTase reaction stage
314 (Fig. 1B). The unreacted dextran would be next digested by dextran glucosidase into free glucose
315 and completely removed by dialysis as well as the remained α -cyclodextrin. The 1 H NMR spectrum
316 of purified N-IMS with the integration data is shown in Fig. S1. The obtained yield was 0.97 or
317 97%, w/w. N-IMS has an α -(1 \rightarrow 4)-linkage composition of 29.0% in the chain of an average
318 molecular weight of 8,100 Da, with a total DP of 50 comprising 15 and 35 units for α -(1 \rightarrow 4)- and
319 α -(1 \rightarrow 6)-glucose, respectively. The structure of N-IMS was confirmed by treatment with the three
320 enzymes of α -amylase, β -amylase, and dextranase. α -Amylase catalyzes the internal hydrolysis of

321 α -(1 \rightarrow 4)-glycosidic segments containing three or more α -(1 \rightarrow 4)-glucosyl moieties, resulting in the
322 production of the mixture of maltose, maltotriose, and maltotetraose. β -Amylase is an exo-
323 hydrolase to successively release maltose units from the nonreducing ends of α -(1 \rightarrow 4)-linked chain.
324 Dextranase is an endo-hydrolase that randomly hydrolyzes the α -(1 \rightarrow 6)-linked substrate, resulting
325 in the production of α -(1 \rightarrow 6)-linked glucosaccharides. Resultant hydrolysates of N-IMS (Fig. S2A)
326 digested with α -amylase and β -amylase were nearly identical. The peak area of N-IMS (retention
327 time = 48.517 min; DP = 50) decreased by approximately 30%, and the retention time shifted to
328 smaller values of 49.653 min (DP = 37; α -amylase) and 49.442 min (DP = 39; β -amylase). Results
329 indicate that α -(1 \rightarrow 4)-segment with at least DP = 13 (or 11) binds to the nonreducing end of α -
330 (1 \rightarrow 6)-segment, which almost agrees with DP = 15 expected previously for α -(1 \rightarrow 4)-chain.
331 Dextranase treatment displayed the loss of the original peak of N-IMS by the digestion of the α -
332 (1 \rightarrow 6)-main chain. The peak corresponded to DP = 15 of the α -(1 \rightarrow 4)-segment was not clearly
333 seen. It was likely eluted at leading-shoulder peak (about 53 min) of the main peak of
334 isomaltooligosaccharides (59.575 min), or/and the maltodextrin with DP of 15 retrograded and was
335 subsequently removed from sample solution. Fig. S2B shows the three enzymes-associated
336 digestion models of N-IMS. Interestingly, CGTase preferentially reacts with smaller acceptor
337 substrate of dextran (DP = 35) even for using dextran T 10 (average DP = 48). Actual size of
338 dextran T 10 ranges from DP = 11 to DP = 250.

339 The proposed model structure of N-IMS was shown in Fig. 1A where the structural feature
340 of N-IMS was subsequently discussed as follows. Iodine staining was used to qualify the inclusion
341 property emerging from the new α -(1 \rightarrow 4)-segment since it would be a negative color for the iodine
342 complexed with both substrates of α -cyclodextrin and dextran but positive for amylose. Swanson
343 (1948) suggested that the iodine color was related to the length of amylose in which iodine
344 molecules were held lengthwise within the helix of six glucose residues per turn. The bathochromic
345 shift from 490 to 568 nm (red to blue) was observed when dextrin/amylose chains are lengthened
346 from 14.5 to over 45 glucose units (Bailey & Whelan, 1961). Consequently, the short-amylose with
347 DP = 28 was used as a reference in this study since it maintains pure α -(1 \rightarrow 4)-glycosidic linkage
348 resulted in a purple color with a λ_{\max} of 526 nm (Fig. 1C). Not surprisingly, the spectrum of N-
349 IMS containing 15 units of α -(1 \rightarrow 4)-glucosyl segment formed one-third of the maximum
350 absorption of the short-amylose complex because the α -(1 \rightarrow 4)-chain of N-IMS was shorter, and
351 the λ_{\max} was a slight shift to 530 nm. Furthermore, the fluorescence emission spectra of TNS were
352 increased corresponding with the increasing concentrations of N-IMS and the K_c determined by the
353 double-reciprocal plots (Fig. 1D and 1E) of N-IMS/TNS complexes was 125 M^{-1} , significantly
354 higher than 14.8 M^{-1} of the L-IMS/TNS complex (Lang et al., 2022b). N-IMS thus possessed a
355 sufficient hydrophobicity and we proposed its structural feature in merits with the lipophilic azo
356 dye solubility in the next study.



358

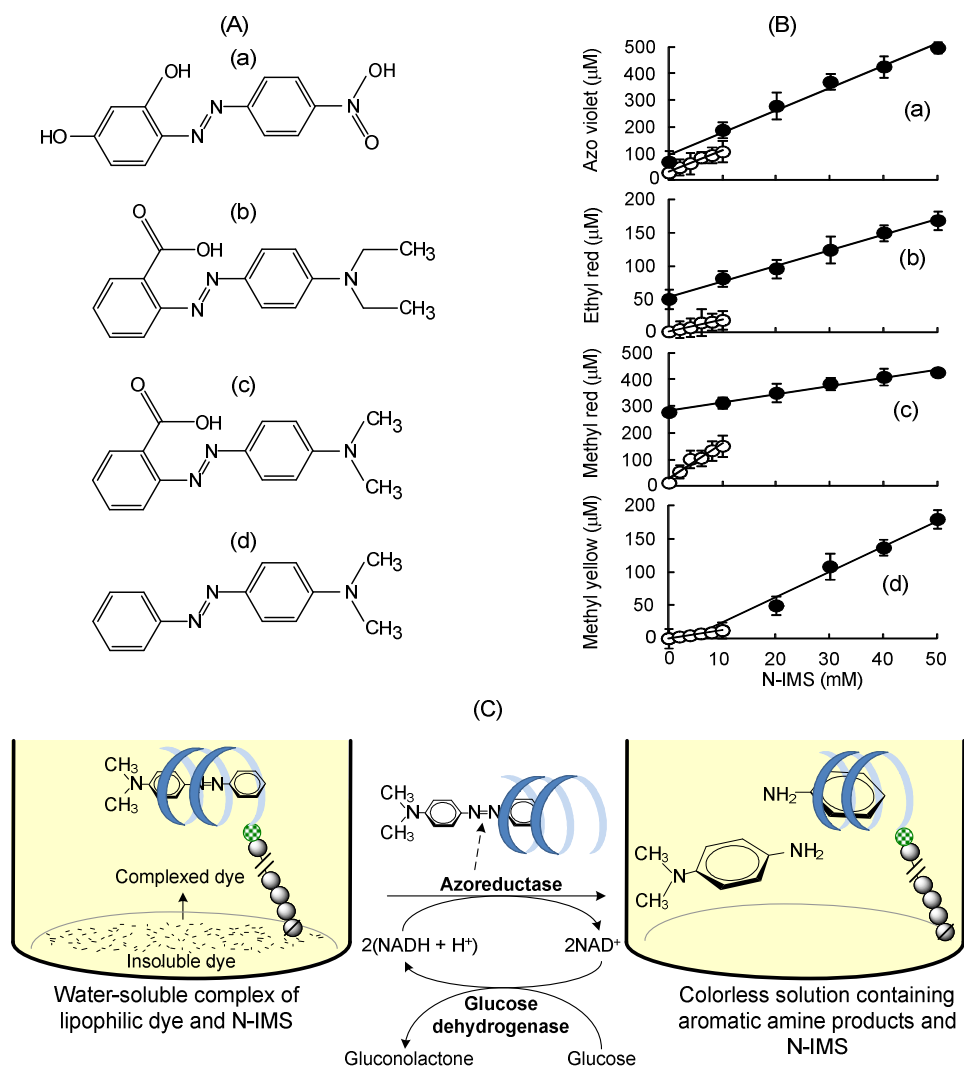
359

360 **Fig. 1.** (A) Model structure of nonreducing terminal isomaltomegasaccharide (N-IMS). Green
 361 glucosyl residue represents the connecting part of dextran main chain to α -(1 \rightarrow 4)-segment. (B) Gel
 362 filtration chromatograms of the enzymatic reactions at 0 and 3 h [incubated with α -cyclodextrin (α -
 363 CD) and dextran T 10 at 20 °C] and purified N-IMS. (C) UV-vis absorption scanned spectra of the
 364 iodine staining solution of α -cyclodextrin (i), N-IMS (ii), and short-amylose DP = 28 (iii) and their
 365 resulted color (inset). (D) Fluorescence spectra of TNS (0.5 μ M) in the presence of N-IMS (0
 366 mg/mL, dashed line; 1–10 mg/mL, green lines). Black arrow indicates the direction of the
 367 increasing concentrations of N-IMS. Dotted line depicts the emission wavelength at 454 nm. (E)
 368 The Benesi-Hildebrand double-reciprocal plots of the emission intensity at 454 nm against the
 369 series of N-IMS concentrations where the I_0 value was 87.5. K_c was calculated from the values of
 370 y-intercept/slope.

371

372 **3.2 Solubility enhancement and inclusion ability.** We evaluated the phase solubilities of four
 373 lipophilic dyes with increasing concentrations of N-IMS, and the results are shown in Table 1 and
 374 Fig. 2B. The K_s values determined with N-IMS concentrations of 0–10 mM in water were 1,786,
 375 851, 491, and 242 M^{-1} for MY, ER, MR, and AV, respectively, depicting a stoichiometric ratio of
 376 1:1 (N-IMS:dye), as it was the linear function corresponding to the A_L -type profile (Fig. 2B). N-
 377 IMS markedly improved the solubility of ER (with the K_s value of 851 M^{-1}) compared with the
 378 binding affinity with L-IMS in which the K_s value of 10 M^{-1} was previously determined (Lang et al.,
 379 2014a). According to buffers maintaining a constant pH for enzyme activity, we thus investigated
 380 the inclusion ability of N-IMS at higher concentrations in Britton-Robinson buffer at pH 6.0, as
 381 these constitute vital data for modeling enzymatic biotreatment. The results indicated that the
 382 solubilities of AV, ER and MR were slightly promoted by the buffer, as seen in the y-intercept

383 areas of Fig. 2B (a–c) and the values depicted in Table 1. Upon increasing the N-IMS concentration,
384 the solubilities of AV, ER, and MR still increased proportionally with the A_L -type relationship, but
385 the K_s values decreased to 89, 44, and 11 M^{-1} for AV, ER, and MR, respectively. In aqueous
386 solution, free dyes can have different resonance structures and tautomer forms depending on the pH
387 of the medium. Khouri, Abdel-Rahim, & Shamaileh (2013) reported the four forms of *m*-MR as *m*-
388 MR^- , *m*-HMR, *m*- H_2MR^+ , and *m*- H_3MR^+ which are for the anionic (basic), mono-protonated
389 (neutral), di-protonated (acidic), and tri-protonated (strongly acidic) forms, respectively. The basic
390 forms are the most highly soluble for AV, ER, and MR but opposite to MY. MR is red in pH values
391 under 4.4, yellow in pH values over 6.2, and orange in between: (red 4.2–6.2 yellow). The other
392 dyes were also ionized and changed the colors: ER, red 4.9–7.0 yellow; AV, yellow 11.0–12.0
393 violet; and MY, red 2.9–4.0 yellow. In Fig. S3, the influence of medium pH (pH 6.0–9.0) on the
394 phase solubility enhancement of MR indicates that the inherent solubility increases remarkably
395 from 0.265 mM (pH 6.0) to 11.8 mM (pH 9.0) following our previous statement. However, the
396 solubility enhancement by N-IMS was effective only at a pH range of 4.0–6.0, and the detracting
397 was mainly seen at pH 9.0 (yielding a form of hydrazone). A similar relevant phenomenon was
398 observed in Fig. 2B (a–c). It is suggested that AV, ER, and MR can adopt their hydrazone
399 tautomeric forms under neutral conditions, resulting in slight solubility compared with their
400 solubility in water, but this weakened the hydrophobic interaction inside the inclusion interior of N-
401 IMS, resulting in decreases in the above K_s values. In contrast, MY solubility was never susceptible
402 to neutral pH, but the phase solubility diagram likely followed the broad-bottom exponential A_P -
403 type in the presence of increasing N-IMS concentrations (Fig. 2B (d)), yielding a higher K_s of 5,746
404 M^{-1} (Table 1). At high concentrations of N-IMS, in principle, more than one stoichiometry can be
405 proposed for the binding of MY to the N-IMS cavity. Consequently, the basic intermolecular
406 characterization by Job's plot and 2D NOESY was further discussed in the following section. The
407 above result emphasized that the large free dyes that were always generated in the highly ionized
408 state preferred to stay outside the N-IMS cavity. Despite this, MY could form a high-affinity
409 complex and dissolve in neutral conditions only with N-IMS binding.
410



411
 412 **Fig. 2.** (A) Chemical structures of the four lipophilic azo dyes used in this study: a, azo violet; b,
 413 ethyl red; c, methyl red; d, methyl yellow. (B) Phase solubility diagrams of the dyes upon the
 414 addition of N-IMS (0–10 mM) in water (○) and N-IMS (0–50 mM) in 50 mM Britton-Robinson
 415 buffer, pH 6.0 (●). (C) Scheme of reactions taking place between methyl yellow and N-IMS and
 416 digestion with an azoreductase coupling reaction.

417

418 **Table 1.** Equilibrium constants (K_s) of lipophilic dyes with N-IMS inclusion and the maximum
 419 aqueous solubilities of lipophilic azo dyes in water and 50 mM Britton-Robinson buffer, pH 6.0.

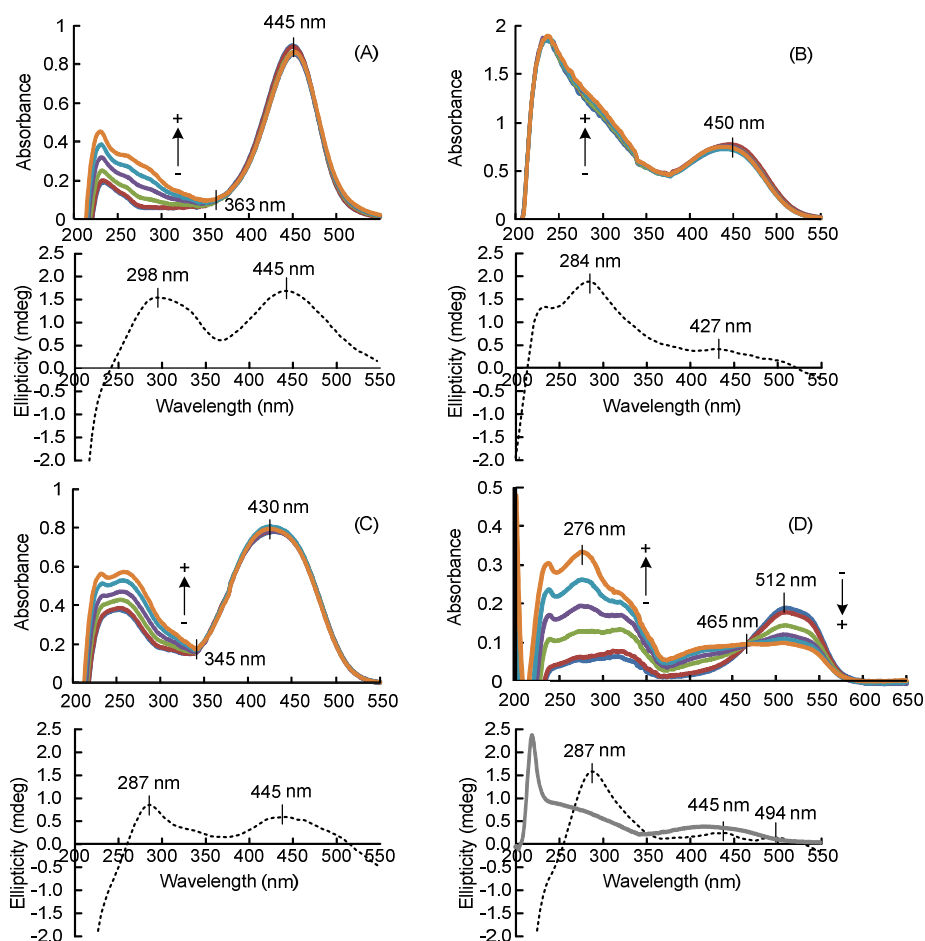
Lipophilic dye	Water		Britton-Robinson buffer, pH 6.0	
	K_s (M^{-1})	Maximum solubility (μM)	K_s (M^{-1})	Maximum solubility (μM)
Azo violet	242	29.8 ± 3.6	89	95.2 ± 1.6
Ethyl red	851	2.1 ± 0.3	44	53.2 ± 3.2

Methyl red	491	14.0 ± 0.5	11	284.2 ± 7.3
Methyl yellow	1,786	0.7 ± 0.2	5,746	0.7 ± 0.2

420

421 The inclusion ability of N-IMS examined in terms of UV-vis spectroscopic titration (0–20
 422 mM) using four lipophilic dyes as a guest compound is shown in Fig. 3 (A–D, top panels). The
 423 absorption maxima at visible wavelengths among AV, ER, and MR at pH 6.0 (with visible λ_{max} at
 424 445, 450, and 430 nm, respectively) remained unchanged when increasing the N-IMS
 425 concentrations, but increasing intensities in UV regions were clearly observed for AV and MR, and
 426 those isosbestic points were formed at 363 and 345 nm, respectively. Khouri et al. (2013) revealed
 427 a decrease in the maximum absorption at 450 nm and a blueshift of approximately 20 nm in the
 428 UV-vis spectrum of *m*-MR with the addition of α -cyclodextrin in basic solution at pH 10.39, and
 429 the absorption in the UV region at 268 nm also decreased without any shift. Hence, their patterns
 430 are completely different from our MR incorporated in the N-IMS cavity.

431



432

433 **Fig. 3.** Spectroscopic examination of the inclusion ability of N-IMS and the four lipophilic dyes:
 434 (A), azo violet; (B), ethyl red; (C), methyl red; (D), methyl yellow. Solvents: Britton-Robinson

435 buffer pH 6.0 (A–C) and pH 2.0 (D). (Upper panel) UV–vis spectra of the dyes in the presence of
436 N-IMS (N-IMS concentration: 0, 1, 5, 10, 15, and 20 mM, read from - to +). (Lower panel)
437 Circular dichroism spectra of the dyes (dashed lines) in the presence of N-IMS (50 mM in Britton-
438 Robinson buffer, pH 6.0). Gray line in (D) is only the absorption spectrum of methyl yellow at pH
439 6.0.

440

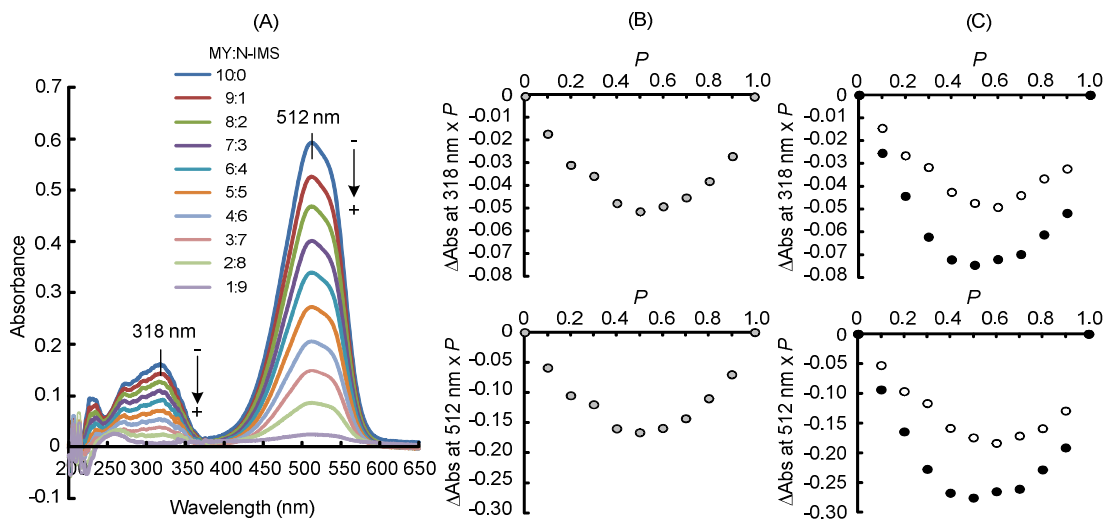
441 Interestingly, the addition of N-IMS to an aqueous solution of MY at pH 2.0 resulted in a
442 marked decrease in the intensity of the visible spectra of MY at 512 nm without any shift in its λ_{\max}
443 value (Fig. 3D, upper). In addition, the intensity in the UV region of 276 nm with a shoulder over
444 318 nm was significantly increased with the formation of an isobestic point at 465 nm. Ferreira and
445 Ando (2012) assigned the bands of MY in acidic pH at 318 and 512 nm to azo and hydrazone
446 tautomers, respectively, where hydrazone species are predominant. After inclusion with α -
447 cyclodextrin, MY adapted the structure to be an azo tautomer, as the interior cavity precludes its
448 protonation. It is clearly seen that the incorporation of MY with N-IMS inclusion has the same
449 trend of spectrum quenching at 512 nm, suggesting that the inclusion of the azo bond penetrated
450 deeply inside of the N-IMS cavity and kept the dimethylamino moiety outside as the proposed
451 inclusion mechanisms of α -cyclodextrin. However, another absorption pattern of MY in the N-IMS
452 cavity is completely different compared with the spectra of α -cyclodextrin. Similarly to amylose
453 structure, the cavity of N-IMS may adopt a left-handed single helical conformation with its specific
454 helix dimensions of 6–8 glucopyranose units per turn upon the complexation with ligands.
455 Accordingly, it can be considered for approximately 2.5, 2.14, and 1.87 cyclic turns of the α -
456 (1→4)-helical structure of DP = 15 segment for 6, 7, and 8 glucopyranose units, respectively, while
457 the cavity of cyclodextrin is rigid with 1 turn. We explained the particularly different binding using
458 the absorption spectra of MY derivatives measured at two pH values of 2.0 and 6.0, assigning 278
459 nm to aniline and a shoulder at approximately 312 nm to dimethyl-*p*-phenylenediamine bands (Fig.
460 S4). Although the inclusion complexes of MY were performed at pH 2.0, the spectra observed in
461 Fig. 3D were likely the combination of aniline and dimethyl-*p*-phenylenediamine spectra
462 monitoring at pH 6.0, as seen in Fig. S4 (azo tautomer became predominant). The binding of N-
463 IMS is more responsible for aniline because the 2.14 turns of the helix cavity may envelop the
464 whole aniline molecule, resulting in an increase in the absorption intensity at 276 nm, where the
465 aniline molecule may be almost released from the narrow rim of the α -cyclodextrin cavity when the
466 azo bond deeply penetrates.

467 Next, the inclusion ability of N-IMS towards four dyes was revealed by the large positive
468 induced circular dichroism effect observed at the main bands in the visible and UV regions. In the
469 UV regions, the spectra were induced at 298, 284, 287, and 287 nm for AV, ER, MR, and MY,
470 respectively, corresponding to the $n-\pi^*$ transition of the phenyl group. The Cotton effect (positive

471 chirality) ascribed to the π - π^* transition of the azo linkage between the two benzene rings was
472 presented at visible wavelengths of 445, 427, 445, and 445 nm for AV, ER, MR, and MY,
473 respectively, corresponding to their visible absorption spectra with different degrees of ellipticity
474 dependent on the induced fit of phenyl ring substituents. All of the data confirmed that the
475 azobenzene dyes were incorporated into the N-IMS cavity with an axial orientation similar to the
476 spectral pattern in the visible regions of the ER in the β -cyclodextrin cavity observed previously
477 (Lang et al., 2014a). Variation in ratio of MY and N-IMS caused the energy transition of
478 absorption maximum at 318 and 512 nm (Fig. 4A), coinciding with aniline and dimethyl-*p*-
479 phenylenediamine bands, respectively. Stoichiometric ratio between MY and N-IMS showed the
480 maximum variation of 0.5 at both λ_{\max} (Fig. 4B), supporting the formation of 1:1-complex, which
481 agreed with those of MY-amylose complex (0.5; Fig. 4C). Based on this manner, MY may
482 penetrate deeply into the cylindrical cavity of N-IMS (2.14 helix turns) or short-amylose (DP = 28;
483 4 helix turns). But α -cyclodextrin exhibited the different maximum variation of 0.6 (Fig. 4C),
484 implying the formation of 2:1-complex of MY and α -cyclodextrin. We have no clear explanation
485 about this 2:1-complex conformation, but cavity of α -cyclodextrin might include the
486 dimethylamino moiety of one MY and the phenyl ring of another MY.

487 As shown in Fig. 5A, the ^1H NMR assigns two types of MY protons: aliphatic protons H_a
488 and aromatic protons H_b - H_f , and their chemical shifts of free or N-IMS-complexed state are shown
489 in Table S1. By formation of MY-N-IMS complex, chemical shifts change in the following order:
490 $\text{H}_b > \text{H}_f > \text{H}_c > \text{H}_d > \text{H}_e > \text{H}_a$ with $\Delta\delta$ values of 0.2233, 0.1530, 0.1518, 0.1406, 0.1020, and -0.0040
491 ppm, respectively, indicating that anomeric protons H_b - H_f interact with N-IMS and H_a of
492 dimethylamino group has no contact with N-IMS. The 2D NOESY estimated interaction sites of
493 MY and N-IMS (Fig. 5B): the NOE correlations were observed between MY protons (H_b , H_c , H_e ,
494 and H_f) and N-IMS proton (H_5), whereas this evidence was not detected clearly in the aliphatic
495 protons of dimethylamino group of H_a (Fig. S5) and the adjacent aromatic proton of H_d (Fig. 5B).
496 Proton of H-5 is located inside of N-IMS similarly to β -CD (Nishizawa et al., 2014), and therefore
497 this study demonstrates that an MY moiety exists inside of cavity of N-IMS to forms 1:1-complex.
498 Conformational rearrangement of complex might accept deep penetration of azo bond and two
499 phenyl rings. Two phenyl moieties and azo bond of MY molecule are included with N-IMS, but
500 dimethyl group is located outside of complex.

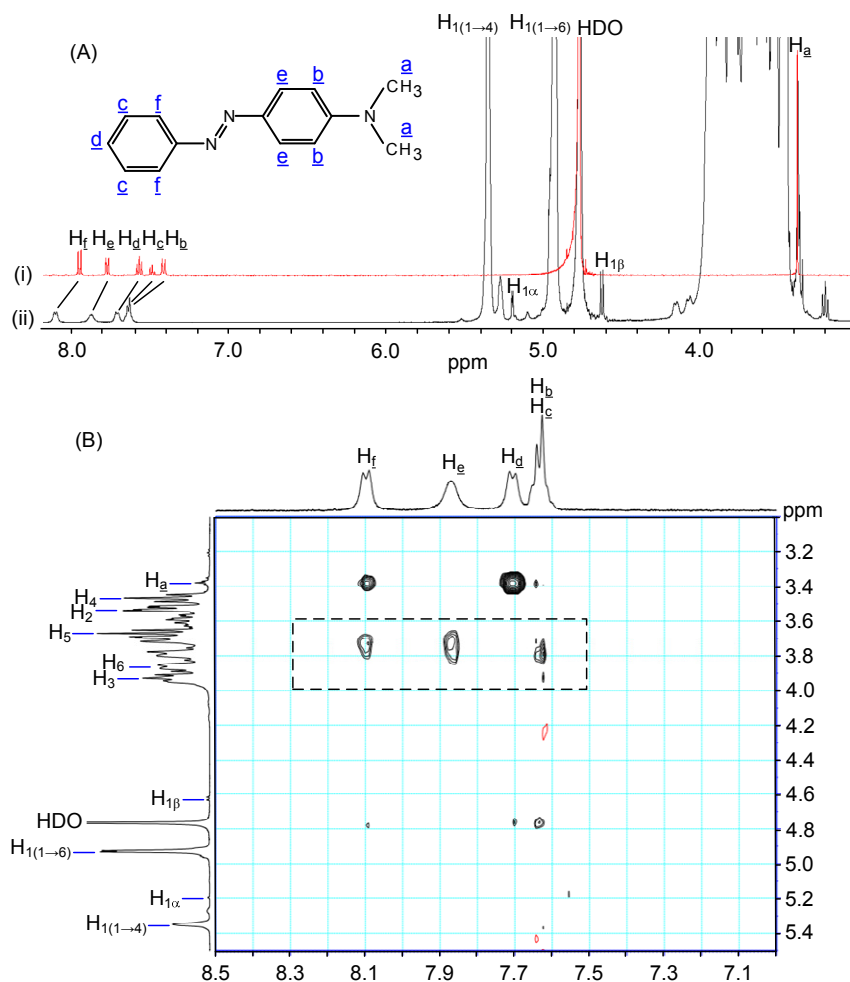
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502

503 **Fig. 4.** (A) UV-vis spectra of methyl yellow (MY) in the presence of N-IMS. Concentration ratio of
 504 MY:N-IMS changed from 10:0 to 1:9, read from – to +. (B and C) Continuous variations of Job's
 505 method for the inclusion complex of methyl yellow and N-IMS (○), α-cyclodextrin (○), and short-
 506 amylose with DP = 28 (●) in 0.1 M Britton-Robinson buffer, pH 1.0. Upper and lower panels were
 507 obtained from Δ Abs at 318 and 512 nm, respectively, where the x-axis value corresponds to the
 508 mol ratio of MY: i.e., $P = [\text{MY}]/([\text{MY}] + [\text{N-IMS}])$.

509



510

511 **Fig. 5.** (A) ¹H NMR spectra of MY (i) and its complex (ii) formed from 20 mM MY and 20 mM N-
 512 IMS under acidic conditions with 3.5 mg/mL deuterium chloride in D₂O. The a–f indicate the
 513 protons of MY. Solid lines between (i) and (ii) represent the protons, whose chemical shifts change
 514 by forming complex. (B) NOESY spectrum of the complex of MY and N-IMS (20 mM each) in the
 515 same solvent as show in Fig. 5A. Intermolecular NOEs are presented in the square.

516

517 **3.3 Soil washing treatment.** In this study, we considered the discharges of untreated effluents
 518 containing lipophilic dyes when dealing with their native medium. For example, alkali and organic
 519 agents are mainly supplied for anionic dyes, while organic media practically have general classes.
 520 However, unlike the binding of water-soluble dyes into soil, in which both substances can be
 521 incorporated in water systems (Imran et al., 2015), the incorporation of lipophilic dyes into soil in
 522 organic systems significantly minimizes their hydrophobic interaction. Moreover, the misbalance in
 523 the presence of incompatible solvent polarity and diluents may lead to reduced solubilization power
 524 and consequent precipitation. In our trials, we treated soils with acidic buffer (pH 3.0) and water
 525 (pH 6.8), which are always attractive electrostatic and hydrophobic interactions to the binding with

526 lipophilic dyes that were predissolved in 1 M NaOH and ethanol, respectively. As shown in Table 2,
 527 anionic dyes (AV, ER, and MR) potentially bound to the acidic soils (0.65–0.77 mg/g) with a
 528 slightly higher affinity than those same dyes (ethanol-based) amended to the neutral soils (0.42–
 529 0.70 mg/g). One of the possible reasons is that the functional groups of organic components of the
 530 soil surface (e.g., humic substance) can be protonated and deprotonated (e.g., –COOH, –OH, amine
 531 groups). At a consequent low pH, the binding of anionic species to soil charges becomes more
 532 positive (Nagy & Kónya, 2007). Not surprisingly, washing soil with the alkaline KOH effectively
 533 removed the dyes due to an increase in the electrostatic repulsion force, coincident with the
 534 previous desorption of anionic textile dyes by 0.1 M NaOH from the fungal beads (Lang et al.,
 535 2013b). However, the organic matter and oxides of silica and alumina also eluted spontaneously, as
 536 observed by the yellow–red color (Jozefaciuk, Muranyi, & Alekseeva, 2002). Moreover, the
 537 alkaline extraction of cationic lipophilic dye (i.e., MY) was unprofitable, as only 0.07 ± 0.01 mg
 538 MY/g was eluted, while the same sample extracted 0.89 ± 0.24 mg MY/g by CWMA solution
 539 without those interfering colors.

540

541 **Table 2.** Desorption of lipophilic azo dyes from acidic and neutral soils.

Desorbent	Dye	Acidic soil	Neutral soil
		Dye (mg/g)	Dye (mg/g)
Potassium hydroxide (0.1 M)	Azo violet	0.69 ± 0.04	0.70 ± 0.02
	Ethyl red	0.65 ± 0.11	0.62 ± 0.09
	Methyl red	0.77 ± 0.04	0.42 ± 0.00
	Methyl yellow	N.D.	0.07 ± 0.01
CWMA ^a (1:1:1:1, v/v)	Azo violet	0.84 ± 0.03	0.78 ± 0.09
	Ethyl red	0.73 ± 0.04	0.65 ± 0.05
	Methyl red	0.66 ± 0.04	0.61 ± 0.02
	Methyl yellow	N.D.	0.89 ± 0.24

542 N.D. = not determined because methyl yellow is insoluble in alkaline solution.

543 ^a Immiscible solution of chloroform + water + methanol + acetone.

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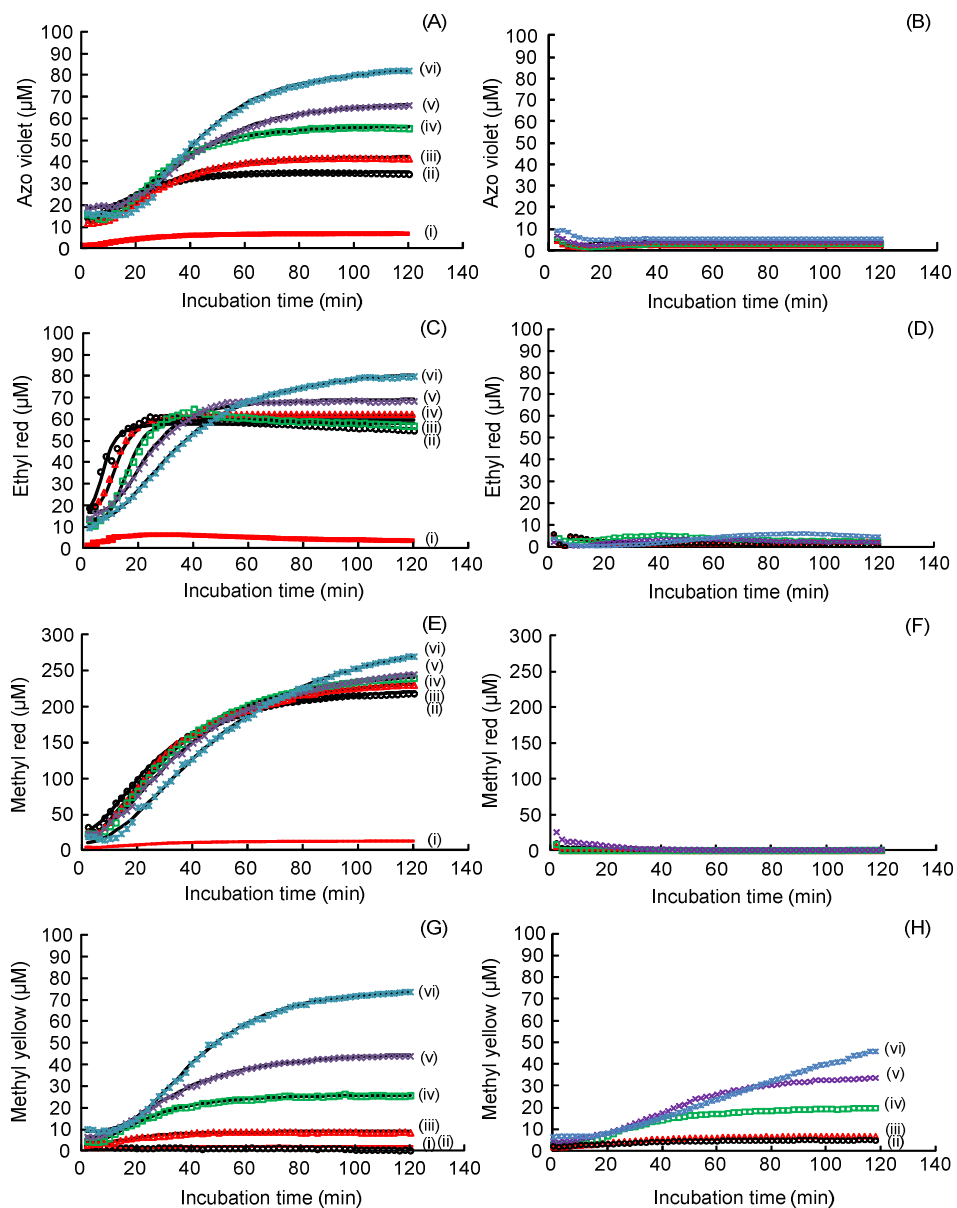
545 Our attempt at alkaline extraction might be profitable in the sense of green treatment
 546 concepts to avoid organic disposal. In this concept, the alkaline extraction solution of those anionic
 547 dyes was able to be further neutralized to the optimum biological pH values by a strong acid, and
 548 after that, the enzymatic ingredients, including buffer and cofactor, could be formulated directly.
 549 However, the poor aqueous solubility of the extracted dyes was the major issue likely found in the
 550 case of high dye concentrations (typically ER, 2 mM), in which the precipitation was immediately
 551 visible at pH values lower than 7.68 (which could not eventually adjust to pH 6.0) because water

552 could not maintain solubilization. In addition, the fraction contaminated with unknown alkali-
553 soluble substances may inhibit the enzyme activity. Furthermore, the adverse effects of soil after
554 extreme acid and alkali treatments are permanent changes in coating minerals and organic matter
555 content and result in a decrease in soil buffering capacity (Jozefaciuk et al., 2002). Taking into
556 account these foregoing facts, we then selected CWMA as the aqueous organic model to wash the
557 lipophilic dyes from soils because it was universal, and the solvents could be removed by
558 evaporation (unlike surfactants), although the solvent efficiency with safety criteria is suggested to
559 be more carefully considered. The following sections describe the dissolution kinetics by N-IMS
560 assistance subsequent to the model treatment by enzymes (Fig. 2C).

561

562 **3.4 Decolorization of dyes in N-IMS complexes.** Wettability is the first step of any dissolution
563 process to determine the interaction of solid and liquid surfaces, in which the latter is always water
564 in the case of cyclodextrin-drug complexes, and both negative exponential and sigmoid type curves
565 can be observed depending on the rate and degree of the wetting process (Szente & Szejtli, 1987).
566 The dissolution patterns of the four dyes are presented in Fig. 6 (left column). N-IMS complexes
567 carried the dyes from the solid-state at the bottoms of the well plates to the soluble form until the
568 plateau of the dye saturation was reached at its maximum concentration (C_M). The solubility of
569 solid dyes with N-IMS complexes was explained by Equation (3), as it gave rise to a sigmoid-
570 shaped adsorption kinetic profile. In this equation, two parameters, b (rate) and c ($t_{1/2}$), can
571 determine the rate-limiting step of the wettability, in which fast dissolution agrees with a large b
572 and a small c . As seen in Table S2, among the four dyes, ER, with the largest b , 3.3–2.3 $\mu\text{M}/\text{min}$,
573 and the smallest c , 7.4–35.3 min, was the rapidly soluble dye, whose b values decreased and c
574 values gradually increased in the presence of N-IMS as the dyes needed the driving force to be
575 incorporated. In addition, a higher N-IMS concentration provided more dissolved dye molecules,
576 representing concentration capacity. All dyes showed increasing a , i.e., AV, 20.5–70.3 μM ; ER,
577 40.2–73.1; MR, 207.6–310.0 μM ; and MY, 0–67.8 μM , in accordance with increasing N-IMS
578 concentration from 0–20 mM. Fig. 6C clearly supports the trends of b and c as mentioned.
579 However, at the same time, a also increased with different levels. The degree of increase of a likely
580 influenced Equation 3, resulting in a non-smooth tendency of some data in Table S2. The initial
581 velocity of dye decolorization determined for BrAzo was in the order of ER > MR > AV > MY
582 ($196.5 \pm 9.0 > 121.5 \pm 0.5 > 26.3 \pm 0.4 > 1.3 \pm 0.0$ U/mg). These degrees of enzyme activity
583 reflected the invisible residual dye in the assay well plates because the addition of the BrAzo/GDH
584 system could maintain the dye decolorization substantially, especially for AV, ER, and MR (Fig.
585 6B, 6D, and 6F). The incorporated dyes might react slowly with the enzyme, whereas the
586 dissolving dyes might react more smoothly. Even in slow reaction, the process could be eventually
587 completed with the appropriate incubation time and stirring control. Decolorization of MY (Fig. 6H)

588 in the presence of N-IMS was the slowest because BrAzo showed less specificity (151-fold lower
 589 than the ER activity). Moreover, the azo bond of MY penetrated deeply into the N-IMS cavity (as
 590 discussed in Section 3.2), consequently accessible to BrAzo only after the dye azo bond was
 591 released to the outside of complex (see equilibrium between two states shown in the left and center
 592 panels in Fig. 2C). This is a dynamic process whereby the guest molecule continuously associates
 593 and dissociates from the host molecules.
 594



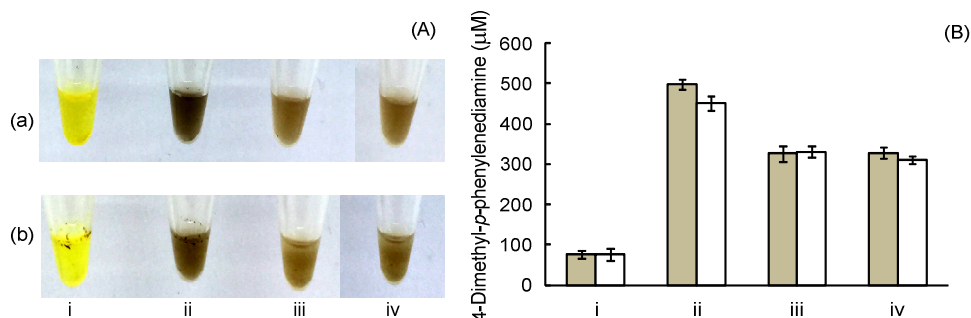
595
 596 **Fig. 6.** (Left column) Sigmoidal dissolution kinetics of the four solid lipophilic dyes, (A), azo
 597 violet; (C), ethyl red; (E), methyl red; and (G), methyl yellow in water (i) and in the presence of N-
 598 IMS from 0 (ii); 1 (iii); 5 (iv); 10 (v); and 20 (vi) mM dissolved in 50 mM Britton-Robinson buffer,

599 pH 6.0. Lines of (ii)–(iv) represent the nonlinear curve fit from Equation (3). (Right column)
600 Coupling enzymatic decolorization kinetics of the four solid lipophilic dyes, (B), azo violet; (D),
601 ethyl red; (F), methyl red; and (H), methyl yellow in the presence of N-IMS from 0–20 mM (ii–vi)
602 in 50 mM Britton–Robinson buffer, pH 6.0.

603

604 After incubating the enzymes and 20 mM N-IMS in the tube containing dried extracted
605 MY with stirring, it was observed that the clear, bright yellow color arose from the tube bottom,
606 while the yellow color in the tube without N-IMS was less visible as certain dye still bound to the
607 tube. MY has two aromatic amine metabolites, aniline (colorless) and dimethyl-*p*-
608 phenylenediamine (brown). After 1 day, the shade of the sample color changed to brown, implying
609 that MY was consumed from both tubes, with a slightly higher color in the N-IMS medium (Fig. 7).
610 After 2 days, the concentrations of dimethyl-*p*-phenylenediamine became almost equal and
611 stabilized, indicating that N-IMS improved the bioavailability of MY digestion in the initial stage.
612 The reason for the unchanged amount of aromatic amine might come from the glucose depletion in
613 the reaction as all glucose molecules might be oxidized to gluconolactone by GDH in a coupled
614 reaction to generate NADH (Fig. 2C). Long-term intermittent glucose feeding is suggested but
615 we cannot explain why dimethyl-*p*-phenylenediamine decreased after some time. Considering the
616 inclusion ability of N-IMS in the presence of high aromatic dye concentrations, the aromatic
617 moieties of the substrate and products could be competitively occupied by the N-IMS cavity
618 corresponding to the degree of hydrophobic attraction, eventually preventing the solubility
619 enhancement activity of N-IMS. We suggest the coupling of one more enzyme that can mineralize
620 aromatic amine products or alter the metabolite shapes to be inappropriate with the N-IMS cavity
621 (e.g., macromolecule products catalyzed by laccase or peroxidase), as the sequential inclusion of
622 the dye substrate can be smoother.

623



624

625 **Fig. 7.** Analysis of the digestion products of methyl yellow extract by the integration of N-IMS and
626 azoreductase from *Brevibacillus laterosporus* TISTR1911 coupled with glucose dehydrogenase (a)
627 at 20 min (i), 24 h (ii), 48 h (iii), and 72 h (iv). The process without N-IMS addition is presented

628 for comparison (b). (A) Color changes in the sampling tubes. (B) Detection of 4-dimethyl-*p*-
629 phenylenediamine with N-IMS (gray bars; a) and without N-IMS (white bars; b).

630

631 **4. Conclusion**

632 In this study, we synthesized N-IMS with a DP of 50 and 29.0% α -(1→4)-linkages by the
633 coupling reaction of CGTase. N-IMS bearing the α -(1→4)-helical segment at its nonreducing end
634 to form cavity was responsible for the specific inclusion ability and improved the aqueous
635 solubility of lipophilic azo dyes. The cavity of N-IMS could incorporate the water-insoluble azo
636 dye ligand and formed the guest-host complex in 1:1 ratio with MY and N-IMS, where MY was
637 located on the inside of cavity. Furthermore, N-IMS contributed to preventing the precipitation
638 formation of dye extracted from soil-contaminated samples. The eco-friendly decolorization system
639 of the dyes was established with N-IMS as pure and safe saccharide solubilizer under the coupling
640 of BrAzo and GDH. Chimeric N-IMS is also profitable for drug delivery approaches and designing
641 foods.

642

643 **Acknowledgments**

644 We thank Assistant Professor Rakrudee Sarthima, Mahasarakham University, Thailand, for
645 the preliminary study of azoreductase, azo dyes, and megalosaccharides. This study was partially
646 supported by a Program for Promotion of Basic and Applied Research for Innovations in
647 Biooriented Industry (BRAIN), Japan [Grant numbers 25001A, 26062B] and the Japan Society for
648 the Promotion of Science KAKENHI [Grant numbers 17H03801, 19KK0147]. The manuscript
649 received an English proof from Elsevier's Author Services.

650

651 **CRedit authorship contribution statement**

652 Weeranuch Lang: Conceptualization, Investigation, Formal analysis, Writing - original
653 draft, review & editing. Sarote Sirisansaneeyakul: Supervision, Validation. Takayoshi Tagami:
654 Resources, Supervision. Hye-Jin Kang: Formal analysis, Investigation. Masayuki Okuyama: Data
655 curation, Resources. Nobuo Sakairi: Conceptualization, Supervision. Atsuo Kimura: Writing -
656 review & editing, Resources, Supervision, Funding acquisition, Project administration.

657

658 **Declaration of competing interest.** The authors have no conflicts of interest to declare.

659

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765

766 **Figure captions**

767 **Fig. 1.** (A) Model structure of nonreducing terminal isomaltomegalosaccharide (N-IMS). Green
768 glucosyl residue represents the connecting part of dextran main chain to α -(1 \rightarrow 4)-segment. (B) Gel
769 filtration chromatograms of the enzymatic reactions at 0 and 3 h [incubated with α -cyclodextrin (α -
770 CD) and dextran T 10 at 20 °C] and purified N-IMS. (C) UV-vis absorption scanned spectra of the
771 iodine staining solution of α -cyclodextrin (i), N-IMS (ii), and short-amylose DP = 28 (iii) and their

772 resulted color (inset). (D) Fluorescence spectra of TNS (0.5 μ M) in the presence of N-IMS (0
773 mg/mL, dashed line; 1–10 mg/mL, green lines). Black arrow indicates the direction of the
774 increasing concentrations of N-IMS. Dotted line depicts the emission wavelength at 454 nm. (E)
775 The Benesi-Hildebrand double-reciprocal plots of the emission intensity at 454 nm against the
776 series of N-IMS concentrations where the I_0 value was 87.5. K_c was calculated from the values of
777 y-intercept/slope.

778

779 **Fig. 2.** (A) Chemical structures of the four lipophilic azo dyes used in this study: a, azo violet; b,
780 ethyl red; c, methyl red; d, methyl yellow. (B) Phase solubility diagrams of the dyes upon the
781 addition of N-IMS (0–10 mM) in water (\circ) and N-IMS (0–50 mM) in 50 mM Britton-Robinson
782 buffer, pH 6.0 (\bullet). (C) Scheme of reactions taking place between methyl yellow and N-IMS and
783 digestion with an azoreductase coupling reaction.

784

785 **Fig. 3.** Spectroscopic examination of the inclusion ability of N-IMS and the four lipophilic dyes:
786 (A), azo violet; (B), ethyl red; (C), methyl red; (D), methyl yellow. Solvents: Britton-Robinson
787 buffer pH 6.0 (A–C) and pH 2.0 (D). (Upper panel) UV–vis spectra of the dyes in the presence of
788 N-IMS (N-IMS concentration: 0, 1, 5, 10, 15, and 20 mM, read from - to +). (Lower panel)
789 Circular dichroism spectra of the dyes (dashed lines) in the presence of N-IMS (50 mM in Britton-
790 Robinson buffer, pH 6.0). Gray line in (D) is only the absorption spectrum of methyl yellow at pH
791 6.0.

792

793 **Fig. 4.** (A) UV-vis spectra of methyl yellow (MY) in the presence of N-IMS. Concentration ratio of
794 MY:N-IMS changed from 10:0 to 1:9, read from - to +. (B and C) Continuous variations of Job's
795 method for the inclusion complex of methyl yellow and N-IMS (\circ), α -cyclodextrin (\circ), and short-
796 amylose with DP = 28 (\bullet) in 0.1 M Britton-Robinson buffer, pH 1.0. Upper and lower panels were
797 obtained from Δ Abs at 318 and 512 nm, respectively, where the x-axis value corresponds to the
798 mol ratio of MY: i.e., $P = [\text{MY}]/([\text{MY}] + [\text{N-IMS}])$.

799

800 **Fig. 5.** (A) ^1H NMR spectra of MY (i) and its complex (ii) formed from 20 mM MY and 20 mM N-
801 IMS under acidic conditions with 3.5 mg/mL deuterium chloride in D_2O . The a–f indicate the
802 protons of MY. Solid lines between (i) and (ii) represent the protons, whose chemical shifts change
803 by forming complex. (B) NOESY spectrum of the complex of MY and N-IMS (20 mM each) in the
804 same solvent as show in Fig. 5A. Intermolecular NOEs are presented in the square.

805

806 **Fig. 6.** (Left column) Sigmoidal dissolution kinetics of the four solid lipophilic dyes, (A), azo
807 violet; (C), ethyl red; (E), methyl red; and (G), methyl yellow in water (i) and in the presence of N-

808 IMS from 0 (ii); 1 (iii); 5 (iv); 10 (v); and 20 (vi) mM dissolved in 50 mM Britton-Robinson buffer,
809 pH 6.0. Lines of (ii)–(iv) represent the nonlinear curve fit from Equation (3). (Right column)
810 Coupling enzymatic decolorization kinetics of the four solid lipophilic dyes, (B), azo violet; (D),
811 ethyl red; (F), methyl red; and (H), methyl yellow in the presence of N-IMS from 0–20 mM (ii–vi)
812 in 50 mM Britton-Robinson buffer, pH 6.0.

813

814 **Fig. 7.** Analysis of the digestion products of methyl yellow extract by the integration of N-IMS and
815 azoreductase from *Brevibacillus laterosporus* TISTR1911 coupled with glucose dehydrogenase (a)
816 at 20 min (i), 24 h (ii), 48 h (iii), and 72 h (iv). The process without N-IMS addition is presented
817 for comparison (b). (A) Color changes in the sampling tubes. (B) Detection of 4-dimethyl-*p*-
818 phenylenediamine with N-IMS (gray bars; a) and without N-IMS (white bars; b).