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Production of Cyclodextrins in Ultrafiltration Membrane Reactor Containing Cyclodextrin Glycosyltransferase from *Bacillus macerans*

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An enzyme reactor installed with ultrafiltration membrane was developed to produce α-, β-, and γ-cyclodextrins (CDs) from soluble starch by *Bacillus macerans* cyclodextrin glycosyltransferase (CGTase) tagged with 10 lysines at its C-terminus (CGTK10ase). Ultrafiltration membrane YM10 with 10,000 of molecular cutoff was chosen for membrane modification and CD production. A repeated-batch type of the enzyme reaction with free CGTK10ase resulted in a α-CD yield of 24.0 (±1.5)% and a productivity of 4.68 (±0.88) g/l-h, which were 7 times higher that those for CGTK10ase immobilized on modified YM10 membrane. Addition of 1-nonanol increased CD yields by 30% relative to the control, which might be due to prevention of the reversible hydrolysis of CDs.

Keywords: Cyclodextrin glycosyltransferase, immobilization, ultrafiltration membrane, enzyme reactor, α-cyclodextrin

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) catalyzes the conversion of starch or starch derivatives into cyclodextrins (CDs), cyclic oligosaccharides composed of six, seven, or eight α-1,4-glycosidic-linked glucosyl residues (α-, β-, or γ-CD). CGTases generally produce a mixture of CDs, glucose, maltose, and other oligosaccharides from starch [6]. The content of CDs depends on reaction conditions, substrate concentration, enzyme amount, and CGTase source [12]. *Bacillus macerans* CGTase mainly produces α-CD from starch with up to 75% selectivity [9, 11, 14]. The advantages of enzyme immobilization include the repeated use of an expensive enzyme, easy separation of products, minimization of inhibitory effects, and greater variety of reactor design [8, 15]. Immobilization of CGTase has been carried out using SP-Sepharose, IRA-900, and polyethylene film [5, 12, 14]. A recombinant *Escherichia coli* system, expressing *B. macerans* CGTase fused with 10 lysine residues at its C-terminus (CGTK10ase), was constructed for the purpose of simple ion-exchange purification of CGTase with high purity [13]. A packed-bed reactor with the immobilized CGTase onto a SP-Sepharose cation exchanger was developed for the production of CD mixture from starch [19]. Small particles of SP-Sepharose in a column-type of enzyme reactor led to a considerable increase in back pressure and a low productivity of α-CD conversion, suggesting that the large cross-sectional area of an enzyme reactor might overcome the reduction of α-CD production. Enzyme reactors equipped with ultrafiltration (UF) membrane have the advantages of high volumetric surface area, minimization of enzyme loss by molecular cutoff, high reaction yield by tangential flow, and membrane reuse [7]. In this study, an enzyme reactor using *B. macerans* CGTK10ase was developed with an ion-exchange UF membrane. After the selection of UF membrane for CD production, the UF membrane surface was modified for CGTK10ase immobilization. A repeated-batch type of CD production was carried out with free and immobilized CGTK10ases in a stirred enzyme reactor equipped with UF membrane.

Selection of Ultrafiltration Membrane for CD Production

Two UF membranes with different molecular cutoff values were subjected to the analysis of the penetration of dextrins, CDs, and CGTK10ase. UF membranes of YM1 with 1,000 molecular cutoff and YM10 with 10,000 molecular weight cutoff (Amicon, Bedford, MA, U.S.A.) were installed into an UF stirred cell with 10 ml of maximum process volume (Model 8010, Cat. No. 5121; Amicon, U.S.A.). A reaction mixture containing 10 g/l dextrin (Type 1, average DP 40; Sigma Co., St. Louis, U.S.A.) and 5 g/l each CD or 0.2 g/l CGTK10ase solution was added into the cell and pure nitrogen gas flowed at

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4.5 kgf/cm^2 of pressure. *E. coli* BL21(DE3):pLysE containing plasmid pTCGTK10 was used for CGTK10ase expression [13]. Culture conditions for the recombinant *E. coli* strain and procedures for the preparation of crude and purified CGTK10ases have been written in detail in our previous report [13]. The retentate and permeate solutions were subjected to the analysis of carbohydrates and CGTK10ase using thin layer chromatography (TLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively [4]. A precoated silica gel glass plate (K5F Silica Gel 150 Å, 20×20, 250 μm pore size, Cat No. 4851-820; Whatmann, U.S.A.) was heated at 110°C for 2 h before use [20]. Two μl of samples was loaded on the edge of the plate, followed by drying the spots. The plate was placed in a tank with running solvent [nitromethane-water-*n*-propanol (2:1.5:4, v/v)]. After running two times, the plate was dried fully and submerged in developing solvent [methanol-sulfuric acid (95:5, v/v) containing 3 g/l of α-naphthol]. Incubation of the plate at 110°C for 10 min revealed the spots of samples. Spot density was quantified with a densitometer (GS-700; Bio-rad, Hercules, CA, U.S.A.) and Molecular Analyst (Bio-rad, U.S.A.). Long-chain dextrins and most of the CDs were unable to pass through YM1 (data not shown). For YM10, similar spot densities of dextrins and CDs between permeate and retentate solutions were observed in TLC (Fig. 1A), indicating that CDs produced by CGTK10ase could pass through YM10 moderately. SDS-PAGE analysis exhibited that CGTK10ase did not penetrate the UF membrane because its molecular weight was higher than the molecular cutoff value of YM10 (Fig. 2). As a result, UF membrane YM10 was chosen for its modification and CD production using free and immobilized CGTK10ase enzymes.

Fig. 2. SDS-PAGE analysis of CGK10ase permeate and retentate through UF membrane YM10.
Lanes SM, P, and R indicate protein size marker, permeate, and retentate, respectively. This experiment was carried out in triplicate.

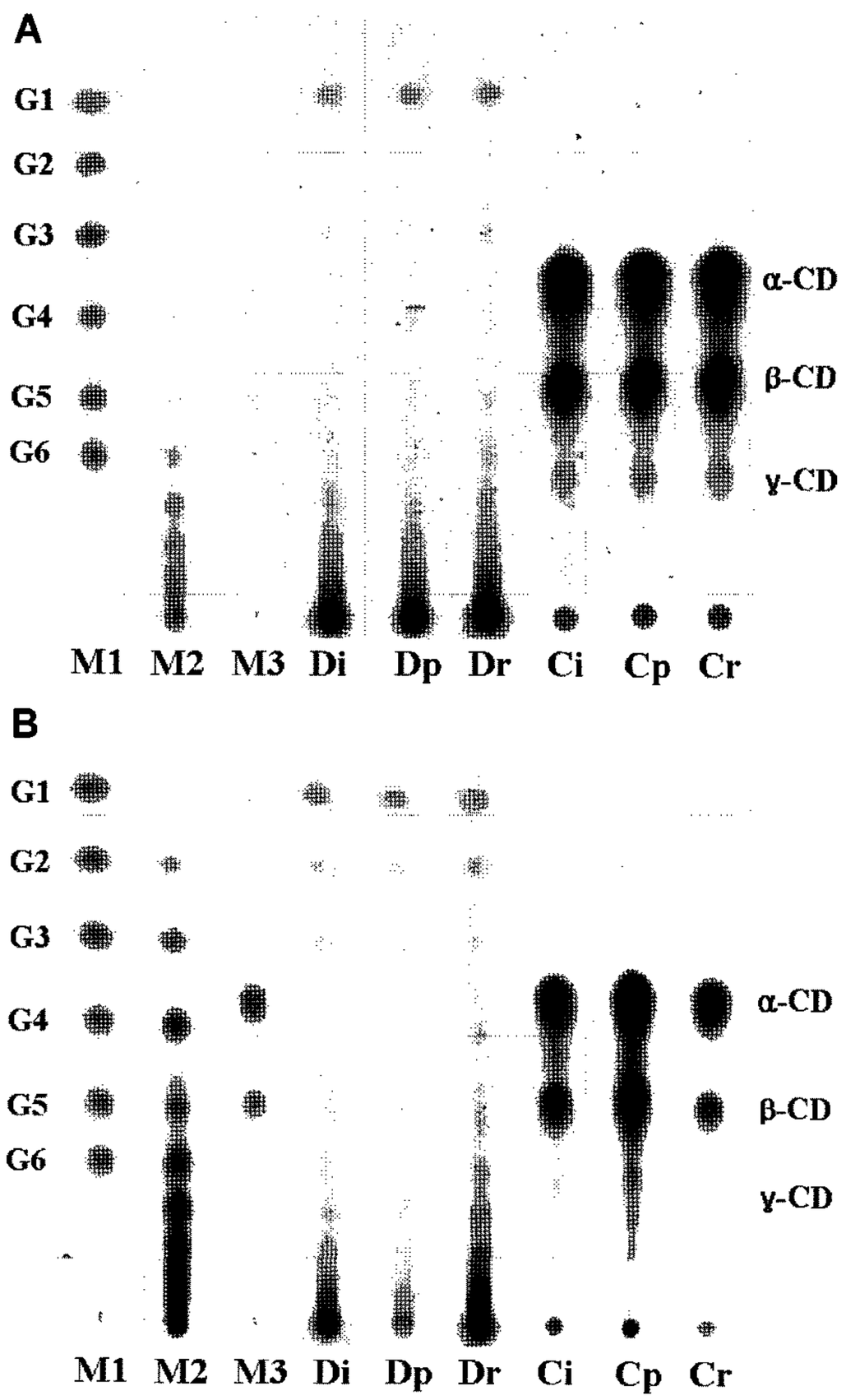

Fig. 1. Thin layer chromatography for the analysis of carbohydrate penetration through UF membrane YM10 without (**A**) and with (**B**) chemical modification.
G1 to G6 mean glucose oligomers containing 1 to 6 units. The analyzed samples contained pure or mixed carbohydrates as follows; M1, G1–G6; M2, G2–G10; M3, CDs; Di, 10 g/l of dextrin initially added; Dp, permeate of dextrin; Dr, retentate of dextrin; Ci, 5 g/l of CDs initially added; Cp, permeate of CDs; Cr, retentate of CDs.

Modification of Ultrafiltration Membrane

By the epoxy activation and ligand coupling of YM10, the hydroxyl group of cellulose was combined with a long-chain hydrocarbon containing a negative-charged sulfur trioxide. Epoxy activation was carried out as done in the previous report [17]. Briefly, after triple washing YM10 with 20 ml of deionized water, the membrane was immersed in 75 ml of 0.6 N NaOH solution containing 150 mg of sodium borohydride diglycidyl ether, which was slowly stirred at room temperature for 10 h. The epoxy-activated YM10 was washed extensively with deionized water to remove excess reagents until there was no oily film on the membrane surface. For ligand coupling, the epoxy-activated YM10 was immersed into a mixture of sodium sulfite/isopropyl alcohol/water (10/15/75, w/w/w) at 37°C for 10 h. Treatment with 0.5 M sulfuric acid at 65°C for 2 h led to the hydrolyzation of remaining epoxy groups into a diol group [1]. The same TLC analysis was carried out to investigate the change of membrane characteristics by chemical modification (Fig. 1B). A low spot density of the dextrin permeate exhibited that dextrins with long chains did not pass through modified YM10. Spots of CDs in the permeate solution showed higher

density than in the retentate solution. In addition, the spot density of the CD retentate for modified YM10 was lower than using original YM10 (Fig. 1A, lane Cr). Membrane modification allowed that more dextrins as substrates for CGTK10ase reaction were kept inside the reactor cell and more CDs passed through the membrane easily. It was reported that UF membranes retained long-chain starch for the sufficient reaction of enzyme and removed inhibitory products from the reaction media [7]. For the determination of membrane capacity, it was assumed that one copper ion (Cu^{2+}) bound with two sulfonic groups. After the incubation of modified UF membrane YM10 in 0.1 M $CuSO_4$ for 12 h, it was installed into the UF stirred cell. The membrane was washed twice with 3 ml of 0.2 M acetate buffer (pH 4.5) and three times with 3 ml of 10 mM sodium phosphate buffer (pH 6.0) containing 58.4 g/l NaCl by flowing nitrogen gas at 4.5 kgf/cm^2 of pressure. The attached copper ions were eluted with 3 ml of 0.1 M Na_2EDTA solution. The concentration of copper ion was determined with an atomic adsorption emission spectrophotometer (Shimadzu, Japan) as described before [10]. Analysis of copper ion attached on modified YM10 resulted in 25 mm of membrane diameter, 4.91 cm^2 of surface area, 2.96×10^{-8} mol/cm^2 of ionic exchange capacity, and 1.45×10^{-7} mol of sulfonic group per one membrane. If there is no steric hindrance and one molecule of CGTK10ase (MW 74335.8) binds ten sulfonic groups, then 1.08 mg of CGTK10ase can theoretically be immobilized onto one modified YM10.

CD Production Using Free and Immobilized CGTK10ase

The structural analysis of *B. macerans* CGTase and kinetic identification of CGTK10ase characteristics showed that the 10 lysine residues at the C-terminus of CGTK10ase did not mask the active sites of the catalytic domain and hence specific enzyme activity did not decrease even when CGTK10ase was immobilized onto a cation-exchange resin [19]. The repeated-batch type of CD production was carried out by free CGTK10ase in the stirred enzyme reactor equipped with original UF membrane YM10. Immobilized CGTK10ase onto modified YM10 was subjected to the same process for CD production. For the immobilization of CGTK10ase, modified UF membrane YM10 was soaked in 5 ml of purified CGTK10ase solution for 24 h at 4°C. By triple washing the membrane with 5 ml of buffer A [0.01 M sodium phosphate buffer with 0.5 mM $CaCl_2$ (pH 6.0)], free CGTK10ase unbound on the membrane was removed. To determine the concentration of immobilized CGTK10ase after CD production, the membrane harboring CGTK10ase was treated twice with 5 ml of buffer A and immersed in 5 ml of buffer A containing 1 M NaCl at 4°C for 24 h. The released CGTK10ase solution was subjected to the determination of protein concentration. Repeated-batch production of CDs was carried out as follows. After the installation of unmodified UF membrane YM10 into the UF stirred cell, 10 g/l of purified CGTK10ase and 1% soluble starch solution in buffer A were added into the cell of which the working volume was 4 ml. After covering the cell, nitrogen gas flowed at 4.5 kgf/cm^2 of pressure. Buffer A was added twice after the solution had run out completely. CD production using CGTK10ase immobilized on modified YM10 followed the same procedure except for enzyme addition. The amounts of CDs were determined by both TLC and high-performance liquid chromatography (HPLC). HPLC analysis was performed as described elsewhere [19]. The results of CD production are summarized in Table 1. Free CGTK10ase produced α-CD with 24.0 (±1.5)% of yield and 4.68 (±0.88) g/l-h of productivity, which were 7 times higher values compared with those for immobilized CGTK10ase. The reason for the low yield of CD production by immobilized CGTK10ase was ascribed to the low concentration of CGTK10ase attached on modified YM10. The amount of immobilized CGTK10ase was determined at 16.8 μg per membrane, which was 50 times lower than the 0.8 mg per batch of free CGTK10ase. In our previous report, an immobilization process onto a cation-exchange resin caused no structural deterioration of CGTK10ase [19]. The immobilization yield of CGTK10ase varied from 0.01 to 0.08 depending on the concentration of

Table 1. The results of the repeated-batch type of CD production by free and immobilized CGTK10ases in an UF enzyme reactor at 25°C.

	Yield (%)[a]				Productivity (g CD/l-h)				CD ratio (%)[b]		
CD type	α	β	γ	Total	α	β	γ	Total	α	β	γ
Free CGTK10ase[c]	24.0 (1.5)	14.7 (1.6)	10.8 (0.5)	49.7 (3.4)	4.68 (0.88)	2.92 (0.54)	2.01 (0.45)	9.60 (1.60)	48.3 (2.4)	30.9 (3.3)	20.8 (2.9)
Immobilized CGTK10ase[d]	3.41 (0.11)	1.86 (0.16)	1.02 (0.08)	6.29 (0.51)	0.67 (0.03)	0.36 (0.02)	0.17 (0.01)	1.20 (0.10)	55.9 (2.3)	30.4 (4.7)	13.7 (2.4)

Number in parenthesis shows the standard deviation.
[a]Iindicates each and total CD concentration per added soluble starch concentration.
[b]Denotes each CD concentration divided by total CD concentration.
[c]Purified CGTK10ase was added into the enzyme reaction cell equipped with unmodified UF membrane YM10.
[d]Modified UF membrane containing immobilized CGTK10ase was installed into the enzyme reaction cell.

added CGTase, because the adsorption profile followed the Langmuir isotherm-like saturation curve. The immobilization of CGTK10ase on modified YM10 improved the selectivity of α-CD by 20%, compared with that of free CGTK10ase. The selectivity of *B. macerans* CGTase for α-CD synthesis varied with immobilization methods [5, 16]. By the immobilization of CGTK10ase on a cation exchanger, the relative content of α-CD was obtained from 43% to 52%, which was higher than that of other immobilization methods using Amberite IRA-900 and chitosan [19].

Effects of Alkyl Alcohols on CD Production

Because CDs contain a fairly polar exterior and a relatively nonpolar cavity, CDs have an ability to form stable complexes with various alkyl alcohols in the solid and aqueous phases [2]. The effects of alkyl alcohols on CD production were investigated with the repeated-batch type of immobilized CGTK10ase reaction (Fig. 3). Alkyl alcohols of 13% concentration including nonan-1-ol, decan-1-ol, and undecan-1-ol were added into buffer A. Addition of alkyl alcohols did not reduce CGTK10ase activity for 90 min of the reaction. Production of CDs was elevated by the addition of nonan-1-ol, decan-1-ol, and undecan-1-ol. Nonan-1-ol supplementation resulted in 4.56% of α-CD yield, corresponding to a 3-fold increase compared with that of the control. A thermodynamic study for the association process involving α-CD and alkan-1-ols from C_3 to C_9 showed that the association constants were enhanced along with an increment of carbon number in 0.5 M phosphate buffer (pH 5.5) [3]. As an example for the elevation of CD yield, glycyrrhizin (CAS No. 1405-86-3) forming a stable complex with γ-CD was added to a reaction mixture containing *B. firmus* CGTase and cornstarch solution, and the yield of γ-CD increased without the change of total yield of CDs [18]. The reason for the enhancement of α-CD yield by alkyl alcohol addition might be attributed to that the formation of a stable complex with α-CD and alkyl alcohols prevented the destruction of α-CD by the reversible and ring opening reaction by CGTK10ase.

For the application of the advantageous characteristics of UF membrane, the repeated-batch production of CDs in the stirred reactor installed with UF membrane was carried out with *B. macerans* CGTase tagged with 10 lysines at its C-terminus. Application, modification of UF membrane, and addition of alkyl alcohols minimized a loss of CGTK10ase and allowed the development of CD production systems using free and immobilized CGTK10ases. More research efforts will be made for increasing the immobilization efficiency of CGTK10ase on modified UF membrane and developing a continuous process for CD production with high α-CD selectivity.

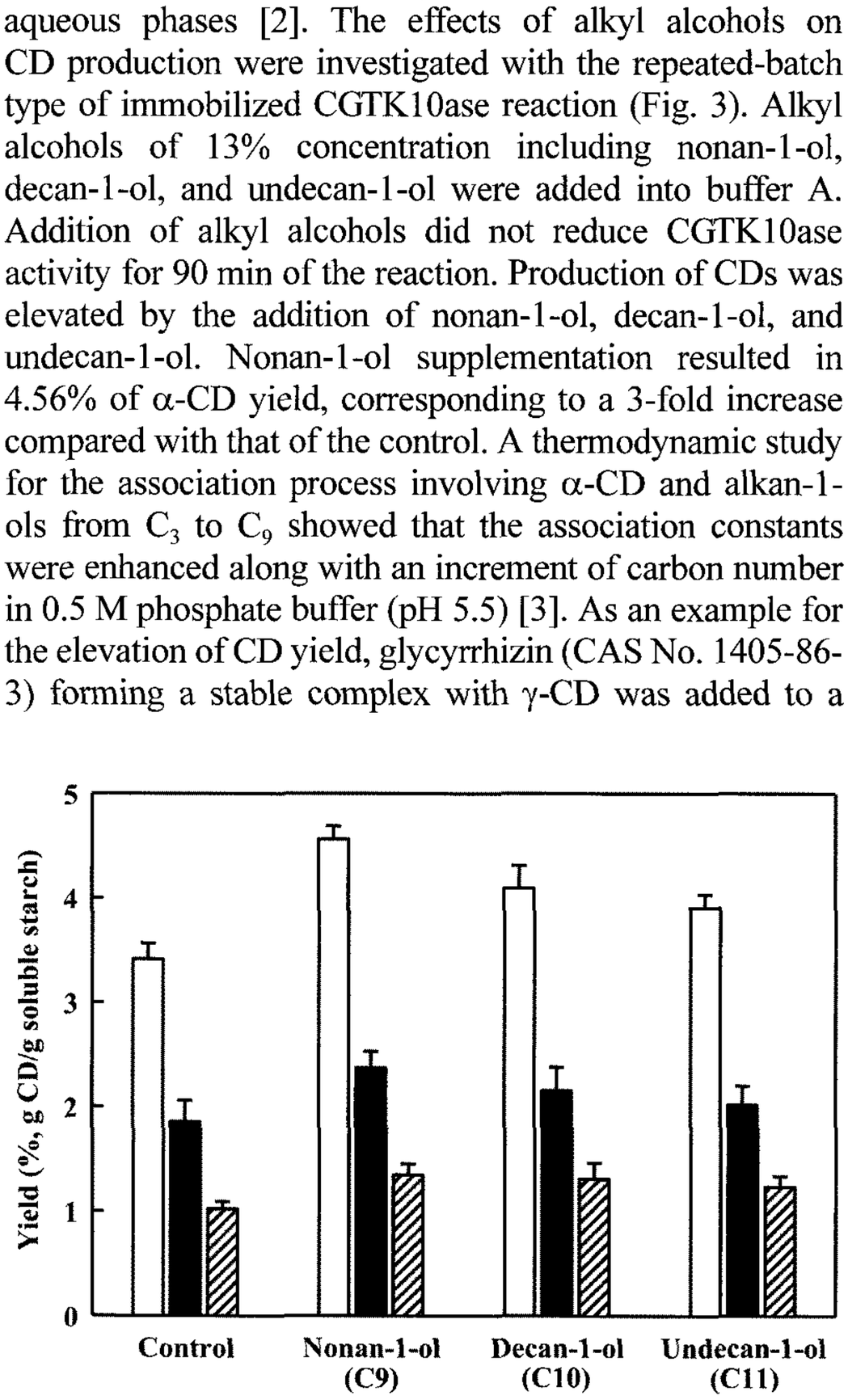

Fig. 3. Effects of alkyl alcohols on the production yield of CDs from 1% starch by CGTK10ase immobilized on modified UF membrane YM10.

Alkyl alcohols of 13% concentration were added into the stirred tank reactor. White, black, and lined bars indicate α-CD, β-CD, and γ-CD, respectively. Letters in parenthesis mean carbon number.

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