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Optimization of Oligosaccharide Synthesis from Cellobiose by Dextransucrase

Misook Kim · Donal F. Day

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Abstract There is a growing market for oligosaccharides as sweeteners, prebiotics, anticariogenic compounds, and immunostimulating agents in both food and pharmaceutical industries. Interest in novel carbohydrate-based products has grown because of their reduced toxicity and low immune response. Cellobiose is potentially valuable as a nondigestible sugar. The reaction of cellobiose, as an acceptor with a sucrose as a donor, catalyzed by a dextransucrase from *Leuconostoc mesenteroides* B-512FMCM, produced a series of cellobio-oligosaccharides. This production system was optimized using a Box–Behnken experimental design for 289 mM of sucrose and 250 mM of cellobiose and 54 U of the enzyme at pH 5.2 and 30 °C, to produce maximum yields of oligosaccharide.

Keywords Oligosaccharide · Cellobiose · Dextransucrase · *Leuconostoc mesenteroides* B-512FMCM · Box-Behnken experimental design

Introduction

Oligosaccharides are carbohydrate polymers, generally of two to ten monomeric residues linked by *O*-glycosidic bonds [1]. Most commercial oligosaccharides were originally developed as sweeteners, but they are currently valued as soluble fiber, which decreases gastrointestinal transit time and moderates constipation and diarrhea. Oligosaccharides are considered to be low-calorie food because they are resistant to attack by digestive enzymes in human and animals and are not absorbed by the host [2]. Oligosaccharides may be produced through microbial fermentation, enzymatic synthesis, or extraction from naturally occurring sources. Currently, commercial oligosaccharides include cyclomaltodextrins, maltodextrins, fructooligosaccharides, galactooligosaccharides, and soy oligosaccharides [3].

Cellobiose is a disaccharide of two glucose molecules linked in a β-1,4 bond produced from enzymatic hydrolysis of cellulose. Enzymes capable of breaking down cellobiose are

M. Kim
Department of Food Science, Louisiana State University, Baton Rouge, LA 70803, USA

D. F. Day (✉)
Audubon Sugar Institute, St. Gabriel, LA 70776, USA
e-mail: dday@agcenter.lsu.edu

absent in the human small intestine. Nakamura et al. [4] confirmed that cellobiose may be hydrolyzed slowly by intestinal lactase in an in vitro study with rat small intestinal brush border membrane vesicles. There are some reports of new classes of sugars, containing cellobiose as a component, produced by transglycosylation reactions [5, 6]. Acarbose analogues containing cellobiose were prepared by the reaction of acarbose and *Bacillus stearothermophilus* α-maltogenic amylase (EC 3.2.1.133; [5]). Cellobiose–acarbose analogues show a potential for use as an inhibitor of β-glucosidase, whereas acarbose does not. Morales et al. [6] produced oligosaccharides with branched chains, using cellobiose as acceptor in the reaction catalyzed by alternansucrase from *Leuconostoc mesenteroides* NRRL B-23192. *L. mesenteroides* B512 FMCM mainly produces extracellularly dextransucrase (EC 2.4.1.5). This dextransucrase synthesizes a dextran that has 95% α-(1→6) linear and 5% α-(1→3) branched linkages and can transfer glucosyl units from sucrose onto the acceptor to produce oligosaccharides [7].

This work reports on optimization of some variables that play an important role in the production of cellobio-oligosaccharides; they are temperature, pH, enzymatic concentration, and the concentrations of sucrose and cellobiose.

Materials and Methods

Dextransucrase Production

L. mesenteroides B-512 FMCM was grown for 16 h at 30 °C in liquid mineral (LM) medium [0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 2% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.001% (w/v) $MnSO_4 \cdot H_2O$, 0.013% (w/v) $CaCl_2 \cdot 2H_2O$] containing 2% glucose. The inoculum development stage required three successive transfers to build sufficient volume for inoculation for the final fermentation. A four-hundred-milliliter culture was inoculated to 14 l of LM medium and incubated for 48 h at 30 °C. The pH and agitation were not controlled during fermentation. After harvesting, cells were removed by centrifugation at 6,000 rpm×*g* for 30 min. The cell-free culture was concentrated tenfold using membrane filtration (100 K cutoff) and washed with 2 vol of sodium citrate buffer, pH 5.2. Tween 80 and NaN_3 were added at concentrations of 1 and 0.2 mg/ml to crude enzyme. The size and specific activity of dextransucrase were 180 kDa and 22.15 (U/mg).

Dextransucrase Assay

Crude dextransucrase was reacted with 100 mM sucrose for 1 h at 30 °C and then boiled for 5 min to terminate the enzyme reaction. One unit of dextransucrase activity was defined as that amount of enzyme releasing 1 μM fructose per min from 100 mM sucrose. The fructose was determined by high-performance liquid chromatography (HPLC) using an Aminex HPX 87K column (300×7.8 mm) and an HPLC analyzer coupled to a refractive index detector. The column was maintained at 85 °C and 0.01 M K_2SO_4 was used as a mobile phase at a flow rate 0.6 ml/min.

Transglycosylation Reaction

To determine optimal reaction conditions, reaction digests were prepared with various sucrose (100–800 mM), cellobiose (50–300 mM), and crude dextransucrase (13–67 U)

concentrations in 20 mM sodium citrate buffer (pH 3.2–6.0) at 20–50 °C for 24 h. The enzyme reaction products were analyzed by high-performance anion exchange chromatography using a Dionex Carbo-Pac PA 100 column (250×4 mm) by gradient elution using 1 M NaOH, water, and 480 mM sodium acetate at a constant flow rate of 0.5 ml/min. Oligosaccharide detection was carried out with an electrochemical detector (ED 40). Relative concentrations of oligosaccharide were analyzed by thin-layer chromatography (TLC). The TLC samples were loaded onto a Whatman K5 silica gel plate. The plate was irrigated three times with 2:5:1.5 volume parts of nitromethane–1-propanol–water. The carbohydrates on the TLC plate were visualized by dipping the plate into a methanol solution containing 0.3% (w/v) *N*-(1-naphthyl) ethylenediamine and 5% (v/v) sulfuric acid, followed by heating at 110 °C for 15 min. The relative percent of carbohydrates were determined using a Scion program from National Institutes of Health (NIH).

Box–Behnken Experimental Design

A three-factor and three-level Box–Behnken design was applied for the optimization procedure using Design Expert 6 software (Stat-Ease). Several factors—the amounts of sucrose, cellobiose, and dextransucrase—used to prepare each of the 17 formulations are given in Tables 1 and 2. These high, medium, and low levels were selected from the preliminary experimentation. Optimization was performed using a desirability function to obtain the levels of X_1, X_2, and X_3.

The behavior of the system has been explained by the following quadratic model equation [8]:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is predicted response, β_0 intercept, β_1, β_2, and β_3 linear coefficient, β_{11}, β_{22}, and β_{33} squared coefficients, and β_{12}, β_{13}, and β_{23} interaction coefficients. A total of 17 experiments were necessary to study the ten coefficients of model.

Results and Discussion

A pH of 5.2 was optimum for production of cellobio-oligosaccharides (Fig. 1). The optimum pH for oligosaccharide production was same as that for dextransucrase activity [9]. The production of cellobio-oligosaccharides was more sensitive to the changes in pH than was dextransucrase activity. Cellobio-oligosaccharide concentrations produced at pH 6.2 were 39% of that produced at pH 5.2.

Table 1 Variables in Box–Behnken design: independent variables.

Independent variables	Levels		
	Low	Middle	High
X_1: sucrose (mM)	100	250	400
X_2: cellobiose (mM)	50	150	250
X_3: dextransucrase (U)	27	40	54

Table 2 Variables in Box–Behnken design: dependent variable (*Y*: relative amount of cellobio-oligosaccharides produced [%])—concentrations (mM) of each independent variable used to prepare the 17 formulations.

Run	X_1	X_2	X_3
1	100	50	40
2	100	150	27
3	100	150	54
4	100	250	40
5	250	50	27
6	250	50	54
7	250	150	40
8	250	150	40
9	250	150	40
10	250	150	40
11	250	150	40
12	250	250	27
13	250	250	54
14	400	50	40
15	400	150	27
16	400	150	54
17	400	250	40

The rate of cellobio-oligosaccharide production sharply decreased above 30 °C and at 40 °C was only 46% of the maximum production (Fig. 2). The effect of temperature on the oligosaccharide production showed the same pattern as the effect of temperature on dextransucrase activity [9].

Optimization of transglycosylation was achieved by employing a Box–Behnken design for the experiments. The response surface methodology was used to study the effect of interaction among independent variables—sucrose, cellobiose, and dextransucrase on the production of cellobio-oligosaccharides. Table 3 depicts the actual and predicted values of cellobio-oligosaccharides on the basis of the experimental design. The predicted values and the observed values agreed reasonably well, as seen from Table 3. The results were

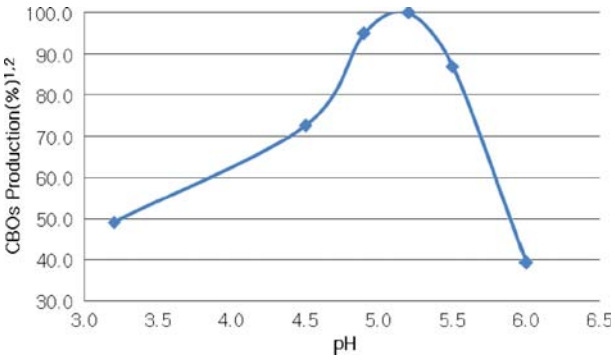

Fig. 1 Production of cellobio-oligosaccharides synthesized by *L. mesenteroides* B-512 FMCM dextransucrase at various pH values. [1]Cellobio-oligosaccharide production was calculated as the percentage concentration of cellobio-oligosaccharides at the selective pH, divided by the highest concentration of cellobio-oligosaccharide over all pH ranges. [2]Reactions conducted at 100 mM sucrose, 100 mM cellobiose, dextransucrase 27 U, and 30 °C for 24 h

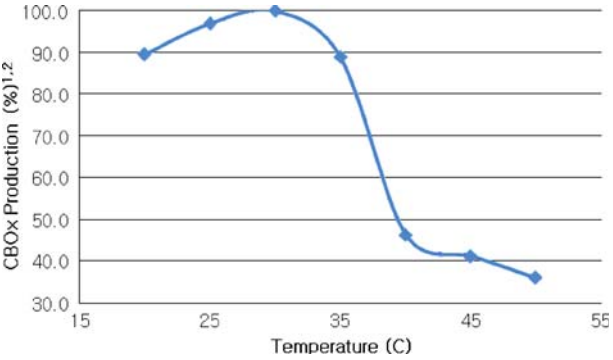

Fig. 2 Oligosaccharides produced as a function of temperature changes. [1]Cellobio-oligosaccharide production was calculated as the percentage concentration of cellobio-oligosaccharides at the selective temperature, divided by the highest concentration of cellobio-oligosaccharides over all temperature ranges. [2]Reactions conducted with 100 mM sucrose, 100 mM cellobiose, dextransucrase 27 U, and 30 °C for 24 h

analyzed using multiple regression. The coefficients of the full model were evaluated and tested for their significance through regression analysis. Concerning the p value of the coefficients, X_1, X_2, X_3, and X_1^2 were found to have significant effects on the performance of the model for the prediction of the cellobio-oligosaccharide production (Tables 4 and 5).

The final estimative response model equation was as follows:

$$Y = 71.91 + 7.64X_1 + 12.36X_2 + 17.82X_3 - 12.98X_1^2 - 3.29X_2^2 - 0.41X_3^2 - 4.25X_1X_2 + 3.31\mathrm{X_1X_3} - 0.52\mathrm{X_2X_3} \quad (2)$$

where Y is the response factor (cellobio-oligosaccharide production, percent) and X_1, X_2, and X_3 represent real values of the independent factors—sucrose (mM), cellobiose (mM), and dextransucrase (U). Table 5 shows the model coefficients and probability values. The

Table 3 Observed and predicted values and variance parameters of cellobio-oligosaccharide yield (%) recorded in experimental setup of response surface methodology.

Run number	Observed Y	Predicted Y	Residuals
1	33.60	30.83	2.77
2	35.72	36.37	−0.65
3	63.25	65.39	−2.15
4	64.12	64.09	0.02
5	35.29	37.51	−2.23
6	73.67	74.19	−0.52
7	66.78	71.25	−4.47
8	66.88	71.25	−4.36
9	74.76	71.25	3.51
10	76.06	71.25	4.81
11	71.77	71.25	0.52
12	63.90	63.27	0.62
13	100.00	97.88	2.12
14	54.34	54.37	−0.02
15	47.29	45.04	2.25
16	87.84	87.29	0.55
17	67.86	70.63	−2.77

Table 4 Regression coefficients and significance of regression model.

Factor	Coefficient estimate	*df*	Standard error	95% CI low	95% CI high
Intercept	71.91	1	1.78	67.70	76.12
X_1	7.64	1	1.41	4.32	10.97
X_2	12.36	1	1.41	9.04	15.69
X_3	17.82	1	1.41	14.50	21.14
X_1^2	−12.98	1	1.94	−17.56	−8.40
X_2^2	−3.29	1	1.94	−7.87	1.29
X_3^2	−0.41	1	1.94	−5.00	4.18
X_1X_2	−4.25	1	1.99	−8.95	0.45
X_1X_3	3.31	1	1.99	−1.39	8.01
X_2X_3	−0.52	1	1.99	−5.22	4.18

CI Confidence interval

analysis of variance of this model demonstrated that the model is highly significant, as is evident from the *F* value (F_{model}=35.99) and a very low probability value (<0.0001). A *P* value lower than 0.1 indicates that a model is considered to be statistical significant [10]. Furthermore, the lack of fit *F* value of 0.64 implies that the lack of fit is not significant relative to the pure error according to our results of analysis of factors. Nonsignificant lack of fit is a good indication that the model fits the actual relationships of the reaction parameters within the selected ranges.

The relationship between the independent and dependent variables was elucidated using contour and response surface plots (Fig. 3). Each plot represents the effect of two variables at their studied range with the other one maintained at fixed level. The shapes of contour plots display the nature and extent of the interactions [11]. As shown in Fig. 3a, an increase in sucrose concentration up to 300 mM results in increased cellobio-oligosaccharide production with increases in cellobiose concentration, at 40.5 U dextransucrase. An increase in the amount of cellobiose causes a linear increase in cellobio-oligosaccharide

Table 5 ANOVA for response surface.

Source	Sum of squares	*df*	Mean square	*F* value	Probability>*F*[a,b]
Model	5119.29	9	568.81	35.99	<0.0001
X_1	466.80	1	466.80	29.53	0.0010
X_2	1221.61	1	1221.61	77.29	<0.0001
X_3	2540.51	1	2540.51	160.73	<0.0001
X_1^2	709.29	1	709.29	44.87	0.0003
X_2^2	45.53	1	45.53	2.88	0.1335
X_3^2	0.69	1	0.69	0.04	0.8400
X_1X_2	72.19	1	72.19	4.57	0.0699
X_1X_3	43.83	1	43.83	2.77	0.1398
X_2X_3	1.08	1	1.08	0.07	0.8014
Residual	110.64	7	15.81		
Lack of fit	35.87	3	11.96	0.64	0.6282
Pure error	74.77	4	18.69		
Correlation total	5,229.93	16			

[a] Probability>*F*, level of significance

[b] Values of "probability>*F*" less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate the model terms are not significant.

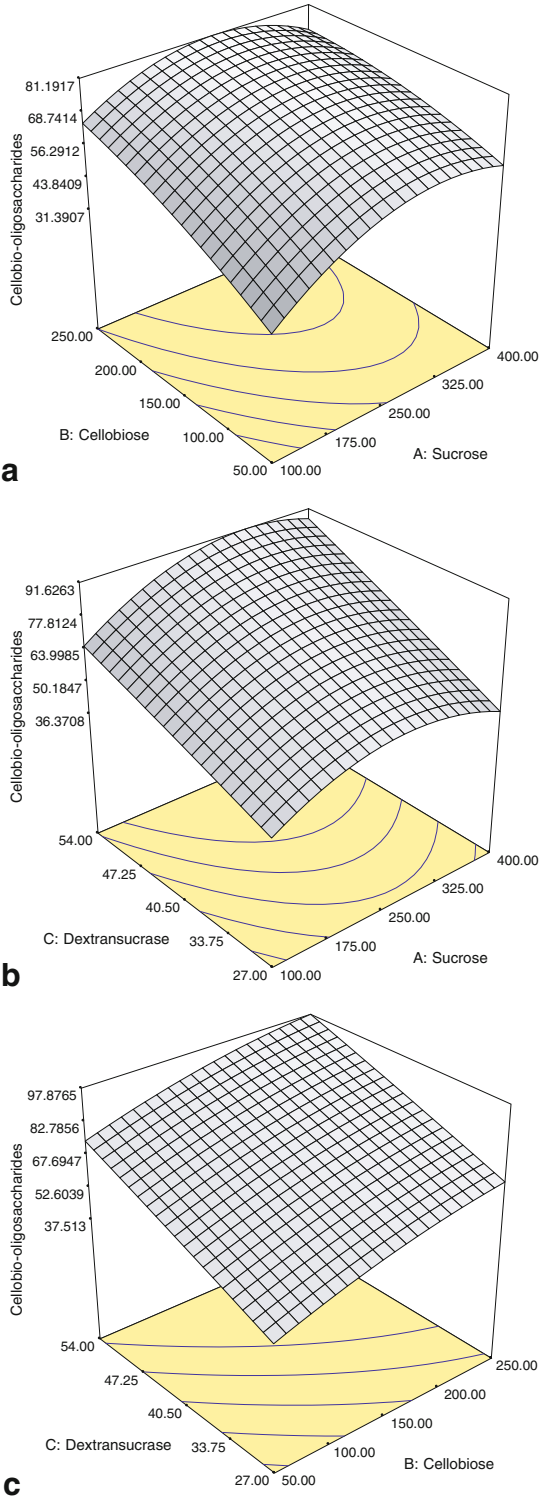

Fig. 3 Response surface plots illustrating the effect of varying concentrations of sucrose, cellobiose, and dextransucrase on their reciprocal interactions with cellobio-oligosaccharide production. Other factors are held constant at 40.5 U of dextransucrase (**a**), 150 mM of cellobiose (**b**), and 250 mM of sucrose (**c**)

produced. However, sucrose concentrations greater than 300 mM interfere with cellobio-oligosaccharide production despite increases in the concentrations of cellobiose. The reaction solutions became increasingly viscous with increases in sucrose concentration due to the concomitant formation of dextran. This finding is similar for the action of sucrose on the dextransucrase production by *L. mesenteroides* B-512 F [12].

The effects of differing dextransucrase and sucrose concentrations on the cellobio-oligosaccharide production with 150 mM cellobiose are shown in Fig. 3b. High production of cellobio-oligosaccharides was achieved at the middle level of sucrose across the entire range of dextransucrase concentrations. A linear increase in dextransucrase in selected ranges does not lead to a continuous increase of cellobio-oligosaccharide production with increased in sucrose concentration. Furthermore, product inhibition was observed because of dextran formation above 54 U of dextransucrase (data were not shown). Dextransucrase catalyzes dextran synthesis as well as oligosaccharide synthesis. Kim and Robyt [13] reported that dextran production showed a slightly sigmoidal increase suggesting an allosteric effect for the dextran.

Figure 3c illustrates the effects of different dextransucrase concentrations and cellobiose concentrations on the cellobio-oligosaccharides production in the presence of 250 mM sucrose. Increases in the amount of both dextransucrase and cellobiose resulted in linear increases in the production of cellobio-oligosaccharides when sucrose concentration was fixed. The highest concentrations were reached using high dextransucrase and cellobiose. However, the addition of cellobiose was limited by the solubility of cellobiose. Figure 3 suggests that high cellobiose and dextransucrase as well as sucrose concentration between 250 and 300 mM achieve the optimal transglycosylation of sucrose to cellobiose.

The optimum values for the selected factors were calculated from the regression equation (Eq. 2). The optimal conditions for the cellobio-oligosaccharides were as follows: X_1=288.96 mM, X_2=250 mM, X_3=54 U (Fig. 4). The theoretical cellobio-oligosaccharide yield predicted under these conditions was Y=98.74%. The prediction value by the model was confirmed using the above conditions. The actual value was 49.9 mM of cellobio-oligosaccharide (relative value to prediction value, 101.5%).

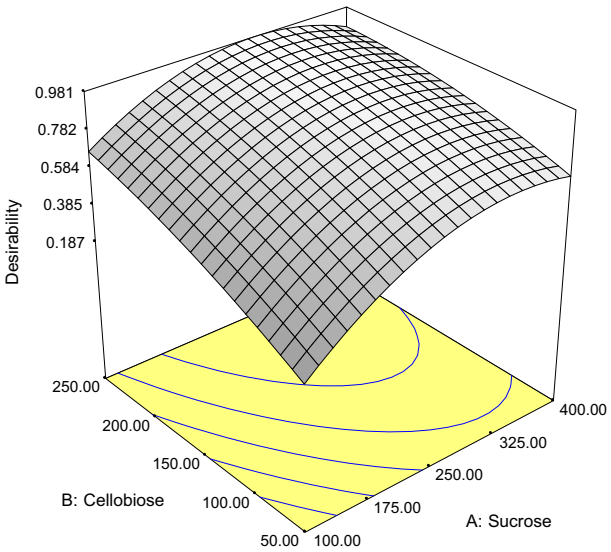

Fig. 4 Response surface plot representing the effect of varying concentrations of sucrose, cellobiose, and dextransucrase on their reciprocal interactions with cellobio-oligosaccharide (dextransucrase 54 U)

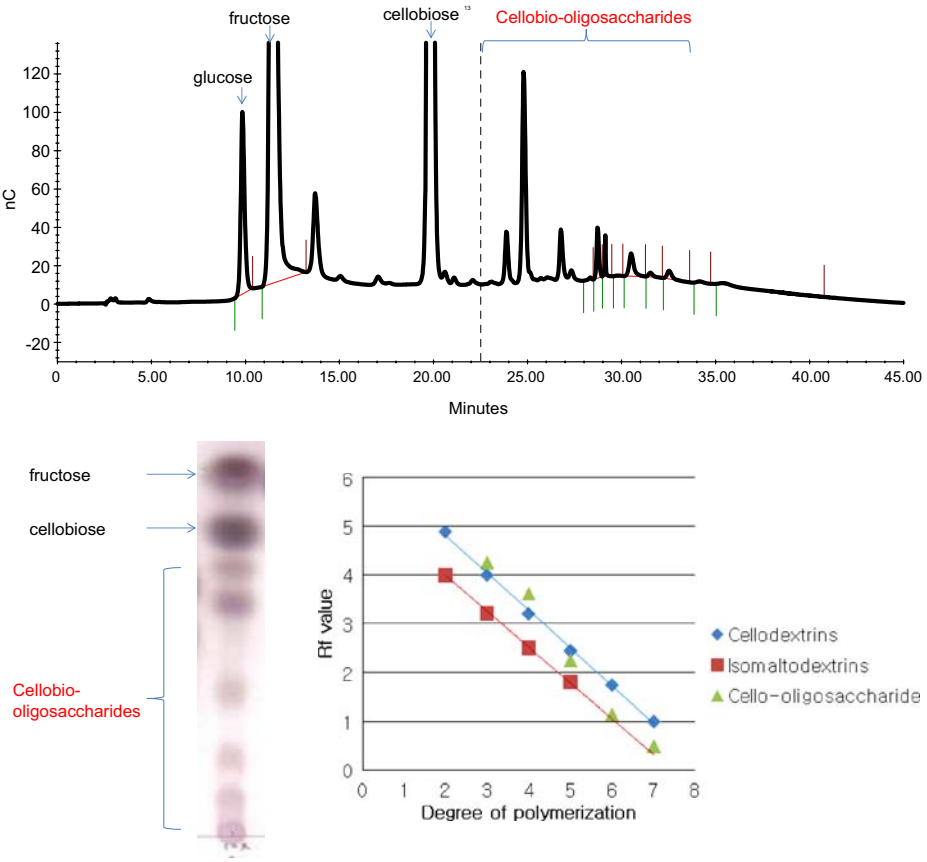

Fig. 5 Chromatogram profiles and R_f values for oligosaccharides

The cellobio-oligosaccharides showed different R_f values on TLC (Fig. 5). The sizes of five cellobio-oligosaccharides detected were compared with commercial oligosaccharide products of cellodextrins linked β-1,4 and with isomaltodextrins linked α-1,6. The degrees of polymerization (DP) of cellobio-oligosaccharides ranged from 3 to 7. Robyt and Mukerjea [14] indicated that the linkage type causes differences in migration. The migration of our cellobio-oligosaccharides with DP 3 and 4 was faster than cellodextrins containing β-1,4 linkages. However, the R_f values of the cellobio-oligosaccharides with DP 5, 6, and 7 were between cellodextrins and isomaltodextrins (α-1,6 linkages).

Conclusions

The optimal conditions for transglycosylation of sucrose onto cellobiose were a pH of 5.2 and a temperature of 30 °C. The concentration of dextransucrase was 54 U, the concentration of sucrose was 289 mM, and the concentration of cellobiose was 250 mM. The cellobio-oligosaccharide yield reached 20% based on initial cellobiose concentration under the above conditions.

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