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Investigation on Cyclodextrin Production with Cyclodextrin Glucanotransferase from *Bacillus megaterium*

Boriana Yordanova Zhekova[1]*, *Ivan Genov Pishtiyski*[1] *and Veselin Stanchev Stanchev*[2]

[1]Department of Biochemistry and Molecular Biology, University of Food Technologies, 26 Maritza Blvd., BG-4000 Plovdiv, Bulgaria

[2]Department of Automatics, Information and Control System, University of Food Technologies, 26 Maritza Blvd., BG-4000 Plovdiv, Bulgaria

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Summary

The process of producing cyclodextrins from starch with cyclodextrin glucanotransferase from *Bacillus megaterium* has been studied. The effect of starch and enzyme concentrations was evaluated using optimal composite designs and the response surface methodology. Mathematical models describing the process in two overlapping areas of variation of the independent variables were developed. The models were used to determine the optimal values of the variables. The increase in starch concentration resulted in an increase in the amount of β-cyclodextrin formed, but lowered the yield. The optimal starch concentration was considered to be 50.0 mg/mL. The critical cyclodextrin glucanotransferase concentration necessary for maximum β-cyclodextrin production was 2.0 U/g. Factors that limit the complete conversion of starch to cyclodextrins were established. The enzyme activity was strongly inhibited by the reaction products. The coupling activity of cyclodextrin glucanotransferase was proved. The enzyme was able to degrade high concentrations of β-cyclodextrin and to transform different types of cyclodextrins one into another. Cyclodextrin glucanotransferase formed α-, β- and γ-cyclodextrins in different ratios, depending on the duration of the process.

Key words: coupling activity, cyclodextrins, cyclodextrin glucanotransferase, optimal composite design, β-cyclodextrin production, product inhibition, starch

Introduction

Cyclodextrins are cyclic oligosaccharides composed of 6, 7, or 8 α-1,4-linked glucose units, respectively classified as α, β, and γ. They are produced by the catalytic action of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19). The enzyme displays its cyclic action on substrates with α-1,4-glycosyl chain, such as starch, amylose, amylopectin, dextrins, or glycogen. However, the most frequently used raw material for cyclodextrin production is starch.

CGTase can catalyze three other reactions (*1*). The enzyme is able to degrade cyclodextrins by intermolecular transglycosylation in the presence of suitable acceptor molecules. This reaction, called coupling, is the reverse reaction of cyclization. CGTase also produces oligosaccharides with different polymerization degree through a reaction of disproportionation and can hydrolyze starch. These activities can have a negative effect on conversion of starch into cyclodextrins. Reaction conditions are essential for directing the enzyme action to cyclodextrin production.

*Corresponding author; Phone: ++359 32 603 879; Fax: ++359 32 644 102; E-mail: zhekova_b@yahoo.com

Substrate and enzyme concentrations have a strong effect on cyclodextrin production (*2*,*3*). According to the literary data the cyclodextrin content of the reaction mixture increases with the increase in starch concentration (*4*,*5*). However, cyclodextrin yield in relation to initial starch used decreases. For these reasons, determination of the optimal starch concentration is difficult and often represents a compromise. Regardless of the low conversion degree, the use of a higher starch concentration is preferred in most cases, since it ensures higher cyclodextrin content of the reaction mixture.

The use of high initial starch concentration is impeded by the high viscosity of the reaction mixture and by the difficult separation of cyclodextrins from byproducts. The use of partly hydrolyzed, water soluble starch enables easier stirring and a better contact between the enzyme and the substrate. However, the conversion degree of the hydrolyzed starch is lower in comparison with the native substrate, as high dextrose equivalence has a negative influence on CGTase action (*6*,*7*).

Maximum cyclodextrin yield at a fixed substrate concentration can be achieved by applying an optimal enzyme amount. Typical of cyclodextrin production is the presence of a critical enzyme concentration, over which no increase in cyclodextrin yield is noticed. Since different methods for CGTase activity determination can be used, the optimal enzyme concentration of different enzymes is hardly comparable. For these reasons its determination is necessary for each case.

The aim of the present work is to investigate the effect of substrate and enzyme concentrations on β-cyclodextrin production by applying mathematical models and to determine the limiting factors of starch conversion in cyclodextrins.

Materials and Methods

CGTase production

A strain of *Bacillus megaterium* from the collection of the Department of Biochemistry and Molecular Biology, UFT, Plovdiv, Bulgaria was used for CGTase preparation. The strain was cultivated on a slant agar medium, containing (in g/L): meat-peptone broth 13.0, starch 10.0, and agar-agar 20.0. The medium pH was adjusted to 7.5. The tubes were incubated at 37 °C for 24 h and stored at 4 °C up to 3 months (*8*).

For inoculum preparation the biomass from a slant-agar tube was transferred to a 500-mL Erlenmeyer flask, containing 50 mL of potato extract, pH=7.5. The strain was cultivated at 37 °C on a rotary shaker at 200 rpm for 22 h.

CGTase biosynthesis was carried out in 500-mL Erlenmeyer flasks, in a medium containing 100 mL of potato extract and 0.5 % (by volume) corn steep liquor, pH=7.5. A volume of 1.0 % inoculum was added and the flasks were incubated at 37 °C on a rotary shaker at 200 rpm for 72 h. Biomass was removed by centrifugation at 5000×*g* for 20 min. The culture liquid was concentrated by vacuum evaporation on a rotary vacuum evaporator until 3-fold volume reduction was obtained. CGTase activity of the crude enzyme concentrate thus prepared was 1.61 U/mL.

Cyclodextrin production

Native corn starch (amylum) and soluble potato starch (Poland) were used as substrates for cyclodextrin production. Substrate solutions were prepared in phosphate buffer, pH=7.0, in a manner allowing the desired concentration to be reached after the addition of the dissolved enzyme. Both types of starches were dissolved in steam water bath. The resultant substrate solutions were cooled to 45 °C and the necessary amount of CGTase was added. The enzyme reaction was conducted in 100-mL Erlenmeyer flasks containing 50 mL of reaction medium, at 45 °C on a reciprocal shaker for 20 h. Samples were taken at certain intervals and CGTase was inactivated by boiling for 10 min in a water bath. The cyclodextrin content of the reaction mixture was determined.

Inhibition of CGTase by cyclic and linear oligosaccharides

Inhibition of CGTase was determined by adding different amounts of α-, β-, γ-cyclodextrins, glucose and maltose to the reaction mixtures of 4.0 mL, containing 50.0 mg/mL of soluble starch in phosphate buffer, pH=7.0, and 2.0 U/g of CGTase. The enzyme reaction was conducted at 45 °C for 10 min. After enzyme inactivation, the concentration of β-cyclodextrin formed by CGTase was determined. The inhibition was expressed as relative CGTase activity, calculated on the basis of the enzyme activity, determined without the addition of oligosaccharides under the same reaction conditions.

Experimental design

The effect of substrate and enzyme concentrations on cyclodextrin production was studied by applying factorial designs and a response surface methodology. The factorial design used was of the type optimal composite design with 'star points' around the centre points. The distance from the centre of the design space to a factorial point was ±1. This approach allowed fitting the data to nonlinear second order polynomials of the type:

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot x_i + \sum_{i=1}^{k} \beta_{ii} \cdot x_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} \cdot x_i \cdot x_j \qquad /1/$$

where the variable $\hat{Y}$ is the predicted response, x_i and x_j are the independent variables, β_0 is the offset term, β_i is the linear effect, β_{ij} is the interaction effect, β_{ii} is the squared term and k is the number of the independent variables.

Real and coded values of the independent variables and their variation intervals are presented in Table 1.

The test for statistical significance of the regression coefficients and the models developed was performed using data analysis software for ANOVA. For determination of the function maxima, MATLAB 6.0 was used.

Assays

CGTase activity was determined with phenolphthalein using Kestner's method (*9*) with modification (*8*).

Table 1. Variation intervals of the independent variables

Independent variable	Native starch						Soluble starch		
	Design 1			Design 2			Design 3		
	–1	0	+1	–1	0	+1	–1	0	+1
x_1 – γ(starch)/(mg/mL)	10.0	30.0	50.0	40.0	70.0	100.0	40.0	70.0	100.0
x_2 – CGTase/(U/g)	0.5	2.0	3.5	3.0	5.0	7.0	3.0	5.0	7.0

Soluble potato starch solution (20.0 mg/mL) in phosphate buffer (pH=6.0) was used as a substrate. A mixture of 2 mL of starch solution and 2 mL of CGTase was incubated at 30 °C for 10 min. At the beginning and the end of the reaction, samples of 0.2 mL were taken and the concentration of β-cyclodextrin was determined. One unit of CGTase activity was defined as the amount of enzyme that forms 1 μmol of β-cyclodextrin for 1 min under the assay conditions.

The concentration of β-cyclodextrin was determined with freshly prepared solution of 1 part of 3.8 mM phenolphthalein in ethanol and 50 parts of carbonate buffer (pH=10.5). Appropriately diluted samples were mixed with 2.0 mL of the phenolphthalein solution and distilled water to a final volume of 5.0 mL. The absorbance was measured at 550 nm in relation to a blank sample, containing a mixture of water and phenolphthalein. β-Cyclodextrin concentration was calculated using a calibration curve. The content of α-cyclodextrin was determined with methyl orange (*10*), and of γ-cyclodextrin with bromocresol green (*11*).

Thin layer chromatography (TLC) was performed on a silica gel plate (Merck, 20×20 cm). A volume of 5.0 μL of the standard solutions of glucose, maltose, maltotriose, maltopentaose and α-, β- and γ-cyclodextrin (1.0 mg/mL each) and 5.0 μL of 20-fold diluted reaction mixtures were applied to the start line. The mobile phase consisted of propanol/ethylacetate/water in a ratio of 7:1:2. The chromatogram was developed once before the mobile phase reached 15 cm from the start line. After being air-dried in a horizontal position, it was sprayed with a mixture containing carbazol 0.5 g, sulphuric acid 5.0 mL, and ethanol 95.0 mL. After heating for 10 min in an oven at 120 °C the carbohydrates appeared as purple spots on a blue background.

Results and Discussion

The effect of substrate and enzyme concentration on β-cyclodextrin production was studied using Eq. 1 in two neighbouring overlapping areas of variation of their values. Native corn starch was used as a substrate in these experiments. In the first experimental design, starch concentration varied from 10.0 to 50.0 mg/mL and the amount of CGTase from 0.5 to 3.5 U/g. In the second experimental design, the effects of both factors were evaluated in the range of 40.0–100.0 mg/mL of starch and 3.0–7.0 U/g of CGTase. The dynamics of β-cyclodextrin formation in the range of the first experimental design is presented in Fig. 1.

Starch concentration had a strong effect on β-cyclodextrin production. Its increase led to a significant increase in β-cyclodextrin content of the reaction mixture. Comparing the results in Figs. 1a and 1c, it can be noticed that a 5-fold increase in starch concentration resulted in 3.0- to 3.6-fold increase in cyclodextrin concentration, depending on the enzyme amount.

Enzyme concentration had an effect on β-cyclodextrin production only up to 2.0 U/g. Further increase led only to an increase in the initial velocity of the enzyme

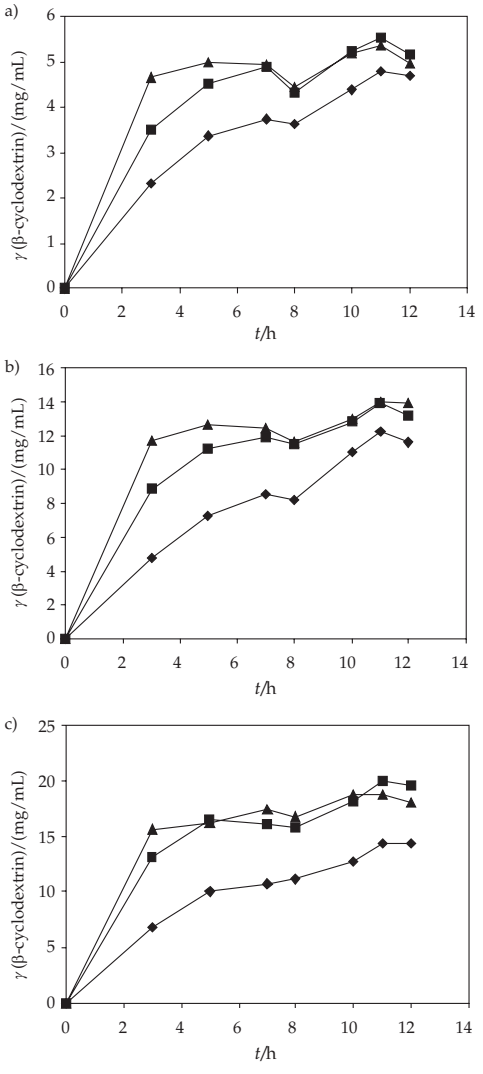

Fig. 1. Dynamics of β-cyclodextrin production from: a) 10.0, b) 30.0, c) 50.0 mg/mL of native starch with (♦) 0.5, (■) 2.0 and (▲) 3.5 U/g of CGTase

reaction (Figs. 1a–c). When the process was prolonged up to 10–12 h, β-cyclodextrin production was the same, not depending on the higher enzyme concentration. This fact may be explained by two hypotheses. On the one hand, CGTase action is strongly inhibited by β-cyclodextrin, and on the other hand, a state of equilibrium may be reached at the end of the process. In this case the higher enzyme concentration just leads to its faster approach and the maximum β-cyclodextrin concentrations are achieved earlier in the process time. Similar results were reported by other investigators (*12,13*).

In all experimental points maximum β-cyclodextrin was formed after 11 h of incubation. These values (marked in Table 2 with Y_1 and Y_2 for design 1 and design 2, respectively) were used for the development of the mathematical models. The regression coefficients and the test for their significance are presented in Table 3. The coefficients with p-value higher than 0.05 were found to be insignificant and were not included. The following mathematical models were obtained:

$$\hat{Y}_1=14.397+6.232\cdot x_1-1.905\cdot x_1^2-1.555\cdot x_2^2 \qquad /2/$$

$$\hat{Y}_2=25.303+5.707\cdot x_1-1.640\cdot x_1^2-0.310\cdot x_2^2 \qquad /3/$$

where $\hat{Y}_1$ and $\hat{Y}_2$ are the predicted values of β-cyclodextrin concentrations, achieved for values of x_1 and x_2 in the boundaries of design 1 and design 2, respectively. The analysis of variance (Table 4) showed that the regression equations are statistically significant at 99.0 % confidence level. The values of the response β-cyclodextrin concentrations $\hat{Y}_1$ and $\hat{Y}_2$ close to the experimental data Y_1 and Y_2 proved the adequacy of the models (Table 2).

After the investigation of Eqs. 2 and 3, their maxima were determined. The maximum value of $\hat{Y}_1$=18.72 mg/

Table 2. Experimental and predicted values of β-cyclodextrin concentration

No.	x_1	x_2	γ(β–cyclodextrin)/(mg/mL)					
			Design 1		Design 2		Design 3	
			Y_1	$\hat{Y}_1$	Y_2	$\hat{Y}_2$	Y_3	$\hat{Y}_3$
1	+1	+1	18.69	17.17	28.25	29.06	25.70	25.41
2	–1	+1	5.36	4.71	17.82	17.65	14.92	15.11
3	+1	–1	14.42	17.17	29.34	29.06	24.66	24.68
4	–1	–1	4.77	4.71	17.93	17.65	14.46	14.38
5	+1	0	19.95	18.72	29.90	29.37	24.43	24.71
6	–1	0	5.54	6.26	17.50	17.96	14.51	14.41
7	0	+1	13.95	12.84	23.86	24.99	21.62	21.72
8	0	–1	12.24	12.84	26.20	24.99	20.92	20.99
9	0	0	13.89	14.40	25.23	25.30	21.19	21.02

Table 3. Analysis for statistical significance of the regression coefficients

Term	Design 1		Design 2		Design 3	
	Value	p-value	Value	p-value	Value	p-value
Intercept	14.397	$3.960\cdot10^{-4}$	25.303	$2.510\cdot10^{-5}$	21.019	$1.380\cdot10^{-6}$
x_1	6.232	$7.980\cdot10^{-4}$	5.707	$3.560\cdot10^{-4}$	5.150	$1.540\cdot10^{-5}$
x_2	1.095	$9.160\cdot10^{-2}$	–0.590	0.155	0.367	0.034
$x_1\cdot x_2$	0.920	0.191	–0.245	0.567	0.145	0.316
$x_1\cdot x_1$	–1.905	$9.070\cdot10^{-2}$	–1.640	0.056	–1.463	0.003
$x_2\cdot x_2$	–1.555	0.138	–0.310	0.606	0.337	0.143

Table 4. Analysis of variance of the mathematical models

	Design 1				Design 2				Design 3			
	Df	SS	MS	F_{sign}	Df	SS	MS	F_{sign}	Df	SS	MS	F_{sign}
Regression	3	245.1	81.7	0.0014	3	200.9	66.0	0.0001	5	164.5	32.9	0.0001
Residual	5	14.2	2.8		5	4.1	0.8		3	0.2	0.1	
Total	8	259.3			8	205.1			8	164.7		

Df=degree of freedom; SS=sum of squares; MS=mean square; F_{sign}=F significance

mL was achieved at x_1=+1 and x_2=0. The analysis of Eq. 2 showed that 2.0 U/g of CGTase are sufficient for maximum β-cyclodextrin production at starch concentrations up to 50.0 mg/mL. According to Eq. 3 the maximum value of $\hat{Y}_2$=29.37 mg/mL was at x_1=+1 and x_2=0. However, the effect of x_2 in Eq. 3 was expressed only by the squared term. Besides, its value is insignificant compared to the other effects. For these reasons the peak value of $\hat{Y}_2$ was calculated at the bottom boundaries of design 2. At x_1=+1 and x_2=–1 the calculated value $\hat{Y}_2$=29.06 mg/mL was close to the maximum value, $\hat{Y}_2$=29.37 mg/mL. The relative error was found to be only 1.07 %. On the grounds of this fact we considered the optimal value of x_2 in the boundaries of design 2 to be x_2=–1. This conclusion was proved by the experimental data in Table 2. The increase of CGTase concentration over 3.0 U/g had no positive effect on β-cyclodextrin.

The effect of both factors in the whole studied area of variation is shown graphically in Fig. 2. The response surfaces fitted well to each other, indicating that both regression equations described adequately the effect of the variables; they also clearly showed that β-cyclodextrin increased with the increase in starch concentration. However, gelatinized starch in concentration above 50.0 mg/mL is characterized with high viscosity and is difficult to handle. One of the approaches to overcome this problem is the use of soluble starch. For these reasons the effect of the variables was studied when soluble starch was applied as a substrate. The variation intervals tested were the same as in the case of native starch (Table 1). In this case, maximum β-cyclodextrins were obtained earlier in the process (after 4 h of incubation). This was probably due to the lower viscosity of the reaction mixture and the easier availability of the substrate to the enzyme. The maximum values of β-cyclodextrin produced (marked with Y_3 in Table 2) were used for developing a mathematical model. The regression coefficients and their test for statistical significance are presented in Table 3. Not considering the insignificant terms, the following equation was obtained:

$$\hat{Y}_3=21.019+5.150\cdot x_1+0.367\cdot x_2-1.463\cdot x_1^2+0.337\cdot x_2^2 \quad /4/$$

The model was found to be significant at confidence level of 99.0 %. The maximum $\hat{Y}_3$=25.41 mg/mL of Eq. 4 was achieved at x_1=+1 and x_2=+1. As in the case of native starch, maximum amount of β-cyclodextrin was formed at 100.0 mg/mL of initial starch concentration. CGTase concentration had insignificant effect on $\hat{Y}_3$. The calculated $\hat{Y}_3$=24.68 mg/mL at x_1=+1 and x_2=–1 was close to the maximum value of $\hat{Y}_3$=25.41 mg/mL. The relative error was found to be 2.9 %. For these reasons the value of x_2=–1 was considered to be optimal. The experimental results in Table 2 proved this consideration. The increase in CGTase over 3.0 U/g was inefficient.

When determining the optimal starch concentration, β-cyclodextrin yield should also be taken into account. The cyclodextrin yield as a function of initial starch concentration at optimal reaction conditions is presented in Fig. 3. Maximum conversion degree (about 55 %) was achieved with 10.0 mg/mL of native starch. The increase in substrate concentration led to a significant decrease in β-cyclodextrin yield. When 100.0 mg/mL of starch were used, the yield was only about 29 %. The lower conversion degree of the higher starch amount may be due to CGTase inhibition by β-cyclodextrin. Decrease in cyclodextrin yield, when high starch concentrations are applied, has been reported by many authors (*14–19*). The same tendency was observed with soluble starch. However, its degree of conversion was lower in comparison with the native substrate. The enzyme transformation of the soluble starch is impeded not only by the inhibitory action of β-cyclodextrin, but also by the high dextrose equivalent (*6,7*). In spite of the lower yield, the soluble starch could be used as a substrate with the aim of application in high concentrations and reduction of process time.

The use of native starch in concentrations above 50.0 mg/mL is not effective and is hardly realizable. For

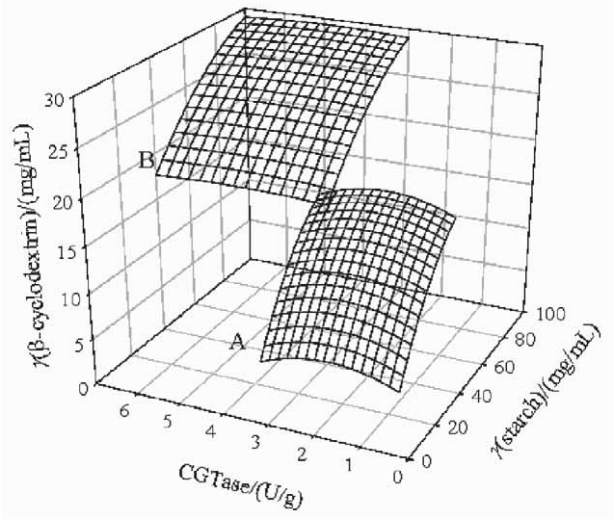

Fig. 2. Effect of starch and CGTase concentrations on β-cyclodextrin production in the whole area of variation of the factors: A – in the range of design 1; B – in the range of design 2

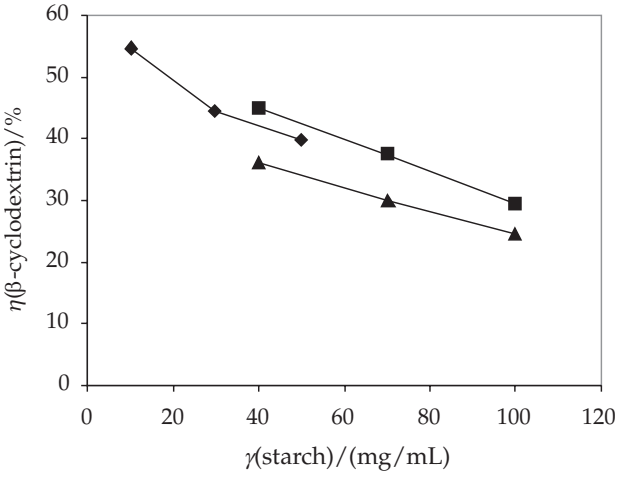

Fig. 3. Effect of starch concentration on β-cyclodextrin yield: (♦) native starch, 2.0 U/g of CGTase; (■) native starch, 3.0 U/g of CGTase; (▲) soluble starch, 3.0 U/g of CGTase

these reasons the concentration of 50.0 mg/mL was considered to be optimal for cyclodextrin formation. In this case, 2.0 U/g of CGTase and 11-hour process time were sufficient for achieving a considerably high cyclodextrin concentration (about 19 mg/mL) at satisfactory yield (about 40 %).

The results of all experiments suggest that CGTase was strongly inhibited by β-cyclodextrin. This assumption was confirmed in another experiment, in which CGTase activity was determined in the presence of different amounts of cyclic and linear oligosaccharides (Fig. 4). All tested compounds reduced CGTase activity. The inhibition pattern of the three types of cyclodextrins was not similar. In the presence of α-cyclodextrin CGTase retained about 80 % of its activity, whereas β- and γ-cyclodextrins caused higher inhibition. In the presence of 12 mg/mL of β-cyclodextrin, a complete loss of activity was observed. The linear oligosaccharides inhibited the enzyme action as well. In the presence of glucose and maltose, CGTase activity was reduced up to 60 and 40 %, respectively. Many authors reported CGTases inhibition by the reaction products. Distinctively, the highest inhibition was caused by the predominant type of cyclodextrins (*12*,*15*,*20*–*22*).

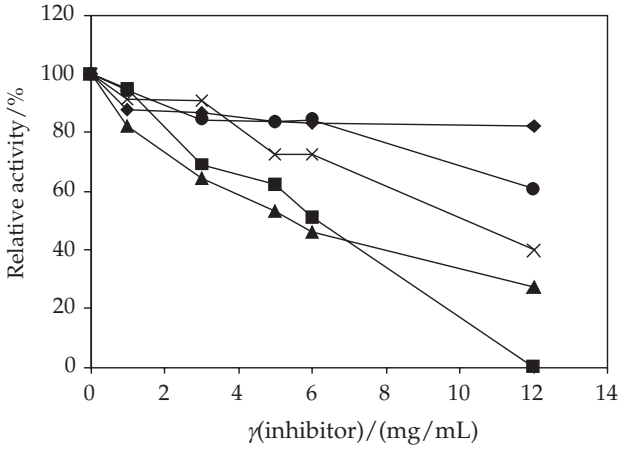

Fig. 4. CGTase inhibition by cyclic and linear oligosaccharides: (♦) α-cyclodextrin, (■) β-cyclodextrin, (▲) γ-cyclodextrin, (●) glucose, and (x) maltose

Except for the product inhibition, another reason for the incomplete conversion of starch in cyclodextrins is the coupling activity of the enzyme. The ability of CGTase to degrade β-cyclodextrin was proved by adding 12.0 mg/mL of β-cyclodextrin to the reaction mixture after 4 h of incubation. After the addition, β-cyclodextrin content of the reaction mixture and of a respective control probe was determined. The results are presented in Fig. 5. In the presence of high β-cyclodextrin concentration, CGTase activity was inhibited and the enzyme formed no more β-cyclodextrin. However, this high concentration favoured the coupling activity of the enzyme. As a result the content of β-cyclodextrin was rapidly reduced and at the end of the process almost the whole added amount was degraded. The predominance of one of the enzyme activities was probably determined by the reaction conditions. The high β-cyclodextrin amount, inhibiting CGTase activity, activated the reverse reaction.

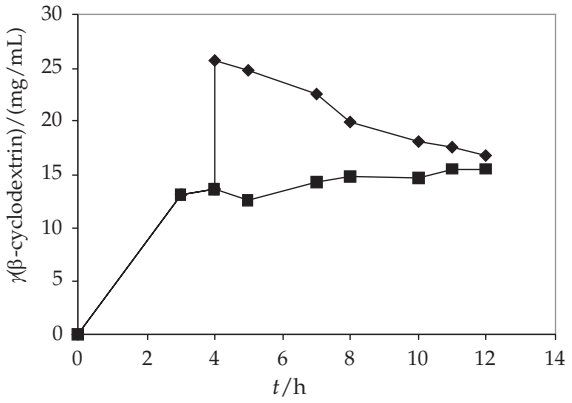

Fig. 5. β-Cyclodextrin coupling by CGTase. The reaction was carried out with 50.0 mg/mL of soluble starch and 2.0 U/g of CGTase (♦) with and (■) without the addition of 12.0 mg/mL of β-cyclodextrin to the reaction mixture after 4 h of incubation

The product inhibition and the coupling activity of CGTase limited the complete conversion of starch in cyclodextrins. Yield increase can be achieved after removing the inhibition action of β-cyclodextrin by the use of specific complexing agents or by ultrafiltration.

All CGTases form a mixture of α-, β-, and γ-cyclodextrins in various ratios (*23*–*25*). The product profile of the process at optimal conditions is shown in Fig. 6. The CGTase from *Bacillus megaterium* formed predominantly β-cyclodextrin. The ratio α/β/γ after 11 h of incubation was 10:79:11. When the reaction time was prolonged, the amount of β-cyclodextrin appeared to decrease and α-cyclodextrin increased. The ratio α/β/γ after 20 h of enzyme reaction was found to be changed to 29:67:4. This is probably due to the ability of CGTase to convert different types of cyclodextrins to one another. This fact was established by other authors as well (*13*,*26*–*28*).

The products formed by CGTase action were analyzed by TLC. The results confirmed the presence of α-, β-, and γ-cyclodextrins in the reaction mixture. Linear oligosaccharides with five or less glucose units were not detected by the TLC.

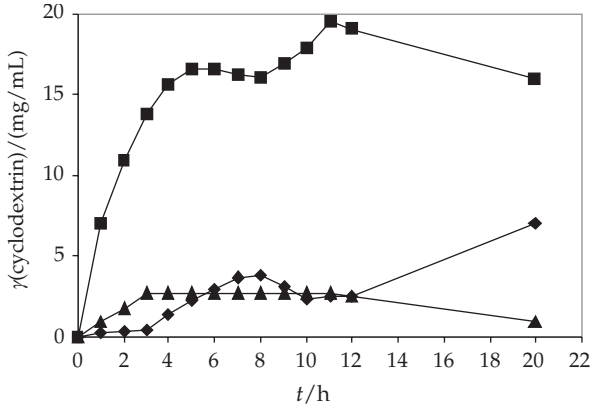

Fig. 6. Formation of (♦) α-, (■) β- and (▲) γ-cyclodextrins under optimal reaction conditions (50.0 mg/mL of native starch, 2.0 U/g of CGTase, pH=7.0, 45 °C)

Conclusion

Mathematical models for determination of the effect of starch and enzyme concentration on β-cyclodextrin production were developed. The models described the process in two overlapping areas of variation of the independent variables and predicted adequately the reaction in the whole studied area. The mathematical description was used to determine the optimal substrate and enzyme concentrations. The optimal starch concentration was considered to be 50.0 mg/mL and the optimal enzyme dosage was 2.0 U/g. The conversion of starch to cyclodextrins depended not only on the reaction conditions. The severe product inhibition, the coupling activity of the enzyme and its ability to transform different types of cyclodextrins into one another impeded the process and lowered β-cyclodextrin yield. Yield enhancement is possible by removing these limiting factors.

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