Cyclodextrins (CD) are cyclic nonreducing oligosaccharides composed of α-1,4-linked glucose units, which are designated α, β and γ, according to the number of glucose units (6, 7 or 8, respectively). CD molecules possess a hydrophobic inner cavity, in which hydrophobic compounds can be encapsulated. As a result, the properties of the guest molecules are altered. This ability of CD determines their broad application in different areas of industry. They are applied in food industry for removal of unwanted flavour and aroma, for protection of guest molecules from degradation under the action of light and heat, for reduction of side effects of drug formulations, for improvement of water solubility of insoluble compounds, for stabilization of volatile substances, etc. (Del Valle, 2004).

CD are produced by enzyme conversion of starch with cyclodextrin glucanotransferase (CGTase, 2.4.1.19). CGTase is a unique enzyme produced only by microorganisms, usually Bacillus species (Tonkova, 1998). All known CGTases form the three types of CD, but in different ratio (Leemhuis et al., 2010; Qi and Zimmermann, 2005). According to the predominant type of CD formed they are classified as α-, β- and γ-CD. The product specificity of CGTases determines their application for production of certain type of CD.

The yield and ratio of CD depend on the properties of CGTase, kind of substrate used (Alves-Prado et al., 2008), its preliminary treatment (Goh et al., 2007; Pishtiyski and Zhekova, 2006; Sakinah et al., 2009) and reaction conditions (Martins and Hatti-Kaul, 2003; Maticoli et al., 2001). A great number of reports, describing the effect of the reaction conditions on the production of the predominant type of CD and determination of the optimal parameters of the process, are available (Charoenlap et al., 2004; Gawande and Patkar, 2001; Rauf et al., 2008; Szerman et al., 2007). However, there is no data for determination of the conditions for maximal production of certain type of CD at the terms of minimal amounts of concomitant types of CD. This is a subject of a certain interest, as the presence of several types of CD in the reaction mixture requires separation and purification of the desired product.

On the other hand the enzyme reaction can be directed to formation of a certain type of CD by selection of proper conditions. Additionally this fact allows the enzyme to be used not only for production of the predominant type of CD, but also for production of the concomitant types of CD. The enzyme from Bacillus

**Reaction Conditions for Maximal Cyclodextrin Production by Cyclodextrin Glucanotransferase from Bacillus megaterium**

BORIANA Y. ZHEKOVA* and VESELIN S. STANCHEV

1Department of Biochemistry and Molecular Biology, University of Food Technologies, Plovdiv, Bulgaria
2Department of Automatics, Information and Control Systems, University of Food Technologies
Plovdiv, Bulgaria

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**Abstract**

The effect of the reaction conditions (substrate concentration, enzyme dosage, and pH) on cyclodextrin production by cyclodextrin glucanotransferase from Bacillus megaterium was investigated by applying mathematical modeling methods. Adequate models were developed and they were used for determination of the optimal conditions for maximal formation of β-cyclodextrins at minimal concentrations of α- and γ-cyclodextrins. The main factor affecting the ratio of the products was pH of the reaction mixture. At pH 9 the enzyme formed mainly β- and γ-cyclodextrins and the ratio α:β:γ was 2.6:83.5:13.9; at pH 5 the ratio changed to 8.6:84.6:6.8. Mathematical models were used for determination of the conditions of maximal conversion of the substrate into cyclodextrins. 45.88% conversion of starch was achieved at 5% substrate concentration, 3.5 U/g enzyme dosage, and pH 7.4.

**Key words:** cyclodextrins, cyclodextrin glucanotransferase, mathematical modeling, Bacillus megaterium

*Corresponding author: B.Y. Zhekova, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria; phone: (+359) 32 603 605; fax: (+359) 32 644 102; e-mail: zhekova_b@yahoo.com
megaterium used in this study formed β-CD as the predominant product of the cyclization reaction (Zhekova et al., 2008; Zhekova et al., 2009).

The aim of the current research was establishment of an optimal working point for the production of CD, which determines maximal amount of β-CD at the conditions of minimal presence of α- and γ-CD, and determination of the conditions for main formation of α- and γ-CD and maximal conversion of starch into CD.

**Experimental**

**Material and Methods**

**Substrate and enzyme preparation.** The substrate for CD production was corn starch obtained from Amilum. The enzyme preparation used was a crude CGTase from *B. megaterium* (from the collection of the Department of Biochemistry and Molecular Biology, University of Food Technologies, Plovdiv) with activity of 2.54 U/ml. The cultivation of the strain and biosynthesis of the enzyme were performed as described in previous research (Pishiysi et al., 2008).

**Enzyme reaction.** Substrate solutions were prepared in citrate-phosphate buffer (pH 5.0–9.0), in a manner allowing the desired concentration to be reached after the addition of the enzyme preparation. The substrate was gelatinized in a steam water bath for 10 min, cooled to 30°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 30°C on a reciprocal shaker for 10 h. CGTase was inactivated by boiling for 10 min in a water bath and the content of α-, β- and γ-CD formed was determined.

**Experimental design.** The effect of starch concentration, CGTase dosage and pH on cyclodextrin production was studied by using optimal composite design with distance from the centre of the design space to a factorial point ±1 (Mason et al., 2003).

The static of the process was described by using nonlinear mathematical models of the type:

\[ Y = β_0 + \sum_{i=1}^{k} β_i x_i + \sum_{i=1}^{k} β_{0i} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} β_{ij} x_i x_j \]

where the variable \( Y \) 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, \( β_{0i} \) 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 I.

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

**Assays.** CGTase activity was determined by Kestner’s method (Kestner et al., 1989) as described previously (Zhekova et al., 2008).

The concentration of α-CD was determined by the method with methyl orange (Lejeune et al., 1989), of β-CD – with phenolphthalein (Kestner et al., 1989), of γ-CD – with brom cresol green (Kato and Horikoshi, 1984).

CD concentrations were confirmed by HPLC system Shimadzu 20 AHT with refractive index detector. For estimation of α-, and γ-CD YMC-Pack ODS-AQ column (YMC Europe) was used. The mobile phase was a mixture of methanol and water in a ratio of 3:97 with a flow rate of 1.3 ml/min, and the column temperature was 30°C. β-CD were analyzed on Ultrahydrogel column (Waters), at 30°C, and bidestilled water as a mobile phase with a flow rate of 0.8 ml/min.

**Results and Discussion**

The effect of the investigated factors in the variation intervals, presented in Table I on α-, β- and γ-CD production was analyzed by using nonlinear regression equations (1).

The optimal composite design, the experimental data for α-, β- and γ-CD (designated \( Y_α \), \( Y_β \) and \( Y_γ \) respectively) and the corresponding predicted values (\( \hat{Y}_α \), \( \hat{Y}_β \), \( \hat{Y}_γ \)) are presented in Table II.

The following regression equations were obtained after removal of the insignificant terms:

\[ \hat{Y}_α = 1.703 + 0.206 x_1 + 0.19 x_2 - 0.376 x_3 - 0.16 x_1 x_3 - 0.13 x_2 x_3 - 0.42 x_1^2 - 0.3 x_2^2 - 0.12 x_3^2 \]  

(2)

\[ \hat{Y}_β = 12.571 + 5.234 x_1 + 3.138 x_2 + 1.669 x_1 x_2 - 1.693 x_2^2 - 1.973 x_1^2 \]  

(3)

\[ \hat{Y}_γ = 1.832 + 0.521 x_1 + 0.332 x_2 + 0.716 x_3 + 0.156 x_1 x_3 + 0.099 x_2 x_3 - 0.249 x_1^2 - 0.249 x_2^2 \]  

(4)

The analysis of variance (Table III) showed that the regression equations are statistically significant at 95% confidence level.

The extremums of equations (2), (3) and (4) are presented in Table IV.

It was noticed that they were achieved at different values of the independent variables. This confirmed the

Table I

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>( x_1 ) starch (%)</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>( x_2 ) CGTase (U/g)</td>
<td>0.5</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>( x_3 ) pH</td>
<td>5</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>
hypothesis that the ratio of α-, β- and γ-CD can be controlled by a change in the reaction conditions.

The mathematical models obtained can be interpreted in several aspects. It is of great interest to determine the working conditions at which equations (2), (3) and (4) achieved their maximal value. This allows the models to be used for selection of conditions at which maximal amount of a certain CD type is produced at beforehand known concentration of other types of CD. The enzyme used in this study formed mainly β-CD. For these reasons the models developed were interpreted in the respect of production of maximal amount of β-CD at minimal concentration of α- and γ-CD.

Minimal values of $\hat{Y}_\alpha$ and $\hat{Y}_\gamma$ were achieved at equal levels of the first and second independent variable $x_1 = -1$ and $x_2 = -1$ (Table IV). However, under these conditions $\hat{Y}_\beta$ also reached its minimum. Consequently, these two factors could not be used for control of the

### Table II
Optimal composite design for three factors and three levels of their variation

<table>
<thead>
<tr>
<th>No</th>
<th>$x_1$</th>
<th>$x_2$</th>
<th>$x_3$</th>
<th>$\alpha$-CD (mg/ml)</th>
<th>$\beta$-CD (mg/ml)</th>
<th>$\gamma$-CD (mg/ml)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
<td>0.38</td>
<td>0.55</td>
<td>2.98</td>
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<tr>
<td>2</td>
<td>-1</td>
<td>-1</td>
<td>1</td>
<td>0.32</td>
<td>0.38</td>
<td>2.46</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
<td>1.24</td>
<td>1.19</td>
<td>5.30</td>
</tr>
<tr>
<td>4</td>
<td>-1</td>
<td>1</td>
<td>1</td>
<td>0.40</td>
<td>0.50</td>
<td>5.52</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>-1</td>
<td>-1</td>
<td>1.48</td>
<td>1.28</td>
<td>10.48</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>-1</td>
<td>1</td>
<td>0.52</td>
<td>0.47</td>
<td>8.66</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>-1</td>
<td>1.83</td>
<td>1.92</td>
<td>17.29</td>
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<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.61</td>
<td>0.59</td>
<td>20.78</td>
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<td>9</td>
<td>-1</td>
<td>0</td>
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<td>1.36</td>
<td>1.08</td>
<td>4.07</td>
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<tr>
<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.32</td>
<td>1.49</td>
<td>15.26</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>-1</td>
<td>0</td>
<td>1.20</td>
<td>1.21</td>
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<tr>
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<td>1</td>
<td>0</td>
<td>1.72</td>
<td>1.59</td>
<td>13.02</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
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<td>-1</td>
<td>1.98</td>
<td>1.96</td>
<td>11.56</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.30</td>
<td>1.21</td>
<td>11.25</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
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<td>0</td>
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<td>1.70</td>
<td>14.88</td>
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<td>0</td>
<td>1.78</td>
<td>1.70</td>
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</tr>
<tr>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.52</td>
<td>1.70</td>
<td>12.81</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.58</td>
<td>1.70</td>
<td>11.97</td>
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</table>

### Table III
Statistical analysis results according to Anova

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<th>Parameter</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>$F_{sign}$</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>$F_{sign}$</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>$F_{sign}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>8</td>
<td>4.83</td>
<td>0.60</td>
<td>8.7E-5</td>
<td>5</td>
<td>441</td>
<td>88.2</td>
<td>1.2E-6</td>
<td>7</td>
<td>10.1</td>
<td>1.44</td>
<td>1.9E-6</td>
</tr>
<tr>
<td>Residual</td>
<td>9</td>
<td>0.28</td>
<td>0.03</td>
<td></td>
<td>12</td>
<td>31.5</td>
<td>2.6</td>
<td></td>
<td>10</td>
<td>0.37</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>5.11</td>
<td></td>
<td></td>
<td>17</td>
<td>473</td>
<td></td>
<td></td>
<td>17</td>
<td>10.4</td>
<td></td>
<td></td>
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</table>

### Table IV
Extremum of equations (2), (3) and (4)

<table>
<thead>
<tr>
<th>Extremum</th>
<th>Equation (2)</th>
<th>Equation (3)</th>
<th>Equation (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value (mg/ml)</td>
<td>$\hat{Y}_\alpha^{max}$</td>
<td>$\hat{Y}_\beta^{max}$</td>
<td>$\hat{Y}_\gamma^{max}$</td>
</tr>
<tr>
<td>Conditions: $x_1$</td>
<td>$-1$</td>
<td>$-1$</td>
<td>$-1$</td>
</tr>
<tr>
<td>$x_2$</td>
<td>$-1$</td>
<td>$-1$</td>
<td>$-1$</td>
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<tr>
<td>$x_3$</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
values of the functions were calculated at the highest
had a negative effect on β-CD. For these reasons the
reaction mixture – the concentration of β-CD was max-
imal at the whole investigated interval of pH (5.0–9.0).

With regard to the third independent variable (pH of
the reaction mixture) there were significant differ-
ences at the values at which Ŷα, Ŷβ and Ŷγ reached their
minimums. Ŷα\text{min} = 0.38 mg/ml was achieved at x1 = 1,
and Ŷγ\text{min} = 0.02 mg/cm² – at x3 = -1 (Table IV). On the
other hand formation of β-CD did not depend on pH of
reaction mixture – the concentration of β-CD was max-
imal at the whole investigated interval of pH (5.0–9.0).
These results allowed the process for CD production to
be performed at reaction conditions which ensured for-
mation of only two types of CD (α and β) or (γ and β).

The minimal values of α- and γ-CD concentrations
were formed at the lowest levels of x1 and x3, which also had a negative effect on β-CD. For these reasons the
values of the functions were calculated at the highest
levels of x1 and x3 and variation of x2.

When the process was performed at x1 = 1, x2 = 1
and x3 = 1, the predicted concentrations of CD accord-
ing to equations (2), (3) and (4) were as followed:
Ŷα = 0.59 mg/ml, Ŷβ = 18.95 mg/ml and Ŷγ = 3.16 mg/ml.
Since at these conditions the concentration of α-CD was
only 3.1% in regard to β-CD concentration, they were
considered as optimal for production of β-CD in the
absence of α. These results were confirmed by per-
forming 4 parallel experiments and the following mean val-
ues of α-, β- and γ-CD were registered: Ŷα\text{exp} = 0.56 mg/ml,
Ŷβ\text{exp} = 18.79 mg/ml, Ŷγ\text{exp} = 3.13 mg/ml. A test for equal-
ity of the mathematical expectation of the experimen-
tal results and the predicted data was conducted. As
the values of t α, (t α, crit = 1.058, t β, crit = 0.392, t γ, crit = 0.821
respectively) were lower than t crit = 3.182 at 95% signifi-
cant level and degree of freedom 3, it was established
that there was no statistically significant difference
between the experimental and predicted results.

It can be concluded that at starch concentration of
5.0%, enzyme dosage 3.5 U/g and pH 9 CGTase from
Bacillus megaterium formed mainly β- and γ-CD and
the ratio α:β:γ was 2.6:83.5:13.9. At these conditions
α-CD were only 2.6% of the total CD amount.

For determination of the conditions at which the
enzyme formed maximal amount β-CD at minimal
γ-CD concentration, the predicted values of equations
(2), (3) and (4) at x1 = 1, x2 = 1, x3 = -1 were calculated.
These were Ŷα = 1.92 mg/ml, Ŷβ = 18.95 mg/ml and
Ŷγ = 1.53 mg/ml, respectively. The experimental results
(mean value of 4 experiments) under these conditions
were as follows: Ŷα\text{exp} = 1.87 mg/ml, Ŷβ\text{exp} = 18.21 mg/ml,
Ŷγ\text{exp} = 1.48 mg/ml. No statistical difference was observed
between the experimental and predicted data
(t α, crit = 1.475, t β, crit = 2.039 and t γ, crit = 1.65 were lower
than t crit = 3.182). The ratio of the three types of CD
was 8.6:84.6:6.8. There was no significant decrease in
γ-CD concentration and its percent ratio was close
to the value of α-CD. The analysis of (2), (3) and (4)
showed that the amount of γ-CD can be decreased only
by a change in x1 and x3. However, these two fac-
tors influenced the concentration of β-CD in a similar
way. For example at the lowest levels of the factors
the enzyme did not formed γ-CD, but the concentration
of the main product was only 2.20 mg/ml (Table IV).
This was probably due to the characteristic feature of
CGTase from B. megaterium to form β- and γ-CD in
a certain ratio independently of the reaction conditions.

The established considerations are presented graphi-
ically in Fig. 1. It can be seen that formation of β-CD
does not depend on pH in the interval from 5.0 to 9.0.
The ratio of the other two types of CD was significantly
influenced by this factor. At low values of pH CGTase
formed predominantly α-CD, and at high values of pH
it produced γ-CD. A change in the ratio of CD depend-
ning on pH of the reaction mixture was reported for
other CGTases as well (Atanasova et al., 2009; Martins

The mathematical models developed can be used also
for determination of the conditions in which CGTase
formed maximal amount of α- and γ-CD, which are not
the predominant product of the reaction. This applica-
tion of the models is not only important in the theoreti-
cal aspect. It has a practical impact if the process for
CD production is performed with the aim of maximal
conversion of the substrate in CD, independently of the
ratio of the products.

The effect of starch and CGTase concentrations on
α- and γ-CD production at optimal pH is presented in
Fig. 2. Data about β-CD was not included, since their
production was investigated in a previous research
(Zhekova et al., 2008).
Cyclodextrin production by cyclodextrin glucanotransferase from \textit{B. megaterium}

The increase in starch concentration led to an enhancement of the two types of CD. With regard to α-CD (Fig. 2a) a presence of a saturation substrate concentration (3.44%) was observed. This may be due to substrate or product inhibition of α-CD forming activity of the enzyme. In the case of γ-CD production no saturation of the substrate was reached in the interval of the experiment.

CGTase concentration had an effect on α-CD formation up to 2.8 U/g, and it influenced γ-CD yield up to 3.47 U/g. In a previous research a similar dependence was established for β-CD. The reason for this fact was found to be product inhibition of CGTase by β-CD (Zhekova \textit{et al.}, 2008). Probably α- and γ-CD forming activities of the enzyme were also inhibited by the corresponding type of product. Similar results were established for other CGTases (Gawande and Patkar, 2001; Tomita \textit{et al.}, 1990).

Maximal sum of the concentrations of the three types of CD 22.94 mg/ml ($\bar{Y}_1 = 1.24$ mg/ml, $\bar{Y}_2 = 18.95$ mg/ml and $\bar{Y}_3 = 2.75$ mg/ml) was achieved under the following conditions $x_1 = 1$, $x_2 = 1$ and $x_3 = 0.202$. This concentration corresponded to 45.88% conversion degree of starch into CD, which was a good yield taking into account that it was obtained without the use of organic solvents.

The mathematical models developed were used for working out a regression equation for the effect of the factors on the conversion degree of substrate in CD (5):

$$\%_{\text{conversion}} = 56.43 - 10.97.x_1 + 14.163.x_2 + 2.06.x_1.x_2 - 3.68.x_1.x_3 + 2.99.x_2^2 - 11.8.x_3^2 - 4.66.x_3^2$$

Maximal conversion of starch 77.19% was achieved at $x_1 = -1$, $x_2 = 0.753$ and $x_3 = 0.221$. However, under these conditions the sum of the concentrations of the three types of CD was only 8.03 mg/ml. This was probably due to the low level of substrate concentration. In a previous work it was established that increase in starch concentration led to enhancement of concentration of CD, but decreased the conversion degree. The reason for this was the product inhibition of CGTase by β-CD (Zhekova \textit{et al.}, 2008).

**Conclusions**

Adequate mathematical models for the effect of starch concentration, CGTase dosage and pH on α-, β-, and γ-CD production were developed. They were successfully used for determination of the conditions, in which CGTase formed maximal amount of β-CD, at possibly minimal concentration of α- and γ-CD. The ratio of the product can be controlled by change in the pH of the reaction mixture. Another application of the models was determination of the conditions for maximal conversion of starch into CD. This approach led to 45.88% degree of substrate conversion, which was a good yield for a process without organic solvents.

**Literature**


