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Studies on enzymatic continuous production of cyclodextrins in an ultrafiltration membrane bioreactor

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Abstract

Studies on simultaneous hydrolysis of starch and synthesis of cyclodextrins by *Thermo-aerobacter* cyclodextrin glucosyltransferase were conducted in an ultrafiltration membrane bioreactor, allowing enzyme recovery and reduction of product inhibition. The influence of various reaction parameters like starch concentration, enzyme dosage and residence time on cyclodextrin composition was tested. A comparison of batch and continuous cyclodextrin production indicates that employing an ultrafiltration membrane bioreactor increases process efficiency.

Keywords: Cyclodextrin glucosyltransferase; Starch; Cyclization; Membrane techniques

1. Introduction

Cyclodextrins (CDs) are cyclic maltooligosaccharides formed by glucopyranose units linked by α-1,4 bonds. α-CD has six glucose units, while β-CD has seven and γ-CD has eight. CDs are ring molecules with a hydrophilic exterior and a hydrophobic cavity. This structure allows them to form inclusion complexes with a wide variety of organic compounds, which makes them suitable for use in the food, pharmaceutical, cosmetic and agricultural industries. They are used for alteration of solubility, masking of unwanted effects, inhibition of some chemical reactions, volatility control, and separation and isolation of compounds (Hedges, 1997; Shieh & Hedges, 1996; Szejtli, 1982, 1996).

CDs are produced from starch by the action of cyclodextrin glucosyltransferase (CGTase). Many CGTases such as those from *Bacillus* strains require a preliminary treatment of starch with α-amylase, and this requires a large amount of energy. However, investigations of direct action of CGTase on native starch (Słomińska & Sobkowiak, 1997) or isolated *Bacillus* sp. BE 101 have also been carried out (Kim, Lee, & Kim, 1993; Lee and Kim, 1991), as have attempts at simultaneous fermentation and cyclization (Lima, De Moraes, & Zanin, 1998). In order to increase CD yield, organic solvents such as isopropanol and tertiary butanol (Lee & Kim, 1992), bromobenzene (Raja & Ramakrsishna, 1994; Raja, Sredharan, Prema, & Ramakrishna, 1990) or cyclohexane have been used (Gawande and Patkar, 2001; Morita, Yoshida, & Karube, 1996).

Membrane processing is a relatively new unit operation to produce starch conversion products. The basic concept of enzymatic membrane reactors is based on the separation of enzyme and products by a semipermeable membrane. Ultrafiltration reactors offer the possibility of converting starch to CDs and reusing the enzyme, which increases the economic viability of the process.

Production may be modified by separately controlling the temperature, pH, substrate and enzyme concentrations, fluid velocity, pressure, reactor volume or membrane surface. Continuous recycle membrane reactors are a time-saving and a low-cost technology, as separation and reaction may be tightly integrated, thus leading to continuous production and enzyme reuse.

The aim of the study was to determine the influence of some selected parameters on cyclization process carried out by CGTase in a membrane recycle bioreactor.

2. Experiments

2.1. Enzymes

Heat-stable CGTase was Toruzyme 3.0 l from *Thermo-anaerobacter* produced by Novo Nordisk (Denmark). Its

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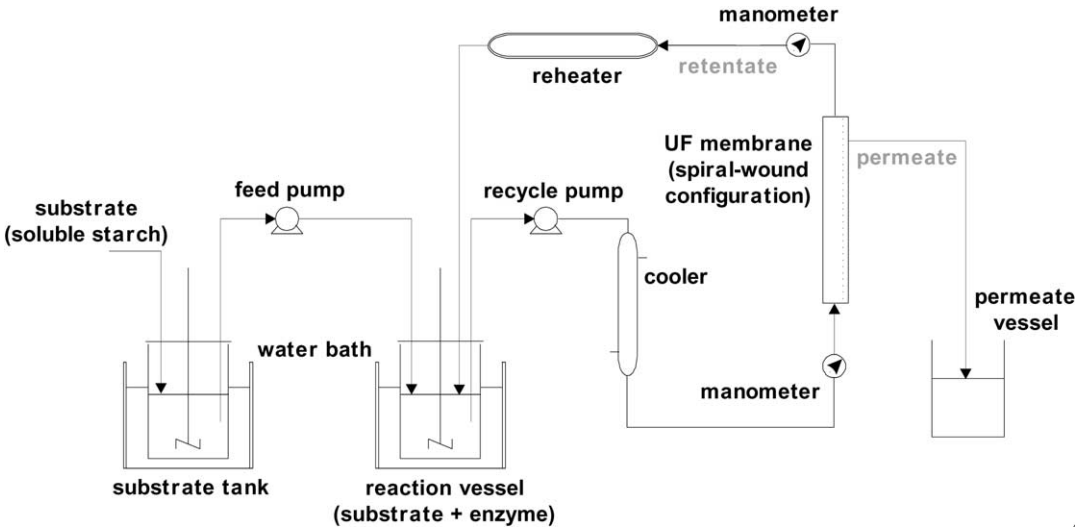

Fig. 1. Schematic diagram of UF reactor system.

activity was 3 KNU/g, where 1 KNU is the amount of enzyme that hydrolyzes 5.26 g starch per hour using Novo's standard method for determination of α amylase (substrate, soluble starch, calcium content of solvent, 0.0043 M, reaction time, 7–20 min, temperature −37 °C, pH 5.6; Novo Nordisk's analitycal method AF 9).

2.2. Substrate

The substrate was a soluble potato starch purchased from Polish Chemical Reagent, Gliwice, Poland (highest purity soluble starch, pH of 2% solution at 25 °C, 5.5–6.5, losses after drying-max 12%, residue after ignition-max 0.3%).

2.3. Batch process for CD production

Soluble starch of 5–25% (w/v) at pH 5.0 (adjusted with citric acid) was incubated with CGTase by shaking at 90 °C for 5 h and samples taken for determination of CD composition. The qualitative and quantitative distribution of CD mixtures was determined by high performance liquid chromatography using a 250 × 6 mm Ostion LG KS column at 85 °C with distilled water as the eluent at a flow rate of 0.6 ml/min.

2.4. Continuous CD production in the membrane bioreactor

An Amicon model 2000 RA ultrafiltration module (a length of 23.8 cm, and a diameter of 9.1 cm) coupled with a stirred tank reactor was used as a membrane bioreactor. Cellulose acetate membranes obtained from Amicon with nominal molecular cut-off sizes of 3 kDa (effective area 0.93 m^2) and 10 kDa (effective area 9.29 m^2) were used. The reaction system, consisting of a glass vessel with mechanical stirrer as the main reactor with the ultrafiltration system attached to it, was filled with soluble starch solution containing CGTase (Fig. 1). The total reaction volume was always kept at 600 ml. Then the reaction mixture was continuously pumped to the membrane module and recycled back to the reaction vessel. A container was used to continuously feed fresh substrate to the reactor. All continuous enzymatic hydrolysis experiments were conducted at pH 5.0 and 90 °C. The temperature stability of the membrane was 55 °C, so the reaction mixture was cooled before reaching and heated after leaving the membrane (cooling and heating jacket). All experiments were conducted at a transmembrane pressure of 55 kPa. After each experiment, the whole system was cleaned and rinsed with distilled water and operated again.

Experiments were carried out with different (i) membrane porosities (cut-off values), (ii) substrate concentrations, (iii) enzyme dosages, (iv) reaction mixture residence times and (v) additional enzyme dosages.

3. Results and discussion

Two main aspects of the research are important:

1. The enzyme does not need liquefied starch as a substrate because it catalyses two reactions, starch hydrolysis and CD synthesis. The high temperature of enzyme action suppresses microbial contamination and the enzyme does not require an extended reaction time.
2. The use of an ultrafiltration bioreactor makes it possible to carry out continuous conversion of starch to CDs when a feed solution is delivered at a moderate rate with the product outflow. The use of a membrane causes a selective separation of molecules according to their physical dimensions. Product separation is achieved with simultaneous recycling of undigested substrate and enzyme. Consequently, enzyme is recovered and can be reused. The loss of enzyme activity can be supplemented by addition of further enzyme.

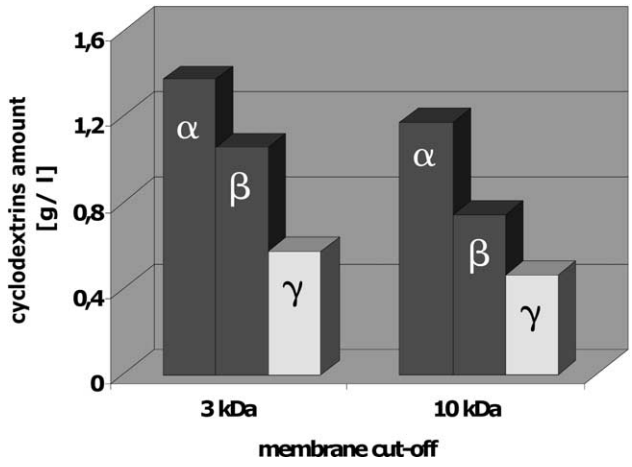

Fig. 2. The effect of membrane cut-off on starch cyclization. Reaction conditions were 10% DS starch concentration, pH 5.0, 90 °C, 0.015 KNU/g enzyme dosage, 5 h reactor residence time, transmembrane pressure 55 kPa.

3.1. Effect of membrane molecular weight cut-off

The influence of membrane cut-off on cyclization composition and concentration is shown in Fig. 2. Membranes with 3 and 10 kDa were used. The lower cut-off yields products with a higher proportion of CDs. This also gives better rejection property; however, a membrane leads to an unacceptable reduction of permeate flux. A sharp decrease of permeate outflow within 2 h was observed with the membrane 3 kDa (Fig. 3). Therefore further experiments were made using the 10 kDa membrane.

3.2. Effect of starch concentration

Conversion and changes in CD composition were observed during CGTase action on different starch concentrations in a batch and in a continuous process with an ultrafiltration membrane reactor (Fig. 4). The process was run for 5 h with an enzyme dosage of 0.015 KNU/g. In both methods the change of substrate concentration from 5 to 15% DS increased the total amount of CDs, but a further increase of substrate concentration to 20 and 25% DS caused a decrease of CD concentration. A comparison of batch and continuous processes indicates that using ultrafiltration membrane bioreactors increases the total amount of CDs by 15–50%. The highest differences of CD content between the batch and the continuous processes occur in the case of 20% DS starch. The continuous method gives higher values by 35% α-CD, 40% β-CD and 23% γ-CD, respectively. The lowest differences are observed in the case of 15% starch solution, which gives 8% α-CD, 12% β-CD and 12.6% γ-CD.

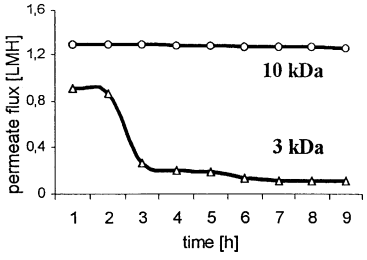

Fig. 3. Distribution ability of membrane and permeate flux. Reaction conditions as in Fig. 2.

The results indicate also that starch concentration influences CD composition. An increase of starch concentration from 5 to 15% DS in the permeate creates a CD solution mainly composed of α-CD, while the highest content of β-CD in CDs occurs with an increase of starch concentration from 20 to 25% DS.

Changes of CD abundance in a 5 h reaction conducted in ultrafiltration membrane reactor with the same enzyme dosage using 5, 15 and 25% DS were also observed (Fig. 5). The results indicate that increase in starch concentration influences the time to reach the maximum yield of α- and β-CDs. Use of substrate with 5% DS gives the highest amounts of α and β-CD after 3 h of reaction. In the case when 15 and 25% DS substrate is used the maximum yield of α and β-CD is reached after 4 h of reaction.

The ratios of α-, β- and γ-CD as part of the CDs produced depend on the substrate concentration. In product obtained from substrate with 5% DS, the proportion of α-, β- and γ-CD is 49:32:19, while with 15% DS it is 42:38:20 but with 25% DS is 35:49:16.

3.3. Effect of reactor residence time

The effect of residence time, either 2, 5, 7 and 9 h, on the cyclization process was also measured (Fig. 6). CD amounts were measured after 5 h hydrolysis time. The increase in residence time from 2 to 5 h increases the CD concentration by 36–47%. A further increase of residence time to 7 h causes small decrease of CD production. The quantity of CDs diminishes by 4–13% when reactor residence time is 9 h. Therefore an increase of residence time beyond 5 h is not effective

Results of the influence of residence time on amounts of CDs are shown in Fig. 7. The reaction with an enzyme dosage of 0.021 KNU/g was monitored for 5 h, with residence times was of 2, 5 and 9 h, respectively. The amount of CD increases moderately with increasing residence time. The steady state was achieved after 2 h. in the case of 2 h residence time. For others (5, 9 h) the steady state was not achieved during 5 h of reaction.

The ratio of α-, β- and γ-CD in the permeate was measured for these experiments. The ratio of α-, β- and γ-CD are 42:38:20, 40:40:20 and 43:37:20 after 1, 3 and 5 h, respectively, showing that it is independent of the residence time and is therefore dependent only on reaction time.

3.4. Effect of enzyme dosage

The influence of enzyme dosage on starch conversion yield indicates that an increase from 0.015 to 0.021 KNU/g influences CD content only slightly. Results indicates also

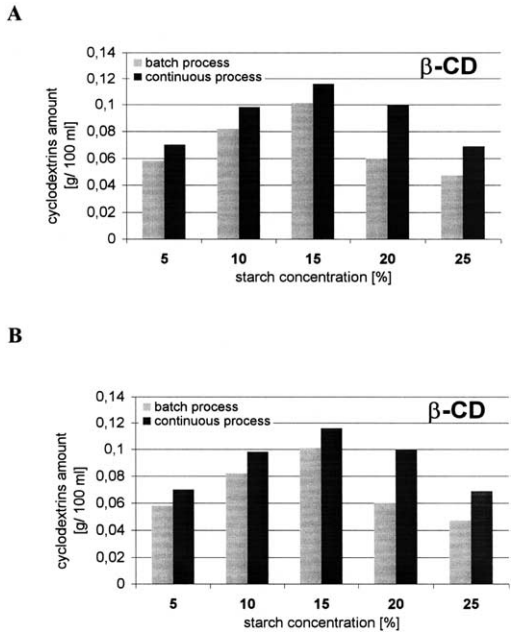

Fig. 4. Influence of starch concentration on cyclization process. Reaction conditions were otherwise as in Fig. 2.

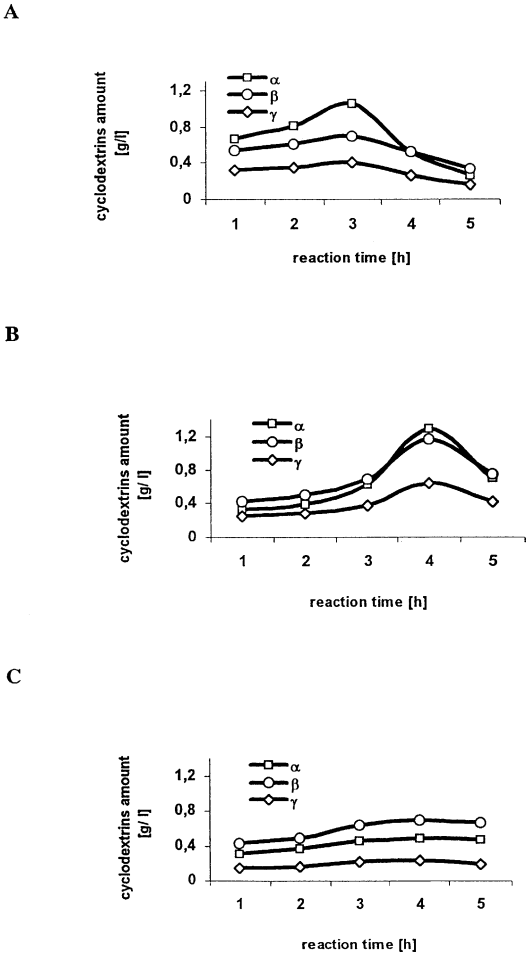

Fig. 5. Changes of cyclodextrin amounts during continuous cyclization process. Reaction conditions were as in Fig. 2 except 9 h reactor residence time and starch concentration: A: 5% DS, B: 15% DS, C: 25% DS.

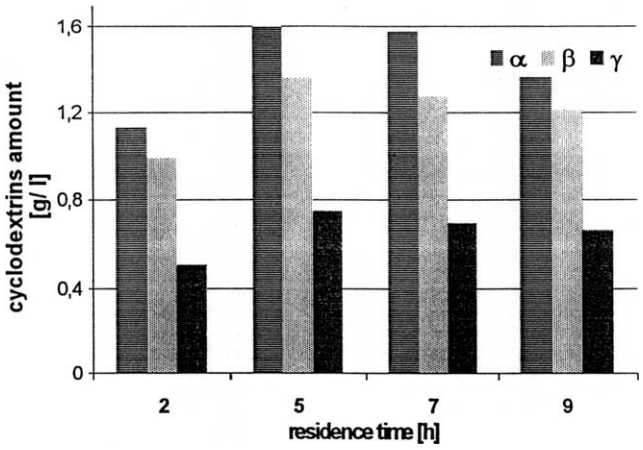

Fig. 6. Effect of reactor residence time on starch cyclization. Reaction conditions were as in Fig. 2 except 15% DS starch concentration and 0.021 KNU/g enzyme dosage.

that the proportion of α-, β-, and γ-CD is 42:38:20 independent of the of enzyme dosage.

Fig. 8 shows the influence of enzyme dosage on the reaction time of cyclization process using 20% DS starch solution. With the lower enzyme dosage the maximal amount of CDs can be obtained after 4 h of reaction, whereas in the case of higher enzyme dosage this occurs after 8 h.

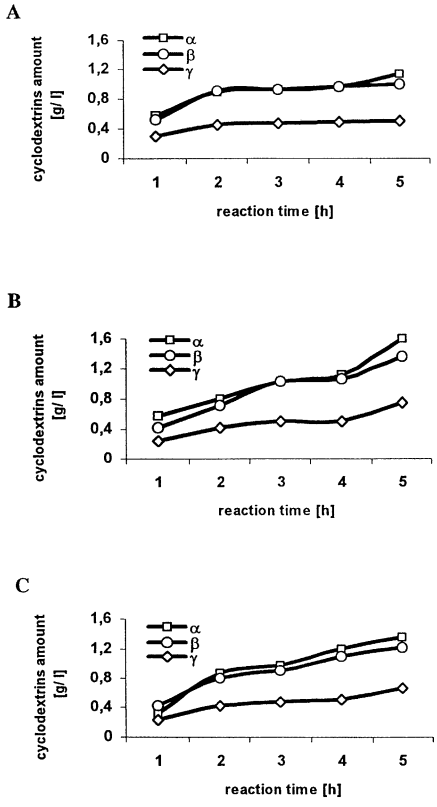

Fig. 7. Influence of residence time on cyclization process. Reaction conditions were as in Fig. 2 except 15% DS starch concentration, 0.021 KNU/g enzyme dosage, and reactor residence time: (A) 2 h, (B) 5 h, (C) 9 h.

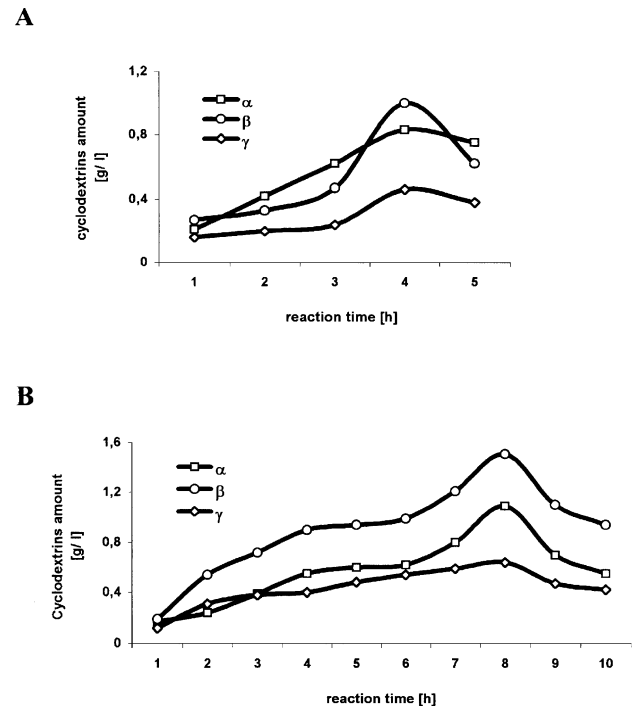

Fig. 8. Quantitative changes of cyclodextrins during cyclization reaction. Reaction conditions were as in Fig. 2 except 20% DS starch concentration, 9 h reactor residence time and enzyme dosage: (A) 0.015 KNU/g, (B): 0.021 KNU/g.

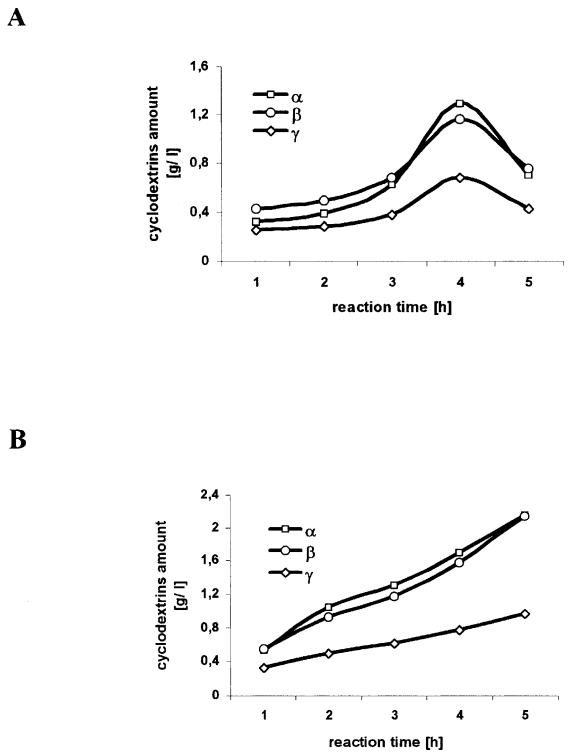

Fig. 9. Influence of additional enzyme dosage on cyclization process. Reaction conditions were as in Fig. 2 except 15% DS starch concentration and enzyme dosage: (A) 0.015 KNU/g, (B) 0.015 + 0.005 KNU/g.

3.5. Additional enzyme dosage

An enzyme dosage in amount of 0.015% KNU/g is sufficient only for reactions of less than 4 h (Fig. 9). It is necessary to supply an additional enzyme before this deadline. The introduction of next enzyme dosage in amount 1/3 of the initial dosage after 3 h of reaction results further increase of CDs. Additional enzyme dosage continuously increases α-CD concentration by 24%, β-CD by 29% and γ-CD by 23% after 5 h compared to 4 h of reaction. Necessity of additional dosage of enzyme can be connected with decreasing of enzyme activity as a consequence of trapping it on the membrane.

4. Conclusions

The results of these investigations indicate that: (1) an ultrafiltration reactor is more efficient than a batch reactors, (2) a membrane with a molecular weight cut-off of 10 kDa is better than one with a 3 kDa cut-off for CD production, (3) an increase of starch concentration causes a decrease of α-CD amount but enhances β-CD production, (4) the concentration of α-, β- and γ-CD depends on starch concentration; on the other hand the reaction time and enzyme dosage have no effect, (5) the most effective substrate concentration is 15% DS with a residence time of 5 h.

References

Gawande, B., & Patkar, A. (2001). Alpha-cyclodextrin production glucosyltransferase from Klebsiella pneumoniae AS-22. *Starch/Stärke*, *53*, 75–83.

Hedges, R. A. (1997). *Properties and application of cyclodextrins. Renewable bioproducts—Industrial outlets and research for the 21st century*, Cerestar USA, Inc.

Kim, T. J., Lee, Y. D., & Kim, H. S. (1993). Enzymatic production of cyclodextrins from milled corn starch in an ultrafiltration membrane bioreactor. *Biotechnology and Bioengineering*, *41*, 88–94.

Lee, Y. D., & Kim, H. S. (1991). Enzymatic production of cyclodextrins from unliquefied corn starch in an attrition bioreactor. *Biotechnology and Bioengineering*, *37*, 795–801.

Lee, Y. D., & Kim, H. S. (1992). Effect of organic solvents on enzymatic production of cyclodextrins from unliquefied corn starch in an attrition bioreactor. *Biotechnology and Bioengineering*, *39*, 977–983.

Lima, H. O. S., De Moraes, F. F., & Zanin, G. M. (1998). β-Cyclodextrin production by simultaneous fermentation and cyclisation. *Applied Biochemistry and Biotechnology*, *70–72*, 789–804.

Morita, T., Yoshida, N., & Karube, I. (1996). A novel synthesis method for cyclodextrins from maltose in water–organic solvent system. *Applied Biochemistry and Biotechnology*, *56*, 311–324.

Raja, K. C. M., & Ramakrishna, S. V. (1994). Improved reaction conditions for preparation of β-cyclodextrin (β-CD) from cassava (*Manhot esculenta* crantz) starch. *Starch/Stärke*, *46*, 402–403.

Raja, K. M. C., Sredharan, V. P., Prema, P., & Ramakrishna, S. V. (1990). Cyclodextrin from cassava (*Manhot esculenta* crantz) starch isolation and characterisation as bromobenzene and chloroform clathrates. *Starch/Stärke*, *42*, 196–198.

Shieh, W. J., & Hedges, A. R. (1996). Properties and applications of cyclodextrins. *Journal of Molecular Structure, Pure and Applied Chemistry*, *A33*, 673–683.

Słomińska, L., & Sobkowiak, B. (1997). Studies on cyclodextrin synthesis by novel cyclodextrin glucosyltransferase. *Starch/Stärke*, *49*, 301–305.

Szejtli, J. (1982). *Cyclodextrins and their inclusion complexes*, Budapest: Akadémiai Kiadó.

Szejtli, J. (1996). Chemistry, physical and biological properties of cyclodextrin. In J. L. Atwood, J. E. D. Davies, D. D. MacNicol & F. Vôgtle, *Comprehensive supramolecular chemistry*, *Cyclodextrins* (pp. 5–40). vol. 3. Oxford: Pergamon Press.