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<th><strong>(51) International Patent Classification</strong></th>
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<td>9 February 1989 (09.02.89)</td>
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<th><strong>(21) International Application Number</strong></th>
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<td><strong>(22) International Filing Date</strong></td>
<td>1 August 1988 (01.08.88)</td>
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<td><strong>(31) Priority Application Number</strong></td>
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<tbody>
<tr>
<td><strong>(72) Inventors</strong></td>
<td>AOKI, Hiroyuki ; 7230 Darcel Avenue #311, Mississauga, Ontario L4T 3T6 (CA). YU, Ernest, Kar-Cheung ; 98 Brentwood Drive, Brampton, Ontario L6T 1R2 (CA).</td>
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<tr>
<td><strong>(81) Designated States</strong></td>
<td>AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</td>
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Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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<tr>
<th><strong>(54) Title</strong></th>
<th>PROCESS FOR PREPARING CYCLODEXTRINS</th>
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<tbody>
<tr>
<td><strong>(57) Abstract</strong></td>
<td>A soil bacterium, namely a previously unreported, novel <em>Bacillus</em>, has been isolated which secretes a cyclodextrin glycosyltransferase enzyme useful in converting starch to cyclodextrin, and yielding predominantly β-cyclodextrin, over a wide range of pH values, and without the requirement of prehydrolysis of the starch.</td>
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Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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FIELD OF THE INVENTION

This invention relates to the production of cyclodextrins, and to novel microorganisms useful therein.

BACKGROUND OF THE INVENTION

Cyclodextrins are cyclic oligosaccharides, common species of which are composed of 6, 7 or 8 glucose residues bound through an α-1,4 linkage. They are called α-, β-, or γ-cyclodextrins depending on the number of glucose residues; 6, 7 or 8, respectively.

Because the torus configuration of the cyclodextrin molecule provides a hydrophobic cavity, a cyclodextrin forms inclusion compounds with a wide variety of "guest" compounds and consequently cyclodextrins have been used in separation processes, extraction processes, as drug delivery enhancing agents in the medical field, as compound stabilizing agents and controlled release agents in the food industry and in a variety of other applications.

While alternative processes for cyclodextrin production are available and described in the art, the conventional process involves biocconversion of gelatinized starch by enzyme action. Enzymes useful for this purpose, termed cyclodextrin glycosyltransferases or CGTase for brevity, are produced by various bacteria. Known CGTase-producing bacteria include Bacillus macerans, B. steàrothermophilus, B. megaterium, B. circulans, B. obbensis and other taxonomically distinct Bacillus spp., Klebsiella pneumoniae M5 and species of Micrococcus such as varians M-849 (ATCC 31606) and luteus B-645 (ATCC 31607). While the CGTase produced by these bacteria all function to convert gelatinized starch to cyclodextrin, they differ in terms of reactivity and stability, indicating a difference also in their primary amino acid structure. Efforts to identify bacterial sources of CGTase capable of producing greater amounts of
cyclodextrin from substrate are on-going. Efforts are focussed particularly on identifying enzymes which are versatile in terms of substrate action, and have stability over wide ranges in pH, temperature and other processing conditions.

Accordingly, it is one object of the present invention to provide a novel bacterial source of CGTase.

It is another object of the present invention to provide a novel CGTase useful in producing cyclodextrin.

It is a further object of the present invention to provide a novel process for producing cyclodextrin.

SUMMARY OF THE PRESENT INVENTION

A novel bacterial isolate has now been found, of the species *Bacillus*, which produces a CGTase having properties which compare favourably with known CGTases. Accordingly, one aspect of the present invention comprises a process for producing cyclodextrin which comprises reacting a source of amylose, such as gelatinized starch, with a cyclodextrin glycosyltransferase produced by this novel* Bacillus* sp. microorganism.

For the purposes of clarity in this disclosure, the novel isolate is referred to herein by the applicant's own internal code, i.e. *Bacillus* IT14. This isolate has the identifying characteristics of the strain on deposit with ATCC under accession number 53605. It will be appreciated that the scope of the present invention extends to include clones of the deposited bacterium and to sub-clones which retain the ability to produce CGTase characteristic of the deposited bacterium. The scope of the present invention extends also to include a culture of the novel isolate. By the term "culture", it is intended to encompass a population of the novel isolate substantially free from the natural soil contaminants and in the substantial absence of foreign microorganisms having a deleterious effect on the
ability of the novel isolate of the present invention to produce CGTase.

CGTase produced by Bacillus IT14 exhibits activity over a wide range of pH and temperature. Notable attributes of this enzyme include stability at high temperature, e.g. above 65°C, even in the absence of stabilizing agents such as calcium ions, stability at pH from about pH 5 to pH 10.0 and conversion of starch preferentially to β-cyclodextrin. It should be noted as well that no amylase pre-hydrolysis is required in the present process of producing cyclodextrin. The CGTase of the present invention actually works better when the amylase substrate has not been pre-hydrolyzed with acid or an amylase, e.g. α-amylase, steps which are required when commercial CGTase preparations are used in cyclodextrin production. The CGTase of the present invention, therefore, provides a viable alternative to enzymes known in the art of cyclodextrin production, and provides an improved process for preparing cyclodextrins which has economic advantages, especially when β-cyclodextrin is the preferred end-product.

According to another aspect of the present invention, there is thus provided an enzyme composition comprising Bacillus IT14 CGTase in a form suitable for converting the amylase component of starch to cyclodextrin and optionally in the presence of an enzyme stabilizing amount of a cationic species such as magnesium, calcium, manganese and cobalt. Forms of the enzyme composition useful herein include raw or concentrated culture broth in which IT14 has been grown, purified enzyme mixed with a buffering carrier, immobilized enzyme etc.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The novel isolate Bacillus IT14 was isolated by screening soil samples obtained in Ontario, Canada for microbial growth on starch and on α-cyclodextrin at 37°C. Cultures of Bacillus IT14 were deposited with the American Type Culture
Collection in Rockville, Maryland on April 6, 1987, under accession number 53605. Samples of the bacterium will be made available while this application is pending only to those entitled access to it by law. After issue of a patent therefor, samples of the bacterium will be available without restriction to all those requesting it from ATCC.

This bacterium can be cultured in a mineral salts medium containing starch 2%, yeast extract 0.5%, peptone 0.5%, K$_2$HPO$_4$ 0.1% and MgSO$_4$ 0.02%, at 37°C. Taxonomic data and other analyses have demonstrated that it has the following characteristics:

A. Morphological Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
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<tr>
<td>Form</td>
<td>Rods</td>
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<tr>
<td>Size</td>
<td>0.6-0.8 x 1.5 - 3 microns</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Positive</td>
</tr>
<tr>
<td>Sporangia swollen</td>
<td>Negative</td>
</tr>
<tr>
<td>Spores</td>
<td>0.8-1.0 x 1.2-1.5 microns</td>
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B. Physiological Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Temperature for Growth</td>
<td>Up to 40°C</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>Utilization of citrate</td>
<td>Negative</td>
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<tr>
<td>Nitrate reduction</td>
<td>Positive</td>
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<tr>
<td>Hydrolysis of starch</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin stab</td>
<td>Negative</td>
</tr>
<tr>
<td>Milk agar streak plate</td>
<td>Positive</td>
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</table>

C. Utilization of Sugars

- Acid from glucose
- Acid from arabinose
- Acid from mannitol
On the basis of this data and in view of the aerobic growth of the organism, it is clear that it belongs to the *Bacillus* genus. Because it does not distend sporangium distinctly, the isolate is considered not to fit within other, known CGTase-producing species of *Bacillus*, including *circulans*, *polymyxa* and *macerans*. It is apparently different as well from *B. stearothermophilus*, another known CGTase-producing species, since IT14 exhibits growth at temperatures as low as 28°C.

For analysis, CGTase produced by this isolate was recovered initially in culture broth filtrate, purified using standard biochemical techniques and ultimately crystallized. The activity of IT14 CGTase, under a variety of conditions, is revealed in the specific examples herein.

The process by which cyclodextrins are produced using *Bacillus* IT14 CGTase is similar to processes known for producing cyclodextrins enzymatically. The amyllose component of starches, such as potato starch, corn starch, wheat starch, rice starch, etc., is ideal as substrate. Because starches usually form crystalline granules of amyllose shrouded by relatively CGTase-inert amylpectin, it is most desirable to free the amyllose component for exposure to CGTase by gelatinizing the selected starch, e.g. by heating it in aqueous solution using known and standard procedures, and then treating the gelatinized starch with the CGTase. Further degradation of the amyllose component of gelatinized starch is typically required when CGTase derived from known sources is used to prepare cyclodextrin. For example, cyclodextrin yields are usually enhanced by pretreating gelatinized starch with an agent which hydrolyses amyllose internally to produce shorter amyllose chains, e.g. by treating with α-amylase or acid, and then subjecting the gelatinized, hydrolysed starch to the action of the CGTase. Gelatinized, hydrolysed starches are suitable substrates for use in the present invention. However, in accordance with a preferred embodiment of the present invention, pre-hydrolysis of the gelatinized starch is unnecessary. *Bacillus* IT14 CGTase is able
to produce cyclodextrin from unhydrolysed, gelatinized starch in yields which approximate and in some instances are higher than corresponding cyclodextrin yields from hydrolysed, gelatinized starch. Accordingly, the cost and time required for hydrolysis of the gelatinized starch is unnecessary according to a preferred aspect of the present invention.

For reaction with the amylose source, i.e. the gelatinized but otherwise untreated starch, preferably corn starch or potato starch (2 to 50% w/v), the IT14 CGTase may be presented in any suitable form. For example, because the enzyme is a secretory product of the bacterium, whole cells may be used, so that the CGTase is actually produced in the presence of the starch to be converted to cyclodextrin. Alternatively, one may use culture broth or a crude or ultra filtrate thereof. For more precise control of reagent proportions, concentrated enzyme preparation comprising enzyme and a carrier such as buffer or culture broth or an immobilized enzyme preparation may be used. Stabilizers for the enzyme, in the preparation or reaction medium, can be unnecessary. While a calcium ion source may be included, as required in some known processes, there is no need to do so in the present process. The IT14 CGTase is substantially equally stable in the presence or absence of calcium at temperatures around 50°C or lower. If desired, however, for example when conducting the reaction at 60°C or above, stabilizing amounts of ions such as calcium, manganese, cobalt, zinc, copper or magnesium may be added. The presence of small amounts of manganese ions gives a significant increase in the relative enzymatic activity of IT14 CGTase. The relative amounts of enzyme and substrate to be used in the reaction may also vary in accordance with established limits. Trials have indicated that an enzyme:substrate weight ratio of $10^{-4}$:1 is suitable but clearly this value can range from about $10^{-6}$:1 to 1:1, i.e. within a range which strikes a balance between efficient enzyme conversion of starch to cyclodextrin and the economic feasibility of the process in general.
Reaction conditions should be designed to accommodate the enzyme to achieve maximum efficiency. Temperatures may range from about 10°C to higher than 70°C but temperatures higher than 70°C may cause some enzyme instability. More preferably, the reaction is conducted at a temperature ensuring suitable enzyme activity and stability, e.g. from about 50°C to about 70°C especially between 50 and 60°C. In terms of pH variation, the reaction may be carried out between about pH 4 and pH 11 but the enzyme stability dictates a preferred pH range of from 5-9, e.g. pH 6 - pH 8.

Preferred reaction times will, of course, depend on the selected processing conditions described above. Usually, the reaction can be terminated 20-48 hours after initiation. CGTase obtained from Bacillus IT14 is able to convert gelatinized starch to cyclodextrin in relatively good yields by comparison with yields using known CGTases. The total cyclodextrin yield can exceed 20% under conditions exemplified herein. Notably, the ratio of β-cyclodextrin to other cyclodextrins in the reaction products approaches 5:1 with no detectable amount of γ-cyclodextrin being produced. The absence of γ-cyclodextrin simplifies the recovery of either α-or, more preferably, β-cyclodextrin from the reaction products.

Embodiments of the invention are described hereinafter by way of example only and with reference to the accompanying drawings in which:

FIGURES 1A and 1B illustrate graphically the activity of the enzyme in terms of pH and temperature, respectively; and

FIGURES 2A and 2B illustrate graphically the stability of the enzyme over ranges of pH and temperature, respectively.
Example 1 - Isolation and Harvest of Strain IT14

CGTase producing strains were screened by using the replicator method. Soil samples collected from various locations in Ontario were pre-soaked in 2% starch broth for 48 hours at 50°C. They were then streaked onto starch plates and onto α-cyclodextrin (α-CD) plates (pH 5-10) and incubated at 37°C for 24 hours. Any colony that showed independent clearance of both starch and α-CD was picked up and transferred into the 4% starch broth for growth. After 48 hours of aerobic growth at 37°C, cells were centrifuged and the supernatant was collected for enzyme activity tests.

Example 2 - Purification of IT14 CGTase

All experiments were carried out at 0-5°C. A culture broth of *Bacillus* sp. IT14 was centrifuged to remove cells and (NH₄)₂SO₄ was added to the supernatant to 15% saturation. The solution was passed through a starch column. The adsorbed enzyme was eluted from the starch column with water, then (NH₄)₂SO₄ was added to the eluate and precipitates formed between saturations of 30 and 55% were recovered. The supernatant, about 0.5 liters, was fractionated by addition of solid ammonium sulfate to 30% saturation and the mixture was allowed to stand in a refrigerator. After centrifugation at 6,000 x g for 30 minutes, solid ammonium sulfate was further added to the supernatant to 55% saturation and the mixture was allowed to stand one hour. The resulting precipitate was collected by centrifugation at 7,000 x g for 30 minutes and dissolved in 10 ml of 0.015 M phosphate buffer, pH 7.5. The enzyme solution was dialyzed twice against 5 liters of the same buffer. The insoluble material formed during dialysis was removed by centrifugation. This dialyzed enzyme solution (30 to 55% ammonium sulfate fraction, Table 1) was applied to a column of DEAE-Zetaprep equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The column was washed first with 1 liter of the same buffer, and then eluted with 0.1 M phosphate, pH 7.5. CGTase active fractions were
combined and concentrated with an Amicon concentrator to a final volume of 30 ml.

Solid ammonium sulphate was added to 30% saturation and continued until the solution became faintly turbid. After standing for a few days, crystals appeared in plate form.

The activities, measured by standard assay (see Nakamura and Horikoshi Agr. Biol. Chem. 40(4) (1976) 753-757) and yield of the enzyme at various stages in the crystallization process are summarized below in Table 1:

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Activity (Units x 10^3)</th>
<th>Specific Activity (Units/mg)</th>
<th>Yield (%)</th>
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<tr>
<td>Culture broth</td>
<td>18.8</td>
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<tr>
<td>Starch adsorption</td>
<td>16.1</td>
<td>12.5</td>
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<tr>
<td>(NH4)₂SO₄ ppt.</td>
<td>13.7</td>
<td>98.6</td>
<td>73</td>
</tr>
<tr>
<td>DEAE-Žetaprep</td>
<td>11.5</td>
<td>264</td>
<td>61</td>
</tr>
<tr>
<td>Crystallization</td>
<td>10.8</td>
<td>278</td>
<td>57</td>
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</table>

The enzyme was purified about 95-fold from the extract with a recovery of about 75% of the original activity. A chromatogram of the last step of the purification run gave only one protein peak. Purified CGTase was judged to be homogeneous by SDS-polyacrylamide gel electrophoresis.

Example 3 - Enzyme Activity Analysis

Purified ITL4 CGTase was assayed for its starch degrading activity in an acetate buffer at pH 3.0 to 5.5, MES Buffer at pH 6.0 - 7.0 and with a Tris-HCl buffer at pH 7.5 to 9.0, for optimum pH. The assay was conducted by mixing 50 ul of starch (0.75 mg/mL from Sigma Chemical Co. Ltd., Missouri, U.S.A.) mixed with appropriate buffer and reacted with 10 ul of diluted enzyme solution for 60 minutes at 50°C. The reaction was
stopped by added 50 ul HCl (0.5N) and activity measured at 620 nm after adding 50 ul of 0.02% Iodine/0.2% potassium iodide.

Figure 1A shows the profile of starch degrading activity of the CGTase over the pH range tested. IT14 CGTase showed strong activity over a wide pH range. The maximum activity (100%) is demonstrated at pH about 5.5-6.0, but over the full range pH 3.5-8.5, at least 75% activity is obtained.

Figure 1B illustrates the enzyme activity as a function of temperature, conducted at pH 6.0 but otherwise as described above. A temperature of about 65-70°C (100% activity) is optimum for IT14 CGTase.

Figure 2A illustrates the results of pH stability analysis conducted on buffer adjusted enzyme solutions held at 40°C for 2 hours, as revealed by starch degrading activity. IT14 CGTase is stable over a pH range of 6.0 to 9.5. Heat stability is shown in Figure 2B. The purified CGTase was allowed to stand at various temperatures for 15 minutes in 50 mM Tris-HCl buffer (pH 7.0) with or without calcium ion solution and starch degrading activity was measured, as plotted in Figure 2B. IT14 CGTase did not lose its activity even at 65°C. Addition of calcium chloride in 1.0 mM to the CGTase produced by IT14 caused a 5°C rise in the limit of heat stability.

Example 4 - Cyclodextrin Production Using IT14 CGTase

For purposes of comparison, IT14 CGTase was compared with a commercial B. macerans CGTase preparation available from Amano Pharmaceutical Co. Ltd., Nagoya, Japan, at equal activity.

In general, starch was gelatinized by heating for 15 minutes at 121°C and then cooling to 60°C. Enzyme was then added and incubated with shaking at 60°C for 20-40 hours. The reaction products were then assayed after glucoamylase digestion for the presence of glucose. Total cyclodextrin produced was calculated
as the difference in the glucose levels in control and CGTase treated solutions.

In the absence of pre-hydrolysis, the results shown in the following Table 2 were obtained:

<table>
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<tr>
<th>Substrate</th>
<th>Total CD Produced</th>
<th>g/L</th>
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<tr>
<td></td>
<td>Substrate Conc’n (%, w/v)</td>
<td>Amano</td>
</tr>
<tr>
<td>Potato starch</td>
<td>4.0</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>45.5</td>
</tr>
<tr>
<td>Corn starch</td>
<td>4.0</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>55.3</td>
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</table>

The effect of pre-hydrolysis on cyclodextrin production was revealed in further experiments. In a first such experiment, starch was pretreated with α-amylase (Termamyl from Novo Industries A/S) using 0.01 units of α-amylase per gram of starch and then incubated for 30 minutes at 90°C and then the α-amylase denatured by heating to 121°C for 15 minutes. Then, the treated starch was cooled to 60°C and CGTase added (100 u/g starch). The results are given in Table 3.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total CD Produced</th>
<th>g/L</th>
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<tr>
<td></td>
<td>Substrate Conc’n (%, w/v)</td>
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</tr>
<tr>
<td>Potato starch</td>
<td>4.0</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>64.4</td>
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<tr>
<td>Corn starch</td>
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<td>13.9</td>
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<tr>
<td></td>
<td>15.0</td>
<td>38.9</td>
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In a similar experiment, the effect of acid pre-hydrolysis was determined. Starch was treated with HCl sufficient to lower the pH to 2.5 and then heated to 121°C for 15 minutes in accordance with standard procedures for pre-treatment. The medium was cooled to 60°C and pH adjusted to pH 6.0 with NaOH. The enzyme was then added (100 u/g starch) and total
cyclodextrin assayed as described above. The results appear in Table 4.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc'n (% w/v)</th>
<th>Total CD Produced</th>
<th>g/L</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Amano</td>
<td>IT14</td>
</tr>
<tr>
<td>Potato Starch</td>
<td>4.0</td>
<td>21.6</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>62.2</td>
<td>54.7</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>4.0</td>
<td>17.0</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>59.6</td>
<td>50.5</td>
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</table>

From the data appearing in Tables 2, 3 and 4, it is evident that pre-hydrolysis is not required for efficient cyclodextrin production using IT14 CGTase.

Subsequent specific HPLC analysis of the cyclodextrins produced by action of IT14 CGTase has revealed that β-cyclodextrin is the dominant reaction product with minor amounts of α-cyclodextrin also being produced. The ratio of β:α-cyclodextrin in the reaction products is about 3:1 to 5:1, with extremely small amounts of γ-cyclodextrin also produced. By contrast the Amano preparation results usually in a β:α ratio of around 2.4:1. If desired, the β-cyclodextrin can be separated from the minor amount of α-cyclodextrin by a process of fractional precipitation.

Example 5 - Effect on Stability of the Presence of Metal Ions

It is known that the stability of some CGTases is affected by the presence of certain metal ion stabilizing agents, notably calcium ions. Accordingly, experiments were conducted to determine the stabilizing effect of a variety of metal ions on IT-14 CGTase.
The experiments were performed by preparing purified enzyme as described in Example 2 above, and pre-incubating the purified enzyme with different metal salts in 0.1 M Tris-HCl buffer at pH 7.5, for 10 minutes at 60°C. Then the amylolytic (starch degrading) activity of each preparation was determined by preparing a substrate mixture of 50 ml of a 0.75 mg/ml solution of starch mixed with 50 ml of 100 mM sodium acetate buffer, pH 6.0. To this were added 10 ml of enzyme, and the mixture was held at 50°C, for 60 minutes. The reaction was stopped by addition of 50 ml of 0.5N HCl. After addition of 50 ml of 0.02% iodine in 0.2% potassium iodide solution, activity of the preparation towards starch degradation was determined from the absorbance read at 620 nm. This is essentially the same process as that used in Example 3.

The nature and amounts of the metal salts, and the activity recorded after this 60 minutes interval, are shown in Table 5. The activities are reported as percentage relative activities, the activity recorded in the case where no metal salt was present during enzyme incubation being assigned the 100% value. A striking retention and increase in activity, indicating significant enhancement of stability of the enzyme, is revealed when manganese ions are used.

<table>
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<tr>
<th>Salt Compound</th>
<th>Amount Present during Incubation (nM)</th>
<th>Relative Activity (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>200</td>
<td>72</td>
</tr>
<tr>
<td>KCl</td>
<td>200</td>
<td>74</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1</td>
<td>169</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>161</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>1</td>
<td>140</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>1</td>
<td>107</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>1</td>
<td>219</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>1</td>
<td>74</td>
</tr>
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</table>
WHAT IS CLAIMED IS:

1. A process for producing cyclodextrin which comprises reacting a source of amylose with a cyclodextrin glycosyltransferase produced by the novel isolate Bacillus IT14.

2. The process according to claim 1 wherein said source of amylose is gelatinized starch.

3. The process according to claim 2 wherein said source of amylose is gelatinized, hydrolysed starch.

4. The process according to claim 2 wherein the cyclodextrin glycosyltransferase is reacted with gelatinized starch in the presence of an enzyme stabilizing amount of manganese ion.

5. A cyclodextrin glycosyltransferase enzyme capable of converting unhydrolysed, gelatinized starch to cyclodextrin, said transferase being an extracellular product of metabolism of the novel bacterial isolate Bacillus IT14.

6. An enzyme preparation comprising the enzyme defined in claim 5 and a carrier acceptable for inoculating a reaction medium.

7. The enzyme preparation of claim 6 which includes an enzyme stabilizing amount of a cation.

8. The enzyme preparation of claim 7 wherein said cation is manganese cation.

9. A microorganism having the identifying characteristics of isolate Bacillus IT14, ATCC 53605, said microorganism being capable of producing a CGTase.
10. The microorganism of claim 9 which is \textit{Bacillus} strain IT14 ATCC 53605 including clones and sub-clones thereof.

11. A culture of the microorganism defined in claim 8.

12. A process for producing $\beta$-cyclodextrin which comprises reacting gelatinized starch with CGTase produced by \textit{Bacillus} isolate IT14 (ATCC 53605) at a temperature in the range from 10$^\circ$C to 70$^\circ$C and at a pH in the range from pH4 to pH11 and recovering $\beta$-cyclodextrin from the products of the reaction.

13. The process according to claim 12 wherein the reaction is conducted at a temperature in the range from 50$^\circ$C to 60$^\circ$C and in the presence of an enzyme stabilizing amount of a cation.

14. The process according to claim 12 wherein the reaction is conducted at a temperature in the range from 50$^\circ$C to 70$^\circ$C, at a pH of from pH6 to pH8 and in the presence of an enzyme stabilizing amount of manganese cation.

15. The process according to claim 14 wherein the gelatinized starch used in the reaction is gelatinized corn starch or gelatinized potato starch.
**INTERNATIONAL SEARCH REPORT**

**International Application No.** PCT/US88/02625

### I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

- IPC(4): C12P 19/18; C12N 9/10, 1/20; C12R 1/07; C08B 37/16
- U.S. CL.: 435/97, 193, 253, 832; 536/103

### II. FIELDS SEARCHED

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Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
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<tr>
<td>Y</td>
<td>US, A, 3,812,011 (OKADA, ET AL.) 21 May 1974, see abstract.</td>
<td>1-15</td>
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<td>Y, P</td>
<td>US, A, 4,748,237 (ROHRBACH, ET AL.) 31 May 1988, see abstract, col. 3, line 49 - col. 4, line 23</td>
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<td>X, P</td>
<td>Chemical Abstracts, Volume 107, No. 17, issued 1987, October 26 (Columbus, Ohio USA), P. PONGSAWASDI ET AL., &quot;Screening And Identification Of A Cyclomaltodextrin Glucanotransferase-Producing Bacterium&quot;, see page 572, column 1, the abstract no. 152777Z, J. Ferment Technol. 1987, 65(4), 463-467 (Eng.).</td>
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* Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

**T** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

**A** document member of the same patent family

### IV. CERTIFICATION

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International Searching Authority: ISA/US

Signature of Authorized Officer: CHARLES L. PATTERSON, JR.
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<td>Chemical Abstracts, Volume 107, No. 5 Issued 1987, August 3</td>
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<td>(Columbus, Ohio, USA), G.U. SO, ET AL. &quot;Preparation of Beta-Cyclodextrin And Its Application&quot;, see page 548, column 1, the abstract no. 38015Z, Choson Minjujuui Inmin Konghwaguk Kwahagwon Tongbo 1986, (3), 42-6 (Korean).</td>
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<td>Y</td>
<td>Chemical Abstracts, Volume 104, No. 1, Issued 1986, January 6</td>
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<td>Chemical Abstracts, Volume 102, No. 11, Issued 1985, March 18</td>
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<td>(Columbus, Ohio, U.S.A), Y. CHEN, ET AL., &quot;Production of Beta-Cyclodextrin&quot;, see page 464, column 2, the abstract no. 94288Z, Shipin Yu Fajiao Gongye 1984, (5), 61-8 (Ch.)</td>
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<td>Y</td>
<td>Chemical Abstracts, Volume 91, No. 26, Issued 1979, July 2 (Columbus, Ohio, U.S.A.) K. Horikoshi &quot;Production and Industrial Applications of Beta-Cyclodextrin&quot;, see page 77, column 1, the abstract no. 212868t, Process Biochem. 1979, 14(5), 26-8, 30 (Eng).</td>
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