

PATENT SPECIFICATION (11)

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(54) XYLOSE ISOMERASE

(71) We, CPC INTERNATIONAL INC., a corporation organised and existing under the laws of State of Delaware, United States of America, of International Plaza, City of Englewood Cliffs, State of New Jersey, 07632, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates generally to enzymatic isomerization using immobilized enzymes. More particularly, the invention relates to improvements in continuous isomerization techniques.

The enzymatic hydrolysis of starch, for the production of corn syrup and corn syrup solids, is routinely conducted on a commercial scale to produce hydrolysates at D.E. values in excess of 95, utilizing what are essentially batch techniques.

The reported investigations of the enzymatic isomerization of dextrose and of starch hydrolysis, for the production of levulose bearing products, have generally utilized batch isomerization techniques. An exemplary batch isomerization, for example, might be conducted in a stirred tank reactor at a temperature of about 70°C and at a pH of about 6.2, for an isomerization period of from about 40 to 60 hours. The enzyme dosage and conversion temperature are selected to obtain a desired levulose content in the end product. The pH of the syrup would be maintained at about 6.2 by either continuous or incremental additions of a basic material, such as sodium bicarbonate, during isomerization.

The selection of optimum operating conditions, for batch isomerization, is not easy. One initial variable that can be fixed, to some extent, is the particular enzyme preparation that is employed. Isomerase enzymes have their own individual characteristics and those derived from different microorganisms cannot be considered to have the same optimum conditions for action. For example, each enzyme preparation ordinarily will be found to have its own optimum pH, opti-

um temperature, metal requirement, temperature stability, and Michaelis constant, among other properties, as is reported, for example, by Dr. T. Sato, in "The Enzyme for the Isomerization of Glucose," in the Japanese publication *Dempunto Gijutsu Kenkyu Kaiho* 32, 81—88 (1965).

When the enzyme preparation is derived from a microorganism of the *Streptomyces* genus, the rate of levulose formation by the enzyme is much greater at 70°C than at 60°C. However, at pH 6.2, some of the advantages of conducting the isomerization at the higher temperature are lost, since the slightly acid pH reduces the rate of levulose formation and reduces enzyme stability. In fact, neither the 70°C operating temperature nor the slightly acidic pH are conducive to enzyme stability.

For effective batch isomerization, continuous mixing of the starch hydrolysate solution (syrup) is necessary. Unfortunately, when cells are used as the source of enzyme activity, the mixing action apparently imposes shear forces on the microbial cells that contain the enzyme. When cells are ruptured, the cellular fluids, including the enzyme, are released into the solution. The enzyme appears to be more susceptible to inactivation under these conditions, than enzyme that remains within intact cells.

Some consideration of the effect of temperature on isomerase has been reported in the literature. For example, two of the pioneer workers in the field, N. Tsumura and T. Sato of the Japanese Food Research Institute, published a series of papers entitled, "Enzymatic Conversion of D-Glucose to D-Fructose". In parts V and VI, which appear in *Agr. Biol. Chem.* 29, 1123—1128 and 1129—1134, respectively (1965), they describe the effects of temperature on an isomerase obtained from *Aerobacter cloacae*. They found that this enzyme lost the majority of its activity at 80°C in the presence of magnesium and at 90°C in the presence of cobaltous ion. Without the presence of stabilizing metal ions, exposure of their enzyme to a temperature of 70°C for a ten-minute period resulted in very substantial

loss of enzyme activity. The addition of stabilizing metal ions enhanced heat tolerance, but even so, enzyme inactivation could be observed at about 80°C.

- 5 An article by Yoshimura, Danno and Natake, Hyogo University, Japan, appearing in *Agr. Biol. Chem.* 30 (10), 1015-1023 (1966), entitled "Studies on Glucose Isomerizing Activity of D-xylose Grown Cells from *Bacillus coagulans*, Strain HN-68", reports the effect of temperature on the isomerase enzyme from their source micro-organism. Maximum activity after two hours incubation was observed at 70°C. Maximum enzyme activity after twenty hours incubation was observed at temperatures in the range from 60°C to 65°C. Above 70°C, substantial inactivation of the enzyme could be observed after two hours, and when operating temperatures for given enzyme dose batches were compared after twenty hours, substantial enzyme inactivation appeared to occur above 65°C.

- 25 In the *Journal of Food Science and Technology* (Nipon Shokuhin Kogei Gakkaishi) 14 (12), 539-540 (1967), N. Tsumura and M. Ishikawa, in "Continuous Isomerization of Glucose by a Column of Glucose Isomerase", described a continuous isomerization technique in which a purified isomerase enzyme sample, derived from a strain of *Streptomyces*, was anchored on a DEAE-Sephadex ('Sephadex' is a Registered Trade Mark) bed in a column, to immobilize the enzyme. A glucose solution was then passed through the column. The column was heated by warm water at 60°C, which was passed through a jacket. An eventual reduction in enzyme activity was observed, although the authors were not certain whether the decrease in observed isomerization was caused by enzyme inactivation or by leaching of the enzyme from the column.

- 45 More recently, in a paper appearing in the text, "Fermentation Advances", edited by D. Perlman, Academic Press, 1969, beginning at page 561, Dr. Takasaki and his associates from the Japanese Fermentation Research Institute have described a continuous column isomerization and have characterized the heat stability of an isomerase enzyme derived from a particular strain of *Streptomyces*. They observed enzyme inactivation after ten minutes at temperatures above 70°C.

- 55 The concept of binding enzymes to solid supports has become the subject of increasing interest. In general, these binding methods may be classified as follows:

- 60 (1) Adsorption—binding to a solid support by physical or electrostatic forces as in the case of activated carbon or clay;
- 65 (2) Ionic—attachment to ion exchange

materials made from synthetic polymers, cellulose, starch, dextran, etc.;

- (3) Covalent Bonding—coupling of enzyme to carrier through a functional group such as diazo, alkyl, peptide or silanyl groups; 70
- (4) Entrapment—immobilization by imbedding or inclusion in a gel structure of a hydrophilic polymeric material such as a polymer of an acrylamide or by the cell wall of a micro-organism; 75
- (5) Cross-linking — insolubilization by cross-linking the enzyme with a bifunctional agent; and 80
- (6) Flocculation—treating microbial cells containing the enzyme with a flocculating agent, such as anionic polyelectrolytes, cationic polyelectrolytes, and mineral hydrocolloids, such as described in Dutch Patent Specification 72/09532, published January 11, 1973. 85

The prior art techniques have not enjoyed widespread acceptance in the industry. For example, activated charcoal, calcium carbonate, etc., are known to have a relatively high binding capacity for enzymes such as xylose isomerase. However, the resulting immobilized enzyme preparations are relatively inactive in respect of ability to convert dextrose-containing solutions to levulose-bearing syrups. In other words, many of the prior art immobilized enzymes have a relatively low effective isomerase activity. Furthermore, these immobilized enzymes of the prior art are generally unstable and typically have very short half-lives. 90 95 100 105

It has been proposed in U.S. Patent No. 3,715,276 to Takasaki et al to utilize calcium carbonate, magnesium carbonate and several ion exchange resins to regulate the pH during prolonged enzymatic isomerization reactions of dextrose-containing solutions to levulose-bearing syrups. Takasaki et al utilized a xylose isomerase enzyme bound within the cells by a heat treatment. The enzyme did not become sorbed onto the pH regulating agent because it was bound within the cells. Furthermore, the pH regulating effect of calcium carbonate, magnesium carbonate and the ion exchange resins is only apparent when the isomerization is conducted over a relatively long period of time, i.e., a residence time of more than about four hours. In the case of enzymatic isomerizations where the residence time of the sugar solution exceeds about four hours, the formation of undesirable compounds occurs, one such compound being, for example, psicose. Since many industrial food uses for levulose-bearing syrups cannot tolerate psicose levels of more than 2% by weight, dry basis, and 125 130

more often, 1% by weight, dry basis, prolonged residence time processes such as are described in U.S. Patent No. 3,715,276 are not commercially feasible.

5 Because of the plethora of terms that are in common use in the art, a few definitions are made to simplify the present specification and permit it to be more concise.

10 **D.E.:** The term "D.E." is an abbreviation for "dextrose equivalent," and these terms are used interchangeably to refer to the reducing sugar content of a material calculated as dextrose and expressed as percent of total solids.

15 **Starch Hydrolysate:** The term "starch hydrolysate" is used in a general way to refer to a syrup or dry product that is made by the hydrolysis of starch. Such a product may be made by acid or enzymatic hydrolysis, or by a combination of acid and enzymatic hydrolysis. A preferred type of starch hydrolysate for use for isomerization in accordance with the present invention is produced by acid or enzyme thinning to a D.E. of 10 or less, followed by enzymatic saccharification to a D.E. of at least 95, and preferably above 97.5.

20 **Glucose and Dextrose:** Medium D.E. Starch hydrolysates are commonly referred to in the art as "glucose", whether the starch hydrolysate is in the form of a syrup or in the form of solids. The term "dextrose" is commonly reserved for the refined crystalline monosaccharide that is recovered from a high D.E. starch hydrolysate, or for D-glucose as a constituent of starch hydrolysates. As used hereafter, the term "dextrose" will be used to embrace this monosaccharide in any form, in solution or dry, as a constituent of a starch hydrolysate syrup, syrup solids, or in refined crystalline form.

25 **Fructose and Levulose:** The terms "fructose" and "levulose" are generally employed interchangeably in the art to refer to a particular isomer of dextrose that is sweeter than dextrose. This isomer is found in honey and in invert sugar, along with dextrose, and it is valuable because of its sweetness. The term "levulose" will be used to refer to this monosaccharide.

30 **The enzyme:** The enzyme that isomerizes dextrose to levulose has been referred to in the art by several names. It is referred to in U.S. Patent Specification No. 2,950,228, as xylose isomerase, because it isomerizes xylose to xylulose. This activity is in addition to its ability to isomerize dextrose to levulose. It has also been referred to in the art as dextrose isomerase and glucose isomerase. The term "xylose isomerase" will be used herein, since investigations have revealed that xylose is the natural substrate of the isomerase.

35 **Enzyme preparation:** The term "enzyme

preparation" is used to refer to any composition of matter that exhibits the desired xylose isomerase enzymatic activity. The term is used to refer, for example, to live whole cells, dried cells, cell extracts, and refined and concentrated preparations derived from cells. Enzyme preparations may be either in dry or liquid form. The present invention is concerned with a particular form of immobilized enzyme and its use in isomerization processes.

40 **Units:** All parts and percentages are by weight, and on as is basis, unless expressly stated to be otherwise.

45 **Isomerase unit:** One isomerase unit is defined as the amount of enzyme activity that is required to produce one micromole of levulose per minute under the isomerization conditions described hereafter under the heading, "Assay of Isomerase Activity".

50 **Streptomyces:** This term refers to a genus of microorganisms of the order of *Actinomycetales*. These microorganisms are aerial mycelium-producing actinomycetes. The genus is well recognised. Some of its important distinguishing characteristics are described, for example, in the text. "The Actinomycetes", by Selman A. Waksman, The Ronald Press Company, New York, 1967, page 135 et seq.

55 The present invention provides a xylose isomerase enzyme preparation comprising a xylose isomerase enzyme sorbed on particulate basic magnesium carbonate. The sorbed enzyme preparation can be made by contacting a cell-free solution of water soluble xylose isomerase with basic magnesium carbonate. Xylose isomerase sorbed this way has a high effective isomerase activity. Other related materials, such as calcium carbonate, do not provide a highly active and stable xylose isomerase. Also, intracellular xylose isomerase does not become sorbed onto basic magnesium carbonate.

60 The immobilized xylose isomerase preparation of the present invention has a very high enzyme efficiency, whereby the sugar contact time necessary to produce a levulose-bearing syrup of at least about 45% by weight levulose from a dextrose-containing solution is generally less than about two hours. Due to the lower contact time occasioned by the improved retained enzyme activity of the composition of the present invention, the resulting levulose-bearing syrups have lower color, lower organic acid, and lower psicose content, i.e., generally less than about 1% by weight, dry basis, and quite often less than about 0.3% by weight, dry basis, psicose. These advantages are quite significant from the standpoint of capital investment and inventory in commercializing a process for enzymatically preparing levulose-bearing syrups from dextrose-containing solutions.

The stabilized xylose isomerase enzyme preparation of the invention can be prepared by contacting cell-free xylose isomerase with particulate basic magnesium carbonate. The particulate basic magnesium carbonate may be either in the form of a powder or a granular structure. However, the granular structure is preferred from the standpoint of flow properties when used in a deep bed converter. The stabilized enzyme preparation can be conveniently recovered by conventional means, such as filtration. Alternatively, the particulate basic magnesium carbonate may be first placed in a converter which is later to be used in the enzymatic isomerization reaction and, secondly, a solution of cell-free xylose isomerase may then be pumped through the column until no more enzyme is sorbed from the solution by the particulate basic magnesium carbonate. Following the preparation of the stabilized enzyme preparation, the converter is ready for use simply by supplying to the converter a dextrose-containing solution.

It will be understood that the xylose isomerase enzyme employed in this aspect of the invention can be either highly purified or crude. The enzyme can be purified by any conventional purification procedure, for example, fractional precipitation with sodium sulfate, ammonium sulfate or other mineral salts, selective adsorption, differential heat inactivation of contaminating proteins by exposure of the product to increasing temperatures at different pHs, isoelectric precipitation, organic solvent precipitation with alcohols, DEAE-cellulose chromatography, and "Sephadex" gel filtration purification procedures and electrophoretic and ultracentrifuge separations.

The xylose isomerase enzyme preparation used is in its soluble form; in other words, the xylose isomerase is freed from the microbial cell where it is formed. The enzyme may be released from its cellular material by any conventional means, and the enzyme in the crude material may be then sorbed on the basic magnesium carbonate. The xylose isomerase thereupon becomes bound to the basic magnesium carbonate. The immobilized enzyme preparation can thereafter be recovered by filtration, centrifugation and the like, and then washed with a buffer. The filtered and washed immobilized enzyme composition can be used in its wet form, as is, or the cake can be dried by conventional drying techniques useful with enzyme preparations, such as by cell, rotary drum or spray drying or freeze drying techniques. Superior results are obtained, however, when the enzyme is purified before it is sorbed.

The invention thus includes a process for the recovery of xylose isomerase from a

fermentation broth containing the enzyme comprising contacting the broth with particulate basic magnesium carbonate and recovering the enzyme in the form of a xylose isomerase enzyme basic magnesium carbonate complex. This process can be carried out by adding basic magnesium carbonate to a broth containing cells containing xylose isomerase and subsequently releasing the xylose isomerase from the cells e.g. by conventional means.

Prior to immobilization, the cell-free enzyme solution is preferably adjusted to a pH in the range from about 7.5 to about 9.5 and, more preferably, in the range from about 8 to about 9. The immobilization can be conducted at room temperature (e.g., generally about 25°C), so as to prevent inactivation of the enzyme. However, higher temperatures can be tolerated if the enzyme solution is mixed with a dextrose-containing solution during immobilization. When the pH of the enzyme solution to be contacted with the basic magnesium carbonate is less than about 7, the basic magnesium carbonate will exhibit a tendency to decompose, and will not be suitable for use. Therefore, it is desirable that the pH of the enzyme solution be maintained at about 7.5 or greater.

The enzyme solution will preferably have an activity of at least about 10 units/ml. (U/ml.) and may have an activity as high as 3,000 U/ml. Generally, the enzyme solution will have an activity of from about 50 U/ml. to about 200 U/ml. of isomerase enzymatic activity.

It has been found that the basic magnesium carbonate has a very high binding capacity for the xylose isomerase enzyme. In this connection, it has been found that the basic magnesium carbonate is capable of binding a quantity of the enzyme that would have an activity, if measured prior to sorption, equivalent to more than 150 units per gram (U/g.) of the bound enzyme (i.e., the isomerase-basic magnesium carbonate complex). Generally, it has been observed that the amount of enzyme that is sorbed is an amount that would have 250 units of activity, if measured prior to sorption, per gram of the complex, and in practice, the number is often more than 500 U/g. of the complex. Unfortunately, as will be demonstrated later in Example 5, not all of the original enzymatic activity is retained after complexing, even though all of the proteinaceous enzyme is in fact sorbed. The terms "bound isomerase" and "binding capacity", as used herein, are defined as units of isomerase, as measured in solution prior to sorption, bound to one gram of the complex, dry basis.

Surprisingly, in view of experience with other carriers, the immobilized xylose iso-

merase preparation of the invention has an extremely high "effective isomerase activity", i.e., the isomerase activity of the immobilized enzyme that will actually enzymatically convert dextrose into levulose. There are many materials which are capable of binding xylose isomerase, such as activated charcoal, calcium and zinc carbonate, and the like. However, when the enzyme is bound to these carriers, the enzyme's effectiveness, or effective activity, is diminished significantly. Quite often, immobilized enzyme complexes, made using these prior art carriers, have an effectiveness of less than 50% of the amount of activity that would be expected, based on the amount of isomerase actually bound or sorbed on the carrier. The immobilized xylose isomerase preparation of the present invention exhibits at least about 70% of the activity of the original isomerase that is found to the carrier, and quite often more than 80%. In other words, the immobilized xylose isomerase exhibits more than 70% of the activity of the amount of isomerase that is actually bound to the basic magnesium carbonate.

The immobilized xylose isomerase preparation of the present invention, therefore, is characterized as having at least about 100 units of effective isomerase activity per gram of immobilized enzyme preparation, dry basis (i.e., 100 U/g. effective isomerase activity), and preferably at least about 175 U/g.

Another striking characteristic of the immobilized and stabilized enzyme preparation of the present invention is its long half-life. The enzyme preparation of the present invention is characterized as being capable of producing 45% levulose, d.b., at flow rates of greater than 0.5 bed volume/hour with an enzyme half-life in excess of 15 days.

The basic magnesium carbonate material employed for preparing the stabilized enzyme preparation of the invention is preferably a granular, porous substance of any desired particle size. The basic magnesium carbonate can be prepared by, first preparing magnesium carbonate trihydrate slurry, dewatering the slurry to at least about 10% solids, and drying the resulting pasty mass. The resulting product has both a strong, crush-resistant structure and a marked affinity for soluble enzymes such as xylose isomerase.

Basic magnesium carbonate can also be prepared by the procedures and techniques described in U.S. Patent Nos. 2,209,752; 2,209,753; and 2,209,754.

Some preferred types of basic magnesium carbonate suitable for the preparation of the immobilized enzyme preparation of the present invention may also be obtained commercially and some of these products have been identified as $4\text{MgCO}_3 \cdot \text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$

(or $5\text{MgO} \cdot 4\text{CO}_2 \cdot 6\text{H}_2\text{O}$) having a molecular weight of about 485.74, an absolute density of about 2.16, a moisture content (110°C) of about 0.5% to about 2.3%, a bulk density, loose, of 3.5 to 8.0 pounds/cubic foot and a Tyler screen mesh size such that at least 99% (wet) passes through a 325 mesh screen, and $3.2\text{MgCO}_3 \cdot \text{Mg}(\text{OH})_2 \cdot 3.9\text{H}_2\text{O}$.

The stabilized, immobilized xylose isomerase enzyme preparation of the invention may be used in any process for the enzymatic conversion of dextrose-containing solutions to levulose-bearing syrups, including the temperature-programmed process described in our copending divisional Application No. 46803/75 (Serial No. 1,447,246). The immobilized enzyme preparation is particularly suited for the continuous conversion of dextrose to levulose. The stabilized and immobilized enzyme preparation of the invention is also applicable to other continuous enzymatic isomerization processes.

The advantages of the stabilized and immobilized enzyme preparation of the invention are particularly evident in terms of the high level of glucose isomerase activity present per gram of material, so that comparatively little contact time is required in preparing levulose-bearing syrups.

A preferred aspect of the invention therefore includes enzymatically converting dextrose to levulose by forming a reaction solution containing preferably from 20% to 70% dextrose by weight and preferably having a pH of greater than 7 and more preferably from 7.5 to 9.5, and a temperature of preferably from 30°C . to 90°C ., and passing the reaction solution over a bed of the immobilized xylose isomerase preferably having an effective isomerase activity of at least 100 U/g. of the enzyme preparation.

More preferably, the reaction solution will contain from 40% to 60% dextrose by weight and the pH of the solution will be in the range of from 8.2 to 9.2. A reaction solution containing about 50% of dextrose, by weight, and a pH in the range of from 8.4 to 8.6 is most preferred.

Typically, the immobilized xylose isomerase preparation will have an effective isomerase activity of at least 175 U/g. of immobilized enzyme preparation. The reaction temperature is preferably in the range of from 50°C . to 75°C . and a temperature of about 60°C . is most preferred.

The xylose isomerase enzyme is preferably derived from a micro-organism belonging to the genus *Streptomyces*, and more preferably, a micro-organism strain that is selected from the group of mutant strains consisting of *S. olivochromogenes*, ATCC No. 21713, *S. olivochromogenes*, ATCC No. 21714 and *S. olivochromogenes*, ATCC No. 21715. The preparation of the latter mentioned strains is more specifically described

in U.K. Patent Specification Nos. 1,411,763, 1,411,764 and 1,411,765.

The dextrose used for preparing the reaction solution may be dextrose in crystalline form, a re-melt, or a starch hydrolysate produced by either sequentially or concurrently enzymatically liquefying and saccharifying a starch-containing solution. Also suitable reaction solution include starch hydrolysates which have been thinned or liquefied with an acid and, in a subsequent step, converted to a dextrose-containing solution, enzymatically by one or more enzymes. Preferred starch hydrolysates are prepared by converting an enzyme liquefied starch-containing solution with a combination of two enzymes, such as glucoamylase and α -1,6-glucosidase enzymes.

The process for enzymatically converting dextrose to levulose employing the immobilized xylose isomerase enzyme preparation of the invention is most suitable in column reactors for the continuous enzymatic isomerization of dextrose to levulose, wherein the immobilized enzyme is packed in the column. The column may optionally contain additional materials to improve the flow rate of the reaction liquor, such as glass beads, filter aids, and the like. However, excellent flow rates are obtained by the use of granular basic magnesium carbonate having particle sizes greater than 40 mesh (Tyler standard). Generally, the particle size of the preferred granular basic magnesium carbonate will be in the range of from about -12 to +20. The basic magnesium carbonate in granular form can be easily obtained by simply compacting and grinding commercially obtainable powdered basic magnesium carbonate in suitable equipment.

A preferred embodiment of the invention comprises utilizing more than one column, preferably at least three columns, containing the immobilized and stabilized xylose isomerase, positioned in series. Employing such a technique, a more efficient utilization of the immobilized enzyme preparation can be obtained. For example, higher flow rates can be employed. Also, when the first column in the series becomes inactivated, the feed liquor can be conveniently shifted to the second column, while the first column is being regenerated by contact with fresh, soluble xylose isomerase enzyme, and when the second column becomes inactivated, the feed liquor can be fed to the third column, initially and subsequently through the regenerated first column. In this manner, the process can be conducted continuously, without interruption. The immobilized xylose isomerase enzyme preparation can also be used in a pressure leaf filter comprising one or more flat filtering elements (leaves) supported vertically or horizontally in a cylindrical tank.

Since the basic magnesium carbonate containing the bound xylose isomerase enzyme must be maintained at an alkaline pH in order to prevent decomposition during the enzymatic isomerization reaction, the feed liquor containing dextrose should preferably be pre-adjusted to the alkaline pH values indicated hereinabove, i.e., a pH of at least 7.5, and preferably, a pH greater than 8.2. Due to the increased enzyme concentration and retained activity of the xylose isomerase-basic magnesium carbonate complex, its use in a continuous conversion reduces the time requirement necessary to produce a levulose syrup having 45% levulose, so that a change in pH due to the formation of organic acids in the converter is not evident. In other words, if the residence time or contact time in a column isomerization is less than two hours, and preferably, less than one hour, and the pH of the initial feed liquor is greater than 8.0, the formation of acid during the enzymatic isomerization is not apparent and, therefore, the pH of the feed syrup does not need to be regulated by an alkaline material.

Assay of Isomerase Activity

The assay procedure involves making a spectrophotometric determination of the ketose produced from a glucose solution under a standardised set of conditions.

A stock solution is made up in the following manner:

Component	Amount
0.1 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1 ml.
0.01 M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1 ml.
1 M Phosphate buffer, pH 7.5	0.5 ml.
Anhydrous D-glucose	1.44 grams
Distilled water	To make up a total volume of 7.5 ml.

The enzyme preparation to be assayed is first sonicated or otherwise treated as necessary to put the enzyme in a form suitable for assay and then is diluted to contain from 1 to 6 isomerase units per ml.

An enzymatic isomerization is conducted by adding 1 ml. of the enzyme preparation to 3 ml. of the stock solution, and incubating for 30 minutes at 60°C. At the end of the incubation period, a 1 ml. aliquot is taken and quenched in a 9 ml. volume of 0.5 N perchloric acid. The quenched aliquot is then diluted to a total volume of 250 ml. As a control, for comparative purposes, a glucose blank is also run by substituting 1 ml. of water for the 1 ml. of the enzyme preparation in solution form, at the beginning of the incubation period.

The ketose is then determined by a cysteine-sulfuric acid method. For the purposes of this assay, one isomerase unit is defined as the amount of enzyme activity that is required to produce one micromole of levulose per minute under the isomerization conditions described.

EXAMPLE 1

Preparation and use of Stabilized Xylose Isomerase

As the isomerization temperature is increased, reaction rate increases, but so does inactivation of the isomerase. At some temperature, the rate of inactivation will exceed the increased reaction rate, and at this temperature, the product produced per unit time will decrease.

To produce stabilized isomerase, a very high purity enzyme is recovered from the cells. A *Streptomyces*-derived enzyme is preferred but the source microorganism may be any satisfactory producer of enzyme. The enzyme is placed in solution and is passed over a bed of particulate basic magnesium carbonate. The enzyme is efficiently absorbed and the product is a very stable, active form of the enzyme.

Operation at higher temperatures is conducive to higher levulose contents because the reaction rate is increased and the equilibrium levulose content is higher at the higher temperature. For example, equilibrium levulose contents at some elevated temperatures are listed below.

TABLE 2

Isomerization Temperatures	Levulose Content, % Dry Basis Based on Initial Dextrose
60°C.	51.2%
70°C.	52.6%
80°C.	54.0%

A column was packed with the stabilized enzyme, and a stream of a dextrose solution was passed through the column, good conversion was observed. Long enzyme life was obtained despite prolonged operations at 60°C. and above, while levulose contents in the effluent were in at least 50% of total sugar present dry basis. The isomerization was conducted at pH 8, and the product had exceptionally good color, was low in organic acid content, and was low in undesirable carbohydrates. The high operating pH promotes enzyme stability despite high operating temperatures, and the high temperatures permit a very short residence time in the column, thus minimizing investment requirements in and storage of process material.

EXAMPLE 2

Continuous Enzymatic Conversion of Dextrose to Levulose Using Immobilized Xylose Isomerase

A granular basic magnesium carbonate material having a particle size in the range of -12 to +20 (Tyler mesh) (55.8 grams) was placed in a column having a volume of 73.3 ml. A substantially pure, soluble, cell-free xylose isomerase enzyme derived from *S. olivochromogenes* ATCC No. 21713, in a 0.01 M $MgSO_4$ solution, having a 50 U/ml. activity, was contacted with the granular basic magnesium carbonate to load the column with 5 million units/ft³ of enzyme. The immobilized xylose isomerase enzyme had more than 100 U/g. of effective activity per gram of immobilized enzyme.

An aqueous dextrose feed liquor was then supplied to the column at 27° Baume (50% d.s.). The feed liquor contained $MgCl_2$ (0.005 M with respect to $MgCl_2$). The pH of the feed was adjusted to the range from 8.4 to 8.8 with 4 N NaOH at 58°C.

The column containing the immobilized enzyme preparation was maintained at a temperature of 58°C. The residence time in the column was less than one hour. The initial bed volume per hour for the production of 45% levulose, dry basis (BVH₄₅) was about one.

The effluent syrup contained about 45% by weight levulose, dry basis, and less than about 0.3% by weight, dry basis, psicose. The following is an analysis of the pH of 100 the feed syrup and effluent syrup after a prolonged continuous conversion campaign:

Hours of Operation	Feed Syrup pH at 25°C.	Effluent pH at 25°C.
261	8.60	8.60

The above experiment was repeated, except that the xylose isomerase was bound on an alumina carrier. The same dextrose feed liquor, pre-adjusted to a pH of 8.3-8.4 with 4 N NaOH, was pumped through the column, which was maintained at a temperature of 58°C., at a rate to provide 45% by weight levulose, dry basis, in the effluent. The residence time was less than one hour. The following is an analysis of the pH of the feed syrup and effluent syrup after a prolonged continuous conversion campaign:

Hours of Operation	Feed Syrup pH at 25°C.	Effluent pH at 25°C.
16	8.3	8.4
376	8.15	8.25
428	8.4	8.3

In the operation of a column where the residence time may exceed about four hours, it is particularly preferred to feed an alkaline material such as sodium hydroxide or $Mg(OH)_2$ solutions or a combination of the

two into the column at different elevations. A suitable means for accomplishing this is to provide alkaline inlets at one or more places along the column, so that the pH of the reaction solution is maintained at a pH of at least about 7.5.

As another embodiment of the invention, the pH of the enzymatic isomerase reaction solution can be programmed by the use of one or more alkaline inlets along the column. In such a technique, the initial pH of the feed liquor can be about 7.5 or greater, preferably in the range of from about 7.5 to about 8.2. By introducing alkaline materials such as sodium hydroxide or $Mg(OH)_2$ or a combination of the two into the reaction solution via inlets along the column, the pH of the reaction solution can be conveniently increased to a value up to about 9.5. Preferably, the pH will be increased by a value of at least about 0.1 from supply liquor to effluent, although more beneficial results in terms of stability of the basic magnesium carrier and quality of the isomerase are realized when the pH is increased by a value of at least about 0.2.

EXAMPLE 3

Continuous Enzymatic Conversion of Dextrose to Levulose Using Immobilized Xylose Isomerase

A glass jacketed column 41 inches in length was used as the converter. The diameter of the enzyme bed was $1\frac{1}{16}$ inches. Water was pumped into the converter at the lower end at approximately two bed volumes per hour. Dry basic magnesium carbonate which had been screened through a 16 mesh and collected on a 30 mesh (Tyler) screen was then added into the converter through the top of the converter column. The particles of the basic magnesium carbonate settled toward the base of the converter, while the fine material in the basic magnesium carbonate was washed out of the top of the column with the ascending water.

After the bed of basic magnesium carbonate particles was built, soluble xylose isomerase enzyme solution which was derived from a microorganism of the genus *Streptomyces* (one liter at an enzyme activity of 100 U/ml.) was pumped through the bed at room temperature. Based on the difference in enzyme activity of the solution before and after being cycled through the bed twice, 79,000 units of isomerase enzyme were bound in the basic magnesium carbonate bed, to provide a xylose isomerase-basic magnesium carbonate complex having an effective activity greater than 175 U/g.

Enzymatic isomerization of a dextrose solution to form a levulose-bearing syrup was conducted by passing through the column a solution containing 600 grams per

liter of dextrose, at an initial pH of about 8.3, and at an initial flow rate of about 1.5 bed volumes per hour (approximately 800 ml. per hour). Aliquot samples were withdrawn periodically for analysis. For the first six hours of operation, the effluent product contained between 45.9% and 48.7% ketose, by weight. The flow rate to make a 45% by weight ketose product, dry basis, remained about one bed volume per hour or greater. The amount of psicose formed was less than 1% by weight, dry basis.

Based upon the results observed from the foregoing experiment, it was quite evident that the xylose isomerase-basic magnesium carbonate complex was quite active. The experiment was also indicative that the bound enzyme was active and also quite stable. Since the quality of a product from a continuous converter is satisfactory at a production rate of 0.5 bed volume per hour (BVH), the production time of this converter was 22 days and the half-life of the converter was 16–18 days. At an average production rate of 1.3 BVH at 45% levulose production for 17 days, the total product on a dry basis was 171,849 grams or 0.46 unit of enzyme per gram of product. This clearly represents a marked advantage of the invention, derived from the savings involved in utilization of the enzyme required to make the desired levulose product.

EXAMPLE 4

A commercially obtained basic magnesium carbonate powder was compacted and ground to a particle size in the range of from about -40 to +30 (Tyler mesh). 31.7 grams of this granulated basic magnesium carbonate carrier were placed in a jacketed column filled with water. The bed had a volume of 53 ml. Water was pumped through the column from its lower end so that the coarser particles of the granular basic magnesium carbonate would settle to the base of the converter, while the fine material was washed out of the top of the column with the ascending water.

In a separate container, a solution was prepared containing a 95 D.E. starch hydrolysate at 50% solids and soluble enzyme. The xylose isomerase enzyme was one derived from *Streptomyces* and having 50 U/ml. of isomerase activity. The solution was brought up to 189 ml. Magnesium chloride ($MgCl_2$) was added to the solution to obtain a 0.005 M content with respect to $MgCl_2$. The pH was adjusted to 8.4 with 4 N sodium hydroxide.

The combination solution (starch hydrolysate and soluble enzyme) was pumped into the column containing the granular basic magnesium carbonate and circulated $2\frac{1}{2}$ times through the column, so as to load the carrier with 5 million units per cubic foot

of enzyme, at a temperature of 60°C. Once the solution containing the soluble enzyme and starch hydrolysate had been circulated into the column, the column thereupon was activated and ready for use to continuously convert a dextrose solution to a levulose-bearing syrup. The xylose isomerase-granular basic magnesium carbonate complex had an effective isomerase activity of greater than 175 U/g. of the complex.

A levulose-bearing syrup was continuously prepared in the column by pumping a solution containing a starch hydrolysate having a D.E. of about 95 and having a solids content of about 50%, dry basis. The starch hydrolysate that was pumped into the column was first treated with $MgCl_2$ to obtain a 0.005 M content based upon $MgCl_2$ and the pH of the solution was adjusted to 8.4 with 4 N NaOH. The isomerization was continuously conducted at a temperature of 60°C. at an initial BVH₄₂ levulose of 1.6.

After 69 hours, the immobilized xylose isomerase in the column continued to convert dextrose into levulose, producing a product having a ketose content of 40.1% dry basis, and a psicose content of 0.15% by weight, dry basis, to obtain an adjusted bed volume per hour equivalent to 1.8 BVH₄₂.

This experiment clearly demonstrates the stability and enzyme efficiency of the immobilized xylose isomerase of the present invention.

EXAMPLE 5

Comparative Example

Several alkaline earth metal carbonates were evaluated to compare their binding capacity ("bound isomerase") and effective

isomerase activity ("retained isomerase activity") with particulate basic magnesium carbonate. In each instance, the carrier tested was contacted with a purified, cell-free xylose isomerase solution containing 2500 to 2600 U/ml. of glucose isomerase activity. The xylose isomerase used was derived from a microorganism of the genus *Streptomyces* (the strain *S. olivochromogenes*, ATCC No. 21713).

Each of the carriers was slurried in a 150 ml. beaker with 30 ml. of a solution consisting of 0.01 M $MgSO_4 \cdot 7H_2O$. The slurry was adjusted to a pH of 8-9 with 0.1 N KOH. The purified xylose isomerase solution was then added to each carrier slurry to provide a total isomerase addition of about 2600 units of glucose isomerase activity. An additional amount of 0.01 M $MgSO_4$ solution was added to each slurry of carrier and enzyme to attain a total volume of 50 ml. The pH of the solution was adjusted (if necessary) to 8 to 9. The beakers were covered and stirred at room temperature for 30 minutes to achieve maximum sorption of the enzyme on the respective carriers. The slurries were filtered and the carriers with the sorbed enzyme were washed with 100 ml. of the 0.01 M $MgSO_4$ solution. The filtrates and washings were combined and assayed for isomerase activity against the original isomerase activity added using a Technicon (Registered Trade Mark) Auto-Analyzer.

The binding capacity or "bound isomerase" for the respective carriers was calculated by difference using the following formula:

$$\text{"Bound Isomerase" (BI), U/g., d.b.} = \frac{\text{Total Units Present} - \text{Total Units in Filtrate and Washings}}{\text{Grams of Carrier, d.b.}}$$

The "effective isomerase activity" (also referred to as "retained isomerase activity") of each of the alkaline earth metal carbonate carrier samples was also determined using the following procedure.

An aqueous solution containing 80 g. of dextrose (d.b.) and 13.3 mg. of $Mg(OH)_2$ per 100 ml. was prepared. The solution was stirred for at least one hour (preferably overnight) to equilibrate. The pH was adjusted to 8.5 at room temperature with 3 N KOH. 193.9 g. of substrate (about 150 ml.) was added to a 1-liter stainless steel beaker covered with a center-holed watch glass containing an additional hole near the edge. The sample was placed in a 60°C water bath, and stirred for 15 minutes, while maintaining a pH of 8.0 with 3 N KOH. The substrate was sparged with nitrogen at a moderate rate using a gas dispersion tube

placed through the peripheral hole of the watch glass. A pre-weighed amount of xylose isomerase-basic magnesium complex containing from 1,000 to 3,000 units of bound isomerase was added using 50 ml. of 60°C. water to transfer the material to the stainless steel beaker. This yielded a reaction mixture containing 60 g. of dextrose and 10 mg. of $Mg(OH)_2$ per 100 ml. and an isomerase dosage level of from 8.3 to 25.0 units per gram of dextrose. The reaction mixture was adjusted to pH 8.0, and digested for exactly two hours after enzyme addition. The material was filtered immediately by vacuum through an 11-cm. Whatman (Registered Trade Mark) 541 paper and the pH of a 500 ml. portion of the filtrate was adjusted to about 2.5 with 1.0 N perchloric acid. The dry substance content of the inactivated sample was determined by refrac-

tive index measurement and the levulose concentration by using spectrophotometric or polarimetric procedures. From the amount of levulose produced, effective iso-

merase ("retained isomerase activity") activity was calculated using the following equation:

$$\text{Effective Activity, U/g, d.b. carrier} = \frac{V}{C} \times 26.46 \times \frac{51.2}{\log 51.2 - L}$$

where:

V=reaction volume, ml.;

C=carrier, g., d.b.; and

L=levulose produced, % d.b.

The details and results of the specific tests are set forth in the following Table:

TABLE 3

Carrier	Substrate		Binding Capacity "Bound Isomerase Activity" (U/g., d.b.)	Effective Isomerase Activity (U/g., d.b.)	% Of Original Activity
	Concentration g., d.s. 100 ml.	Solvent			
ZnCO ₃	60	MgSO ₄	515	252	48.9
CaCO ₃	60	MgSO ₄	368	54	14.7
BaCO ₃	60	MgSO ₄	38	75	—
Basic ⁽¹⁾ MgCO ₃	60	MgSO ₄	412	294	71.4

⁽¹⁾ Basic Magnesium Carbonate have the formula 4 MgCO₃·Mg(OH)₂·5H₂O.

As it can be seen from the above data, there is a great deal of unpredictability in the binding capacity (also defined as "bound isomerase") and effective isomerase activity for the alkaline earth metal carbonates. It is particularly significant that BaCO₃ has an extremely low "bound isomerase" capability. While ZnCO₃ and CaCO₃ complexes with isomerase exhibit reasonable "bound isomerase activity", the complexes of both compounds exhibit a relatively low "effective isomerase activity" or ability to isomerase dextrose to levulose as compared to the xylose isomerase-basic magnesium carbonate complex of the present invention.

Not wishing to be bound to any theory, it is postulated that the relatively low "effective isomerase activity" occurs with respect to these salts (ZnCO₃ and CaCO₃) because the isomerase is bound so tightly that its conformation is charged and/or the isomerase is not accessible to the substrate due to the manner the isomerase is bound to the salt. In any event, it is unexpected that basic magnesium carbonate is capable of having a relatively high binding capacity in addition to a high "effective isomerase activity". The combination of these two features, together, provides a number of important advantages from the standpoint of more stability and longer half-life of the immobilized enzyme and greater enzyme efficiency. The enzyme efficiencies normally

range from 0.30 to about 0.70. Thus, a deep bed column with an enzyme loading of 6 MM units/ft³ (the amount of enzyme activity in the bed (units/g.) divided by the volume of the bed (lbs./ft³)) and an enzyme efficiency of 0.6 would have an initial column production rate of 3.6 BVH at 45% ketose (BVH₄₅), wherein BVH is the supply rate divided by the bed volume.

WHAT WE CLAIM IS:—

1. A xylose isomerase enzyme preparation comprising a xylose isomerase enzyme sorbed on particulate basic magnesium carbonate.
2. A preparation as claimed in claim 1 wherein the xylose isomerase enzyme is soluble in water.
3. A preparation as claimed in either claim 1 or claim 2 wherein the enzyme is derived from a microorganism of the genus *Streptomyces*.
4. A preparation as claimed in claim 3 wherein the microorganism is a strain of the species *S. olivochromogenes*.
5. A preparation as claimed in claim 4 wherein the strain is *S. olivochromogenes* ATCC No. 21713; *S. olivochromogenes*, ATCC No. 21714 or *S. olivochromogenes*, ATCC No. 21715.
6. A preparation as claimed in any one of claims 1 to 5, having an effective iso-



tive index measurement and the levulose concentration by using spectrophotometric or polarimetric procedures. From the amount of levulose produced, effective iso-

merase ("retained isomerase activity") activity was calculated using the following equation:

$$\text{Effective Activity, U/g, d.b. carrier} = \frac{V}{C} \times 26.46 \times \frac{51.2}{\log 51.2 - L}$$

where:

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25	ZnCO ₃	60	MgSO ₄	515	252	48.9
	CaCO ₃	60	MgSO ₄	368	54	14.7
	BaCO ₃	60	MgSO ₄	38	75	—
	Basic ⁽¹⁾					
30	MgCO ₃	60	MgSO ₄	412	294	71.4

⁽¹⁾ Basic Magnesium Carbonate have the formula 4 MgCO₃·Mg(OH)₂·5H₂O.

As it can be seen from the above data, there is a great deal of unpredictability in the binding capacity (also defined as "bound isomerase") and effective isomerase activity for the alkaline earth metal carbonates. It is particularly significant that BaCO₃ has an extremely low "bound isomerase" capability. While ZnCO₃ and CaCO₃ complexes with isomerase exhibit reasonable "bound isomerase activity", the complexes of both compounds exhibit a relatively low "effective isomerase activity" or ability to isomerase dextrose to levulose as compared to the xylose isomerase-basic magnesium carbonate complex of the present invention.

Not wishing to be bound to any theory, it is postulated that the relatively low "effective isomerase activity" occurs with respect to these salts (ZnCO₃ and CaCO₃) because the isomerase is bound so tightly that its conformation is charged and/or the isomerase is not accessible to the substrate due to the manner the isomerase is bound to the salt. In any event, it is unexpected that basic magnesium carbonate is capable of having a relatively high binding capacity in addition to a high "effective isomerase activity". The combination of these two features, together, provides a number of important advantages from the standpoint of more stability and longer half-life of the immobilized enzyme and greater enzyme efficiency. The enzyme efficiencies normally

range from 0.30 to about 0.70. Thus, a deep bed column with an enzyme loading of 6 MM units/ft³ (the amount of enzyme activity in the bed (units/g.) divided by the volume of the bed (lbs./ft³)) and an enzyme efficiency of 0.6 would have an initial column production rate of 3.6 BVH at 45% ketose (BVH₄₅), wherein BVH is the supply rate divided by the bed volume.

WHAT WE CLAIM IS:—

1. A xylose isomerase enzyme preparation comprising a xylose isomerase enzyme sorbed on particulate basic magnesium carbonate.

2. A preparation as claimed in claim 1 wherein the xylose isomerase enzyme is soluble in water.

3. A preparation as claimed in either claim 1 or claim 2 wherein the enzyme is derived from a microorganism of the genus *Streptomyces*.

4. A preparation as claimed in claim 3 wherein the microorganism is a strain of the species *S. olivochromogenes*.

5. A preparation as claimed in claim 4 wherein the strain is *S. olivochromogenes* ATCC No. 21713; *S. olivochromogenes*, ATCC No. 21714 or *S. olivochromogenes*, ATCC No. 21715.

6. A preparation as claimed in any one of claims 1 to 5, having an effective iso-

merase activity of at least 100 units per gram of preparation.

7. A preparation as claimed in claim 6 having an effective activity of at least 175 units per gram of preparation.

8. A preparation as claimed in any one of claims 1 to 7 wherein the basic magnesium carbonate is in granular form.

9. A preparation as claimed in any one of claims 1 to 8 and substantially as hereinbefore described in any one of the Examples.

10. A process for making the preparation claimed in any one of claims 1 to 9 which process comprises contacting a particulate basic magnesium carbonate with a solution containing cell-free water soluble xylose isomerase.

11. A process as claimed in claim 10, wherein the pH of the solution is from 7.5 to 9.5.

12. A process as claimed in either claim 10 or claim 11, wherein the enzyme is in a substantially purified form.

13. A process as claimed in any one of claims 10 to 12, wherein the enzyme is derived from a microorganism of the genus *Streptomyces*.

14. A process as claimed in claim 13, wherein the enzyme is derived from a microorganism of the species *S. olivochromogenes*.

15. A process as claimed in claim 14, wherein the microorganism is *S. olivochromogenes*, ATCC No. 21713; *S. olivochromogenes*, ATCC No. 21714 or *S. olivochromogenes*, ATCC No. 21715.

16. A process as claimed in any one of the claims 10 to 15 and substantially as hereinbefore described.

17. An enzyme preparation whenever made by the process claimed in any one of claims 10 to 16.

18. A process for the enzymatic isomerization of dextrose to levulose which comprises passing a solution containing dextrose into contact with the preparation claimed in any one of claims 1 to 9 and 17.

19. A process as claimed in claim 18 wherein the process is performed at a temperature of from 30°C. to 90°C.

20. A process as claimed in claim 18 or claim 19 wherein the process is performed at a pH greater than 7.

21. A process as claimed in claim 20 wherein the pH is from 7.5 to 9.5.

22. A process as claimed in claim 21 wherein the pH is from 8.2 to 9.2.

23. A process as claimed in any one of claims 18 to 22 wherein the concentration of the solution containing dextrose is from 20% to 70% by weight of dextrose.

24. A process as claimed in claim 23 wherein the concentration is from 40% to 60% by weight.

25. A process as claimed in any one of claims 18 to 24 wherein the preparation is packed in a column.

26. A process as claimed in claim 25 wherein the column contains inlets whereby an alkali is fed to the column to regulate the pH of the isomerization solution.

27. A process as claimed in either claim 25 or claim 26 wherein the isomerization solution is continuously passed through more than one column in series.

28. A process as claimed in any one of claims 18 to 27 wherein the dextrose containing solution is a starch hydrolysate.

29. A process as claimed in claim 28 wherein the starch hydrolysate is produced by either sequentially or concurrently enzymatically liquefying and saccharifying a starch-containing solution.

30. A process as claimed in either claim 28 or claim 29 wherein the starch hydrolysate is a corn starch hydrolysate.

31. A process as claimed in any one of claims 28 to 30 wherein the starch hydrolysate has a D.E. of at least 95.

32. A process as claimed in any one of claims 18 to 31 and substantially as hereinbefore described.

33. Levulose whenever made by the process claimed in any one of claims 18 to 32.

34. A process for the recovery of xylose isomerase from a fermentation broth containing the enzyme comprising contacting the broth with particulate basic magnesium carbonate and recovering the enzyme in the form of a xylose isomerase enzyme basic magnesium carbonate complex.

35. A process as claimed in claim 34 wherein the basic magnesium carbonate is added to a broth containing cells containing xylose isomerase and subsequently releasing the xylose isomerase from the cells.

For the Applicants,
STEVENS, HEWLETT & PERKINS,
Chartered Patent Agents,
5 Quality Court,
Chancery Lane,
London, WC2A 1HZ.

