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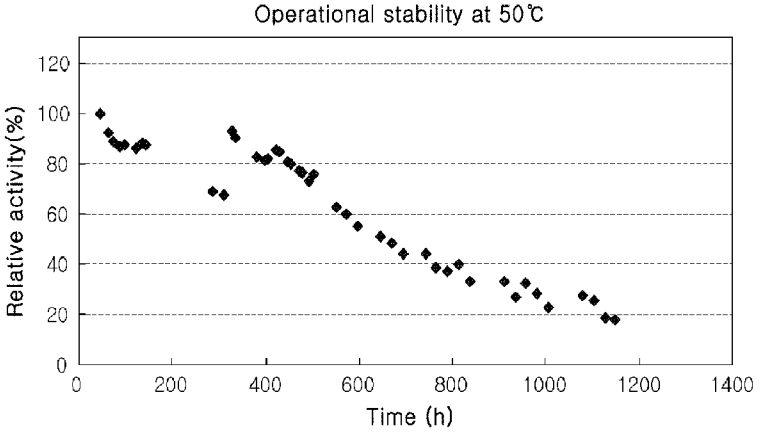
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(54) Title: IMMOBILIZATION OF PSICOSE-EPIMERASE AND A METHOD OF PRODUCING D-PSICOSE USING THE SAME

[Fig. 5]



(57) Abstract: The present invention relates to a method of successively producing D-psicose from D-fructose or D-glucose by using a psicose-epimerase derived from Agrobacterium tumefaciens which is expressed in a food safety form.

WO 2011/040708 A2

Description

IMMOBILIZATION OF PSICOSE-EPIMERASE AND A METHOD OF PRODUCING D-PSICOSE USING THE SAME

Technical Field

[1] The present invention relates to a method of successively producing D-psicose from D-fructose or D-glucose by using a psicose-epimerase derived from *Agrobacterium tumefaciens* which is expressed in a food safety form.

[2]

Background Art

[3] D-psicose has been widely used as a functional sweetener which has a sweet taste similar to sugar but is an ultralow-energy monosaccharide sugar. In particular, D-psicose is known as a "rare sugar" because it is rarely found in nature, and even when found, only in small amounts.

[4] D-psicose is an epimer of D-fructose, and the sweetness intensity and kinds thereof are quite similar to those of D-fructose. However, contrary to D-fructose, since when absorbed into the body, D-psicose is rarely metabolized, it has almost zero calories. Further, due to its action to alleviate abdominal obesity by suppressing enzyme activities regarding to lipid synthesis, D-psicose can be used as an effective ingredient of diet foods. In addition, while sugar alcohols widely used as a substitute for sugar have a problem of causing side effects such as diarrhea when taken in excessive amounts, D-psicose has almost no side effects (Matsue, T., Y. Baba, M. Hashiguchi, K. Takeshita, K. Izumori, and H. Suzuki. 2001. Dietary D-psicose, a C-3 epimer of D-fructose, suppresses the activity of hepatic lipogenic enzymes in rats. Asia Pac. J. Clin. Nutr. 10:233-237.; Matsuo, T., and K. Izumori. 2004. D-psicose, a rare sugar that provides no energy and additionally beneficial effects for clinical nutrition. Asia Pac. J. Clin. Nutr. 13:S127).

[5] Due to such reasons, D-psicose is getting the spotlight in the food industry field as a diet sweetener, and thus, there is a growing need to develop a method of producing D-psicose efficiently.

[6] Conventional methods of producing D-psicose by using a psicose-epimerase had been focused to develop an epimerization process of D-psicose from D-fructose by using a recombinant enzyme massively expressed in a recombinant *E. coli* or a host cell comprising the same. However, the biotechnological production of D-psicose using such a recombinant *E. coli* is unsuited for the production of D-psicose as a food material in terms of food safety. In order to produce D-psicose suitable to be used as a food material, it is required to develop a mass production method using a psicose-

epimerase expressed in a host cell which is a GRAS (Generally Recognized As Safe) strain as well as a strain capable of being industrially mass producing, or using a host comprising the same.

[7] GRAS is granted for substances that are generally recognized to be safe when experts qualified by scientific training and experience evaluate their safety through scientific procedures under the conditions of their intended use. GRAS is a unique system only implemented in USA, but its importance and necessity has been recognized internationally as well as in USA. Therefore, people are paying attention to the development of GRAS.

[8] Meanwhile, for the industrial production of D-psicose, it is required to develop a method of producing D-psicose from a more inexpensive substrate. Currently, due to a high substrate specificity of a psicose-epimerase, only expensive D-fructose is used as a substrate for the production of D-psicose.

[9]

Disclosure of Invention

Technical Problem

[10] The present invention has been actively studied to mass produce D-psicose which can be used as a food material, and found that the successive production of D-psicose from D-fructose or D-glucose can be achieved by using immobilization for providing stable environment for enzymatic activation for the industrially mass production of D-psicose and using a D-glucose isomerase along with a psicose-epimerase.

[11] An object of the present invention is to provide a psicose-epimerase in a food safety form for the preparation of D-psicose which can be used as a food material.

[12] Another object of the present invention is to provide a method of successively producing D-psicose from D-glucose or D-fructose by using a psicose-epimerase and a D-glucose isomerase in an immobilized food safety form.

[13]

Technical Solution

[14] The present invention provides a method of preparing D-psicose from D-fructose by using a psicose-epimerase derived from *Agrobacterium tumefaciens* which is expressed in a GRAS strain.

[15] The method of the present invention comprises the steps of: expressing a psicose-epimerase derived from *Agrobacterium tumefaciens* in a GRAS strain; and preparing D-psicose from D-fructose by using the expressed psicose-epimerase.

[16] The method of the present invention may also comprise the steps of: separating a psicose-epimerase from the culture of a GRAS strain capable of expressing a psicose-epimerase derived from *Agrobacterium tumefaciens*; and preparing D-psicose from D-

fructose by using the separated psicose-epimerase.

[17] The GRAS strain expressing a psicose-epimerase derived from *Agrobacterium tumefaciens* can be a strain transformed with a recombinant expression vector including a gene encoding the aforementioned enzyme.

[18] The psicose-epimerase used in the preparation of D-psicose according to the present invention can be in the form of being contained in a culture of the GRAS strain expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* or being isolated from the culture. The GRAS strain can be a strain transformed with a recombinant expression vector including a gene encoding a psicose-epimerase. The GRAS strain can be *Corynebacterium* sp., preferably *Corynebacterium glutamicum* KCCM 11046.

[19] It is preferable that the psicose-epimerase derived from *Agrobacterium tumefaciens* used in the method of the present invention has the amino acid sequence of SEQ ID NO: 1.

[20] The method of preparing D-psicose according to the present invention may further comprise the step of immobilizing the psicose-epimerase onto a carrier. The carrier suitable for the present invention may include alginate, preferably sodium alginate, but is not limited thereto.

[21] The method of preparing D-psicose according to the present invention can further comprise the steps of packing a column with the carrier on which the psicose-epimerase is immobilized and supplying the packed-bed column with a D-fructose solution.

[22] Also, the present invention further provides a method of preparing D-psicose from D-glucose, comprising the step of immobilizing a psicose-epimerase and a D-glucose isomerase onto carriers. The carrier suitable for the present invention may include alginate, preferably sodium alginate, but is not limited thereto.

[23] In the method as described above, the psicose-epimerase may be contained in a culture of the GRAS strain capable of expressing psicose-epimerase derived from *Agrobacterium tumefaciens* or to be isolated from the culture. The GRAS strain can be a strain transformed with a recombinant expression vector including a gene encoding psicose-epimerase. Further, the GRAS strain can be *Corynebacterium* sp., preferably *Corynebacterium glutamicum* KCCM 11046.

[24] It is preferable that the psicose-epimerase derived from *Agrobacterium tumefaciens* used in the method of the present invention has the amino acid sequence of SEQ ID NO: 1.

[25] In addition, the present invention further provides a method of preparing D-psicose from D-glucose, comprising the steps of: immobilizing a psicose-epimerase and a D-glucose isomerase onto carriers; packing a column with the carriers onto which the

psicose-epimerase and D-glucose isomerase are immobilized; and supplying the packed-bed column with a D-glucose solution. The carriers onto which the psicose-epimerase and D-glucose isomerase are immobilized can be packed into the same column, or respectively packed into two separate columns. However, it is preferable that the carrier is packed into a separate column, and in this case, the two separate columns are mutually connected.

[26] The present invention provides a *Corynebacterium glutamicum* KCCM 11046 strain useful for the preparation of D-psicose from D-fructose or D-glucose.

[27] The term "food safety form" as used herein means that it is safe enough to be used as a food material. Therefore, the term "expressed in a food safety form" as used herein means that a target gene was expressed in a strain which is recognized as being safe and causing no harmful effects although it is used as a food material, for example, a GRAS strain.

[28] In one embodiment of the present invention, a method of transforming the GRAS strain with a recombinant vector may include an electroperforation but is not limited thereto, and may be carried out using any transformation method known to those skilled in the art to which the present invention pertains.

[29] In one embodiment of the present invention, the recombinant vector including the gene encoding the psicose-epimerase derived from *Agrobacterium tumefaciens* may include the psicose-epimerase encoding gene operatably linked to a promoter which is active in *Corynebacterium* sp. bacteria. The promoter can have one of sequences having SEQ ID NOs: 2 to 8 that have been confirmed to express a target gene more efficiently than a tac promoter in *Corynebacterium* sp. bacteria (Korean Patent Publication NO. 2006-0068505).

[30] The term "psicose-epimerase" as used herein means a D-psicose-3-epimerase having a conversion activity of D-fructose to D-psicose.

[31] In one embodiment of the present invention, the psicose-epimerase derived from *Agrobacterium tumefaciens* may have the amino acid sequence of SEQ ID NO: 1 or be functional fragments thereof. The functional fragment means a fragment which includes mutations by substitution, insertion or deletion or the like of some amino acids in the amino acid sequence of SEQ ID NO: 1, while having a conversion activity of D-fructose to D-psicose.

[32] In one embodiment of the present invention, the GRAS strain may be a *Corynebacterium* sp. strain.

[33] The *Corynebacterium* sp. strain is an industrial microorganism producing chemicals which have various uses in an animal feed, medicine, and food fields, etc. including L-lysine, L-threonine and various nucleic acids. Such a *Corynebacterium* sp. strain is a GRAS (Generally Recognized As Safe) strain and has properties of being easy for

genetic manipulation and mass cultivation. Further, the *Corynebacterium* sp. strain has high stability under various process conditions, has a relatively rigid cellular wall structure as compared with other bacteria, thereby having a biological property of existing of cell mass in a stable state even under high osmotic pressure by high sugar concentration, etc.

[34] In one embodiment of the present invention, the *Corynebacterium* sp. strain may be *Corynebacterium glutamicum.*

[35] In one embodiment of the present invention, the GRAS strain expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* may be a recombinant strain *Corynebacterium glutamicum* KCCM 11046.

[36] The psicose-epimerase may be easily modified by mutagenesis conventionally known to those skilled in the art such as directed evolution and site-directed mutagenesis, etc. Therefore, it should be construed that recombinant enzymes having a certain extent of sequence homology, such as sequence homology of 70% or higher, preferably 80% or higher, more preferably 90% or higher to the amino acid sequence of the psicose-epimerase represented by SEQ ID NO: 1, and being expressed as an active form in the GRAS strain; and a host cell comprising the same are within the scope of the present invention.

[37] Culture of the GRAS strain expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* may be obtained by culturing the transformed bacteria in a medium and under culture conditions that are easily selected by those skilled in the art to which the present invention pertains depending on the properties of the strain. The method of culturing may include any method of culturing known to those skilled in the art, such as batch culture, continuous culture and fed-batch culture, but is not limited thereto.

[38] The method of preparing D-psicose according to one embodiment of the present invention may comprise the step of separating the psicose-epimerase from the culture of the GRAS strain which expresses the psicose-epimerase derived from *Agrobacterium tumefaciens.*

[39] In one embodiment of the present invention, cells separated from the culture of the strain which expresses the psicose-epimerase derived from *Agrobacterium tumefaciens* by centrifugation, filtration, etc.; or supernatant obtained by homogenizing and centrifuging the separated cells; or the psicose-epimerase which is separated and purified from the supernatant by fractionation, chromatography, etc. may be used as an enzyme for converting D-fructose to D-psicose.

[40] In one embodiment of the present invention, the psicose-epimerase may be in the form of the cell mass in the culture or the psicose-epimerase separated from the cell mass.

[41] In one embodiment of the present invention, the step of preparing D-psicose may further comprise the step of immobilizing the psicose-epimerase or the cell mass expressing the same onto a carrier.

[42] Since in case of immobilizing the enzyme or the cell mass onto a carrier, there may be provided environment for maintaining the activity for a long time, the immobilization has been used in the industrial production method using an enzyme or an microorganism. The carrier which may be used for the immobilization may include alginates such as sodium alginate, but is not limited thereto.

[43] In one embodiment of the present invention, the immobilization may be carried out by using sodium alginate as the carrier. Sodium alginate is a natural colloidal polysaccharide which is present abundantly in cell wall of algae, consists of -D-mannuronic acid and -L- guluronic acid, and is formed by random -1,4 links in content, and thus, it is advantageous that the cell mass or the enzymes can be stably immobilized onto it.

[44] In one embodiment of the present invention, the immobilization may be carried out by using 1.5% to 4.0% of sodium alginate as the carrier.

[45] In one embodiment of the present invention, the immobilization may be carried out by using 2% of sodium alginate as the carrier.

[46] In one embodiment of the present invention, by using sodium alginate as the carrier for immobilization, the immobilization may be carried out by adding 1- to 2-fold volume of an aqueous sodium alginate solution to a sample containing the enzyme or the cell mass, dropping the resulting mixture to a 0.1 M calcium ion solution to create the enzyme or the cell mass-alginate complex in beads by using a syringe pump and a vacuum pump.

[47] In one embodiment of the present invention, the step of immobilizing the psicose-epimerase onto the carrier may further comprise the steps of packing a column with the immobilized enzyme and supplying the packed column with a D-fructose solution.

[48] The column to be packed with the carrier onto which the enzyme or the cell mass is immobilized and the method of packing the column with the same may be suitably and easily selected by those skilled in the art to which the present invention pertains depending on the enzyme or the cell mass, or the carrier for immobilization used.

[49] In one embodiment of the present invention, it is possible to prepare a packed-bed column by packing the column with the immobilized enzyme. Enzymatic reaction, i.e., conversion of D-fructose to D-psicose may be carried out by supplying the packed-bed column with a D-fructose solution that is a substrate thereof.

[50] In one embodiment of the present invention, when the packed-bed column which is packed with the cell mass including the psicose-epimerase is supplied with the D-fructose solution at a constant concentration, the epimerization reaction proceeds by

the immobilized cell mass, resulting in the conversion of D-fructose to D-psicose. The converted D-psicose is subject to separation and purification by using separation columns, and then, is available as pure D-psicose.

[51] The term "immobilization reactor" as used herein means a reactor wherein the reaction for the production of D-psicose occurs by the cell mass or the enzyme immobilized onto the carrier, or the column packed with the cell mass or the enzyme immobilized onto the carrier. Namely, the immobilization means that a substance providing biological activities, in this case, a psicose-epimerase or a D-glucose epimerase or a cell mass including the same is immobilized onto the carrier.

[52] The term "operational stability" as used herein means that a biological reactor for the successive production of a target product such as D-psicose may be operated while maintaining a suitable level of productivity as compared with initial activity thereof, and generally, is represented by an operation period.

[53] In one embodiment of the present invention, the column packed with the carrier onto which the cells expressing the psicose-epimerase are immobilized is supplied with 300 g/L of D-fructose at an inflow rate of 0.1 min/ml at 50℃, which makes it possible to guarantee 25 days or longer of operational stability.

[54] The present invention further provides a method of preparing D-psicose from D-glucose, comprising the step of immobilizing a psicose-epimerase and a D-glucose isomerase onto carriers.

[55] As described above, since D-fructose used as a substrate for the psicose-epimerase is expensive, it is required to produce D-psicose from a cheap substrate such as D-glucose for the industrial mass production of D-psicose. The D-glucose isomerase is an enzyme to convert D-glucose to D-fructose, and thus, may provide a substrate for a psicose-epimerase by converting D-glucose to D-fructose. Therefore, it is possible to produce D-psicose from D-glucose by using both of these two enzymes.

[56] In one embodiment of the present invention, the psicose-epimerase may be the culture of the GRAS strain expressing the psicose-epimerase derived from *Agrobacterium tumefaciens*, or the cell mass or the psicose-epimerase separated from the culture.

[57] In one embodiment of the present invention, the D-glucose isomerase may be the culture of the GRAS strain expressing the D-glucose isomerase, or the cell mass or the D-glucose isomerase separated from the culture.

[58] In one embodiment of the present invention, the psicose-epimerase may have the amino acid sequence of SEQ ID NO: 1.

[59] In one embodiment of the present invention, the D-glucose isomerase may be commercially available. For example, the D-glucose isomerase or the immobilized D-glucose isomerase commercially available from Novozymes A/S (Bagsvaerd,

Denmark) may be used.

[60] The D-glucose isomerase is a representative enzyme for converting D-glucose to D-fructose and is currently used for the production of fructo-oligosaccharides. Among the enzymes currently used for the industrial production, the D-glucose isomerase is one of the enzymes whose activities and mechanisms have been clearly identified.

[61] In one embodiment of the present invention, the GRAS strain may be a strain transformed with a recombinant vector including a gene encoding the psicose-epimerase and a gene encoding the D-glucose isomerase or a strain co-transformed with recombinant vectors including each of the genes described above.

[62] In one embodiment of the present invention, the GRAS strain may be *Corynebacterium* sp. bacterium.

[63] In one embodiment of the present invention, the GRAS strain expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* may be the recombinant strain *Corynebacterium glutamicum* KCCM 11046.

[64] In one embodiment of the present invention, the carrier for the immobilization may be sodium alginate.

[65] In one embodiment of the present invention, the immobilization may be carried out by using 1.5% to 4.0% of sodium alginate as the carrier.

[66] In one embodiment of the present invention, the immobilization may be carried out by using 2% of sodium alginate as the carrier.

[67] In one embodiment of the present invention, by using sodium alginate as the carrier for immobilization, the immobilization may be carried out by adding 1- to 2-fold volume of the aqueous sodium alginate solution to the sample containing the enzyme or the cell mass, dropping the resulting mixture to the 0.1 M calcium ion solution to create the enzyme or the cell mass-alginate complex in beads by using the syringe pump and the vacuum pump.

[68] In one embodiment of the present invention, the gene encoding the psicose-epimerase and the gene encoding the D-glucose isomerase may be expressed in the same GRAS strain or different two GRAS strains, respectively, and the immobilization step may be carried out by immobilizing the cells of the cultured GRAS strains or a mixture of two enzymes separated from the cells onto the carriers.

[69] In one embodiment of the present invention, the culture of the GRAS strain expressing the psicose-epimerase or the cell mass or the psicose-epimerase separated from the culture; and the D-glucose isomerase may be immobilized onto carriers, respectively.

[70] In one embodiment of the present invention, the method of the present invention may further comprise the step of packing the column with the carrier onto which the cells or the enzymes are immobilized.

[71] The method of preparing D-psicose from D-glucose according to the present invention may further comprise the steps of packing the column with the immobilized psicose-epimerase and D-glucose isomerase and supplying the packed column with the D-glucose solution.

[72] In one embodiment of the present invention, the packed-bed column may be prepared by packing the column with the immobilized enzymes. The enzymatic reaction, i.e., conversion of D-glucose to D-psicose may be carried out by supplying the packed-bed column with the D-glucose solution that is a substrate thereof.

[73] In one embodiment of the present invention, the immobilized psicose-epimerase and D-glucose isomerase may be packed into separate columns, respectively.

[74] The column to be packed with the carrier onto which the enzymes or the cells are immobilized and the method of packing the column with the same may be suitably and easily selected by those skilled in the art to which the present invention pertains depending on the enzymes or the cell mass, or the carrier for immobilization used. In one embodiment of the present invention, it is possible to pack two separate columns with the psicose-epimerase immobilized carrier and the D-glucose isomerase immobilized carrier, respectively, connect these two columns in communication with each other to transfer D-fructose as a product of isomerization from the column packed with the D-glucose isomerase to the column packed with the psicose-epimerase, and induce the conversion reaction of the D-fructose to the D-psicose. When the D-glucose solution as a substrate is supplied to the immobilization reactor consisting of said two communicated columns, the D-glucose is converted to the D-fructose in the column packed with the D-glucose isomerase, and the D-fructose which transfers to the column packed with the psicose-epimerase is converted to the D-psicose. Thus, it is possible to obtain the D-psicose as a final product from the D-glucose.

[75]

Advantageous Effects

[76] The method of preparing D-psicose according to the present invention can massively and successively produce D-psicose in a food safety form which may be used as a food material from D-glucose or D-fructose.

[77]

Brief Description of Drawings

[78] Fig. 1 schematically illustrates a process of constructing a recombinant expression vector pCJ-1-ATPE including a gene encoding a psicose-epimerase derived from *Agrobacterium tumefaciens*.

[79] Fig. 2 illustrates the results of measuring the activity of the psicose-epimerase derived from *Agrobacterium tumefaciens* by HPLC for the method according to one

embodiment of the present invention, wherein Fig. 2a shows a reference standard for each sugar, and Fig. 2b shows the conversion by reaction(s).

[80] Fig. 3 illustrates % conversion of D-fructose to D-psicose by using the recombinant strain for the method according to one embodiment of the present invention under combined conditions of 100 g/L (3a), 300 g/L (3b), or 500 g/L (3c) of D-fructose, and 40℃, 45℃, or 50℃ of a reaction temperature.

[81] Fig. 4 illustrates the productivity of D-psicose depending on an inflow rate of D-fructose as a substrate for the method according to one embodiment of the present invention.

[82] Fig. 5 illustrates the operational stability of an immobilization reactor at a reaction temperature of 50℃ for the method according to one embodiment of the present invention. A relative activity (%) means an activity relative to the activity at the time when the operation is initiated.

[83] Fig. 6 illustrates % conversion of D-glucose to D-psicose by using the immobilized recombinant strain expressing the psicose-epimerase and the immobilized D-glucose isomerase under combined conditions of 100 g/L (6a), 300 g/L (6b), or 500 g/L (6c) of D-glucose, and 40℃, 45℃, or 50℃ of the reaction temperature for the method according to one embodiment of the present invention.

[84] Fig. 7 illustrates the productivity of D-psicose depending on an inflow rate of D-glucose for the method according to one embodiment of the present invention.

[85]

Best Mode for Carrying out the Invention

[86] Hereinafter, the present invention is illustrated in more detail by the following specific examples. However, these examples are for illustration and the scope of the present invention is not limited to the examples.

[87]

[88] **Measurement of psicose-epimerase and D-glucose isomerase activities**

[89] Activities of a psicose-epimerase and a D-glucose isomerase were measured by using D-fructose and D-glucose as a substrate, respectively. The enzymes or samples containing the same were added to a 50 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) buffer (pH 8.0) containing 100 g/L of the substrates, followed by the reaction at 50℃ for an hour and the reaction was then stopped by heating the reaction at 100℃ for 5 minutes. Concentrations of D-fructose, D-glucose and D-psicose were measured by HPLC equipped with a RI (Refractive Index) detector. HPLC analysis was performed by injecting a sample into Supelcogel Pb column set at 80℃ and then passing distilled water as a mobile phase through the column at a rate of 0.5 ml/min. D-psicose was separated at a retention time of around

32 minutes, and its % conversion and productivity were calculated based on the quantitative amount of a D-psicose reference standard. For comparative analysis of enzyme activities, one unit of an enzyme was defined as the amount of the enzyme to produce 1 mole of D-psicose per minute at pH 8.0 and 50℃.

[90]

[91] **Example 1: Recombinant strain expressing a psicose-epimerase**

[92] (1) Preparation of a recombinant strain

[93] The polymerase chain reaction (PCR) was carried out to amplify a gene encoding a psicose-epimerase by using a genomic DNA of *Agrobacterium tumefaciens* ATCC 33970 as a template and oligonucleotides of SEQ ID NOs: 9 and 10 with introduced PstI and XbaI restriction enzyme recognition sties, respectively, as primers. PCR conditions were as follows: after denaturation at 95℃ for 30 seconds, 26 cycles consisting of 30 seconds at 95℃, 30 seconds at 55℃ and 1 minute at 68℃, then extension at 68℃ for 10 minutes. For the mass expression of the psicose-epimerase encoded by the amplified gene, the amplified PCR product was digested with the restriction enzymes PstI and XbaI, and inserted into a shuttle vector pCJ-1 derived from *Corynebacterium* sp. bacteria (deposited with the Korean Culture Center of Microorganisms (KCCM), an international depository institution on Nov. 6 2004, under the deposit number KCCM-10611), to construct a recombinant expression vector pCJ-1-ATPE. Fig. 1 schematically shows the method of preparing the recombinant expression vector pCJ-1-ATPE including the gene encoding the psicose-epimerase derived from *Agrobacterium tumefaciens*.

[94] The recombinant expression vector pCJ-1-ATPE was introduced into *Corynebacterium glutamicum* ATCC 13032 by transformation using electroperforation, to prepare the recombinant strain expressing the gene encoding the psicose-epimerase derived from *Agrobacterium tumefaciens*. The recombinant strain was designated *Corynebacterium glutamicum* ATPE and deposited with the Korean Culture Center of Microorganisms (KCCM, Hongje-1-dong, Seodaemum-gu, Seoul, Korea) under the deposit number KCCM 11046 on Oct. 26, 2009 under the Budapest Treaty.

[95]

[96] (2) Expression of a psicose-epimerase

[97] MB media (Bacto-trypton 10 g/L, Bacto-yeast extract 5 g/L, NaCl 5 g/L, Soytone 5 g/L) including10 ㎍/ml of kanamycin were inoculated with the recombinant strains obtained in the above (1), respectively, at initial concentration of OD600 = 0.1, followed by incubation at 30℃ for 24 hours to induce the expression of the recombination psicose-epimerase. 5 L-jar fermenter containing 3 L of a modification medium (D-glucose 80 g/L, soytone 20 g/L, $(NH_4)_2SO_4$ 10 g/L, KH_2PO_4 1.2 g/L, $MgSO_4$ 1.4 g/L) including 10 ㎍/ml of kanamycin was inoculated with the above obtained culture at

OD600 = 0.6, followed by incubation at 30℃ for 20 hours. In order to measure the activity of the expressed psicose-epimerase, the culture was subject to centrifugation at 8000x g for 10 minutes to harvest cells. The harvested cells were then resuspended in a 50 mM EPPS buffer (pH 8.0) (Sigma-Aldrich) and the resulting suspension was subject to ultrasonication resulting in lysis of the cells. The cell lysate was subject to centrifugation to obtain a supernatant containing the psicose-epimerase, and epimerization reaction of D-fructose was carried out by using the supernatant as an enzyme. Measuring the activity of the psicose-epimerase as described above was used in the epimerization reaction. Fig. 2b shows the HPLC analysis results of epimerization reaction.

[98]

[99] **Example 2: Immobilization of a recombinant strain and preparation of D-psicose**

[100] (1) Immobilization of a recombinant strain

[101] For the mass production of D-psicose, *Corynebacterium glutamicum* ATPE (KCCM 11046) expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* and prepared in the above Example 1 was immobilized onto the carrier in this example. For the immobilization of the recombinant strain, the modification medium for *Corynebacterium* used in the Example 1 was first inoculated with the recombinant strain at an initial concentration of OD600 = 0.6 followed by the incubation at 30℃ for 20 hours. After the completion of the incubation, the resulting culture was subject to the centrifugation to recover the cells, which were resuspended in the 50 mM EPPS buffer (pH 8.0) to be 20%. The resuspended cells of the recombinant strain were added to 2% (v/v) of an aqueous sodium alginate solution. The resulting mixture was dropped to a 100 mM $CaCl_2$ solution by using the syringe pump and the vacuum pump to create a cell-alginate complex in which the cells were trapped in sodium alginate beads.

[102]

[103] (2) Preparation of D-psicose by using the immobilized recombinant strain

[104] XK16 packed-bed column (16 mm X 20 mm, Amersham pharmacia) was packed with the recombinant strain immobilized onto sodium alginate in the above (1) followed by measurement of % conversion pack of D-fructose to D-psicose. In order to determine an optimal reaction temperature of an immobilization reactor and a substrate concentration of D-fructose, the conversion reaction of D-fructose to D-psicose was carried out under combined conditions of 40℃, 45℃ and 50℃ of reaction temperatures, and 100 g/L, 300 g/L and 500 g/L of D-fructose concentrations. According to Figs. 3a to 3c, It was found that the best conversion is achieved at a temperature of 50℃ and 100 g/L of D-fructose concentration, and 300 g/L and 500 g/L of D-fructose concentration also give high conversion. Further, different from other D-glucose

conversion reactions, the epimerization reaction for D-psicose showed low dependency on temperature.

[105]

[106] (2-1) Productivity depending on an inflow rate of D-fructose

[107] The XK16 packed-bed column was packed with the immobilized recombinant strain as described above followed by the measurement of the productivity of D-psicose depending on an inflow rate of D-fructose. At this time, based on the results of the above (2), the concentration of D-fructose and the reaction temperature were set at 300 g/L and 50℃, respectively. The productivity was measured by adjusting the inflow rate of D-fructose, a substrate to be 0.4, 1, 2, 4, and 8 h^{-1} of space velocity (1/h). The results are shown below in Table 1 and Fig. 4.

[108]

[109] Table 1

[Table 1]

[Table]

Productivity and % conversion depending on an inflow rate of D-fructose

Inflow rate (ml/min)	D-fructose (g/l)	D-psicose (g/l)	Productivity (g/l/h)	Conversion (%)
3	281.388	3.693	33	1.30
2	289.215	3.309	20	1.13
1	291.268	7.579	23	2.54
0.5	284.639	12.055	18	4.06
0.25	286.463	20.579	15	6.70
0.1	260.096	38.041	11	12.76

[110]

[111] (2-2) Operational stability

[112] Operation was carried out at 50℃ by using the immobilized recombinant strains which were packed into the XK16 packed-bed column until specific activities thereof become 50%, respectively, in order to measure operational stability of the immobilized recombinant strains which was packed into the column. At this time, the substrate concentration of D-fructose was 300 g/L and the inflow rate thereof was 0.1 min/ml (space velocity 0.4 h^{-1}). It was found that 25 days or longer of operational stability could be maintained under these reaction conditions.

[113] Fig. 5 shows the operational stability for the immobilized recombinant strain(s) at a reaction temperature of 50℃.

[114]

[115] **Example 3: Preparation of D-psicose from D-glucose by using the immobilized recombinant strain/enzyme**

[116] (1) Productivity depending on a reaction temperature and a D-glucose concentration

[117] The XK16 packed-bed columns (16 mm X 20 mm, Amersham pharmacia) were packed with the recombinant strain immobilized onto sodium alginate in the above Example 2 and 10 g of the immobilized D-glucose isomerase (Novozymes, Denmark), respectively, followed by the measurement of % conversion of D-glucose to D-psicose. In order to determine the optimal reaction condition for the two mixed enzymes, the conversion reaction of D-glucose to D-psicose was conducted under combined conditions of 40℃, 45℃, or 50℃ of a reaction temperature, and 100 g/L, 300 g/L, or 500 g/L of D-glucose concentration. Fig. 6 shows % conversion of D-glucose to D-psicose depending on changes in the reaction temperature and the substrate concentration. According to Figs. 6a to 6c, different from the epimerization reaction for D-psicose, the isomerization reaction for D-glucose was a temperature-dependent reaction. That is, the higher the reaction temperature the faster the conversion rate of D-glucose to D-psicose. It was found that this had effect on the productivity of D-psicose finally. The best conversion of D-glucose to D-fructose was achieved at a temperature of 50℃, and there was no significant difference in the reaction rate as the substrate concentration increased higher. These results demonstrate that it is possible to operate the immobilization reactor under the condition of a higher concentration of a substrate and to increase the productivity of D-psicose from D-glucose.

[118]

[119] (2) Productivity depending on an inflow rate of D-glucose

[120] The two packed-bed columns were packed with the immobilized recombinant strain including a psicose-epimerase and immobilized D-glucose isomerase, respectively, and the productivity of D-psicose was measured depending on an inflow rate of D-glucose. Based on the results of the above examples, the concentration of D-glucose and the reaction temperature were set at 300 g/L and 50℃, respectively. The productivity was measured by adjusting the inflow rate of D-fructose, a substrate to be 0.4, 1, 2, 4, and 8 h^{-1} of space velocity (1/h). The results are shown below in Table 2 and Fig. 7.

[121]

[122] Table 2

[Table 2]

[Table]

Productivity and % conversion depending on an inflow rate of D-glucose

Inflow rat (ml/min)	D-glucose (g/l)	D-fructose (g/l)	Productivity (g/l/hr)	Conversion (%)
4	281.465	31.136	374	9.96
3	278.849	40.042	360	12.56
2	267.061	50.196	301	15.82
1	238.615	78.854	237	24.84
0.5	207.168	113.081	170	35.31
0.1	172.826	155.850	47	47.42

Claims

[1] A method of preparing D-psicose from D-fructose by using a psicose-epimerase derived from *Agrobacterium tumefaciens*, and expressed in a GRAS (generally accepted as safe) strain comprising steps of:
expressing the psicose-epimerase derived from *Agrobacterium tumefaciens* in the GRAS strain; and
preparing D-psicose from D-fructose by using the expressed psicose-epimerase.

[2] The method as claimed in claim 1, wherein the psicose-epimerase used in the preparation of D-psicose is contained in a culture of the GRAS strain or separated from the culture.

[3] The method as claimed in claim 1, wherein the GRAS strain is *Corynebacterium* sp.

[4] The method as claimed in claim 3, wherein the GRAS strain is *Corynebacterium glutamicum* KCCM 11046.

[5] The method as claimed in one of claims 1 to 4, wherein the psicose-epimerase derived from *Agrobacterium tumefaciens* has the amino acid sequence of SEQ ID NO: 1.

[6] The method as claimed in one of claims 1 to 4, wherein the step of preparing D-psicose comprises the step of immobilizing the psicose-epimerase onto a carrier.

[7] The method as claimed in claim 6, wherein the carrier is sodium alginate.

[8] The method as claimed in claim 6, wherein the method further comprises the steps of packing a column with the carrier onto which the psicose-epimerase is immobilized and supplying the packed column with a D-fructose solution.

[9] A method of preparing D-psicose from D-glucose, comprising the step of immobilizing a psicose-epimerase and a D-glucose isomerase onto carriers.

[10] The method as claimed in claim 9, wherein the psicose-epimerase is contained in a culture of the GRAS strain expressing a psicose-epimerase derived from *Agrobacterium tumefaciens* or separated from the culture.

[11] The method as claimed in claim 10, wherein the GRAS strain is a strain transformed with a recombinant vector including a gene encoding a psicose-epimerase.

[12] The method as claimed in claim 10, wherein the GRAS strain is *Corynebacterium* sp.

[13] The method as claimed in claim 12, wherein the GRAS strain is *Corynebacterium glutamicum* KCCM 11046.

[14] The method as claimed in one of claims 10 to 13, wherein the psicose-epimerase derived from *Agrobacterium tumefaciens* has the amino acid sequence of SEQ

ID NO: 1.

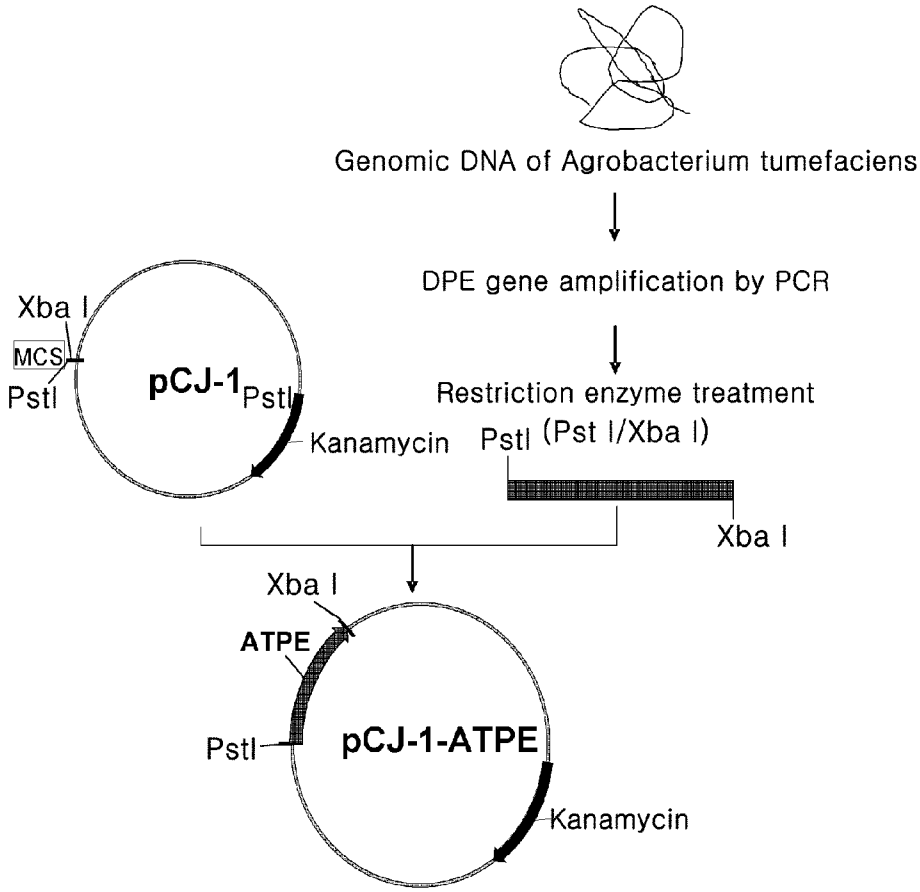
[15] The method as claimed in claim 9, wherein the carrier is sodium alginate.

[16] The method as claimed in one of claims 9 to 13, wherein the method further comprises the steps of packing a column with the carriers onto which the psicose-epimerase and the D-glucose isomerase are immobilized and supplying the carrier-packed column with a D-fructose solution.

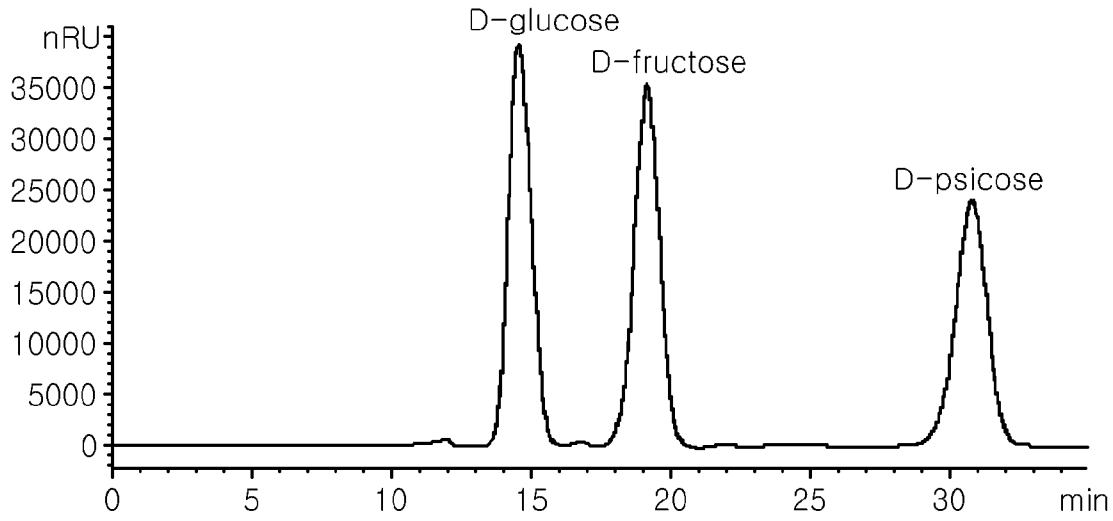
[17] The method as claimed in claim 16, wherein each of the carriers onto which the psicose-epimerase and the D-glucose isomerase are immobilized, respectively, is packed into a separate column, and the two columns are connected in communication with each other.

[18] *Corynebacterium glutamicum* KCCM 11046 strain useful for the preparation of D-psicose from D-fructose or D-glucose.

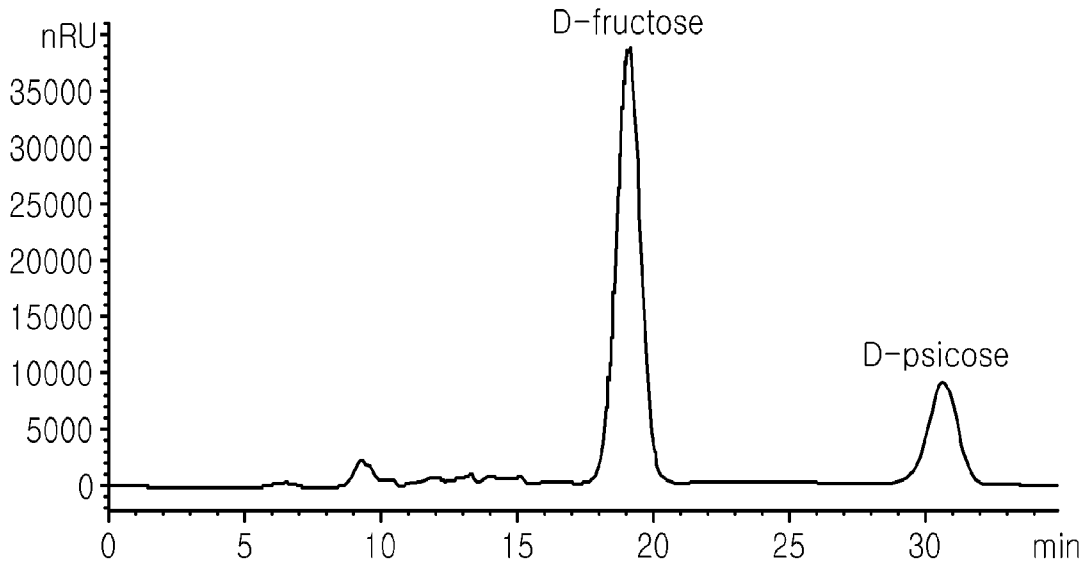
[Fig. 1]


Genomic DNA of Agrobacterium tumefaciens
DPE gene amplification by PCR
Restriction enzyme treatment
Xba I
MCS
PstI
pCJ-1
PstI
Kanamycin
PstI (Pst I/Xba I)
Xba I
Xba I
ATPE
PstI
pCJ-1-ATPE
Kanamycin

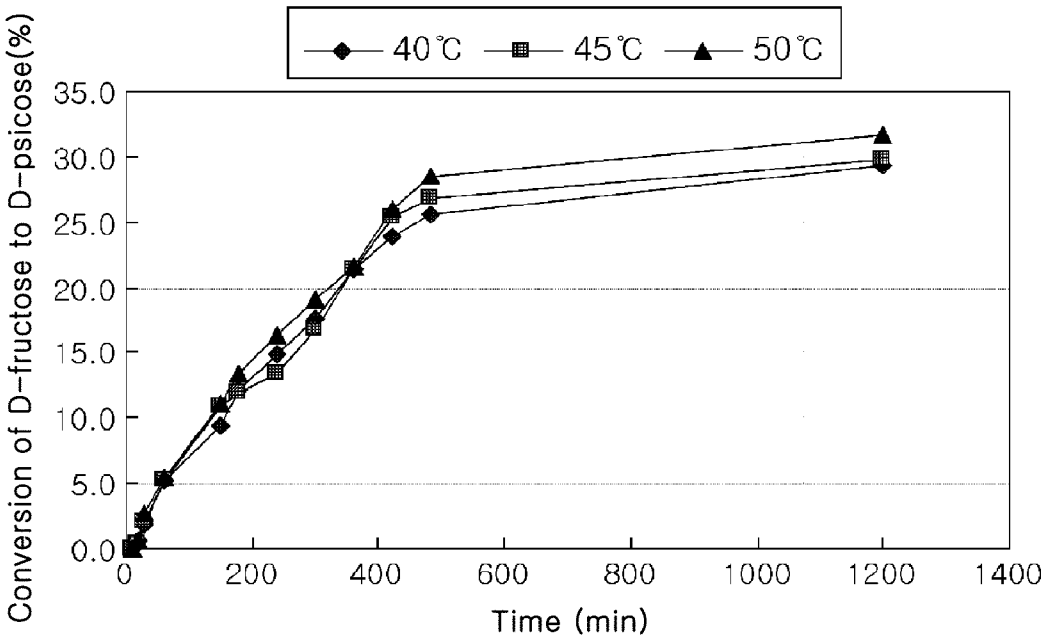
[Fig. 2a]


nRU
35000
30000
25000
20000
15000
10000
5000
0
D-glucose
D-fructose
D-psicose
0
5
10
15
20
25
30
min

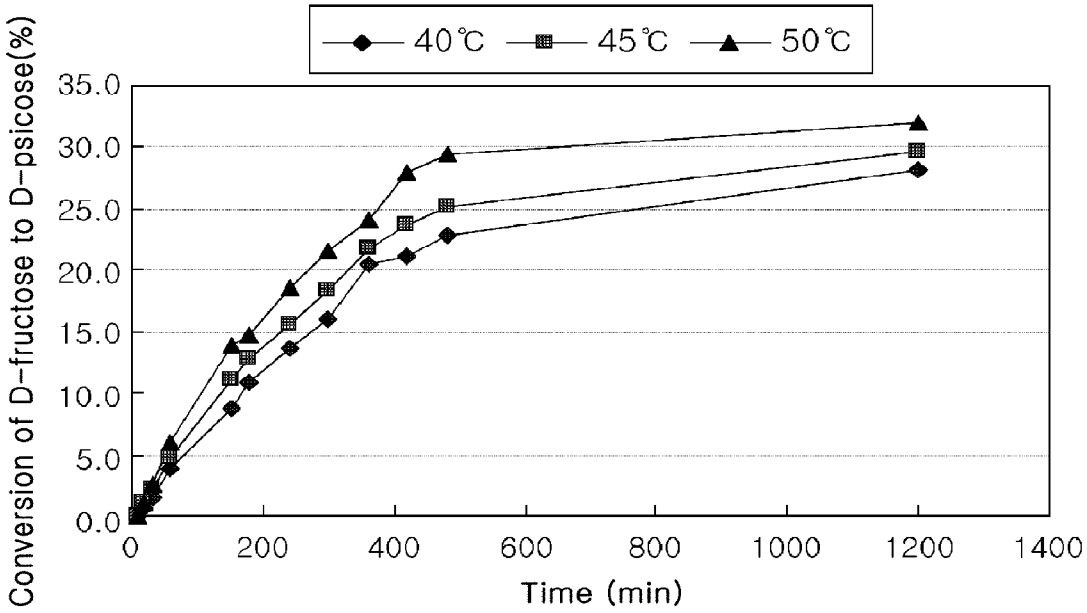
[Fig. 2b]


nRU
35000
30000
25000
20000
15000
10000
5000
0
D-fructose
D-psicose
0
5
10
15
20
25
30
min

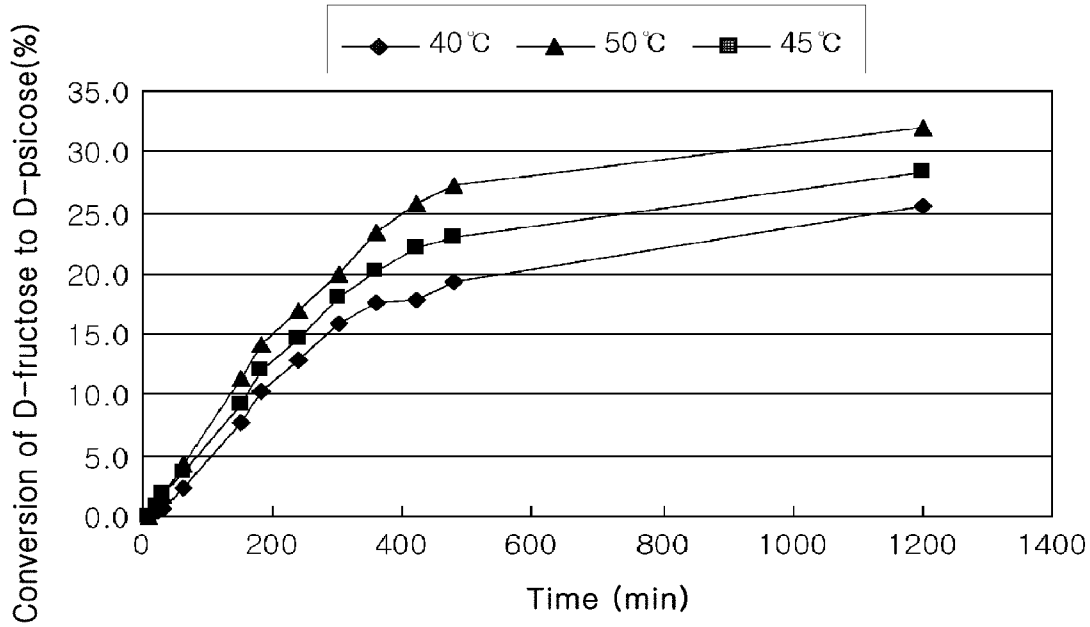
[Fig. 3a]


40℃
45℃
50℃
Conversion of D-fructose to D-psicose(%)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
200
400
600
800
1000
1200
1400
Time (min)

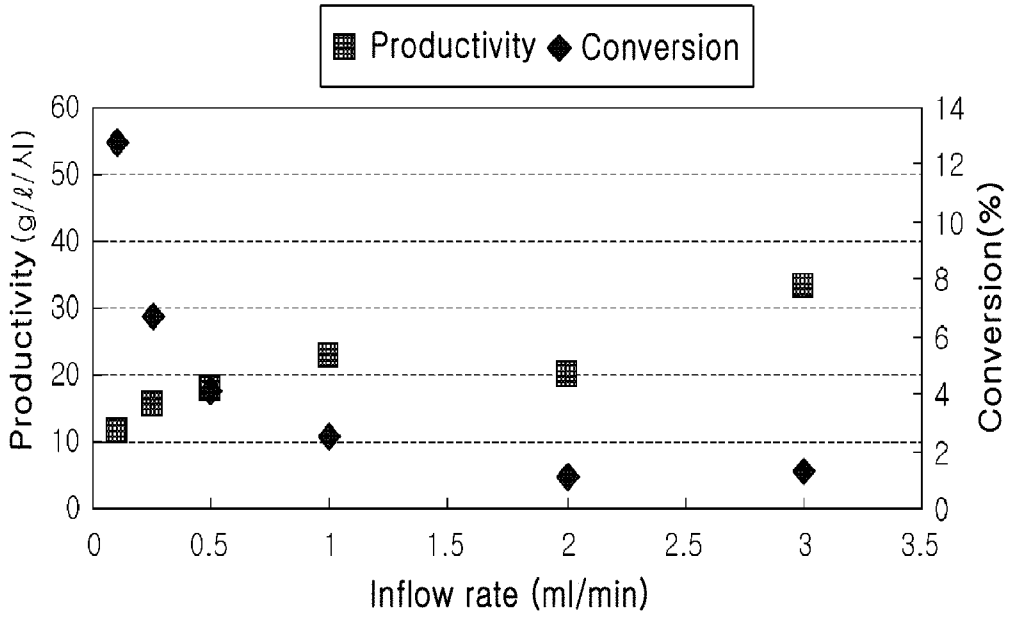
[Fig. 3b]


40℃
45℃
50℃
Conversion of D-fructose to D-psicose(%)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
200
400
600
800
1000
1200
1400
Time (min)

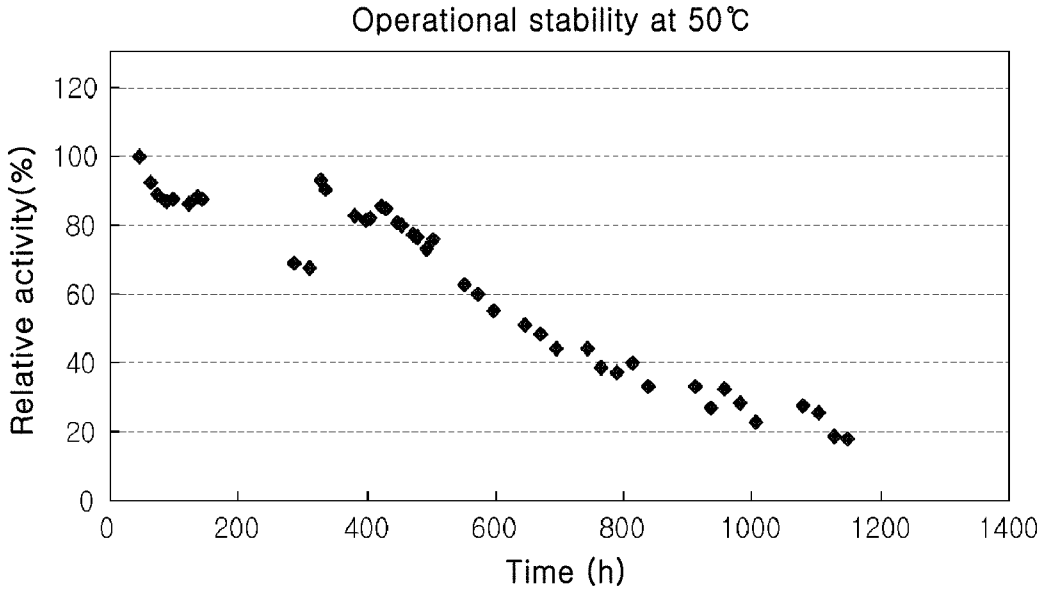
[Fig. 3c]


40℃
50℃
45℃
Conversion of D-fructose to D-psicose(%)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
200
400
600
800
1000
1200
1400
Time (min)

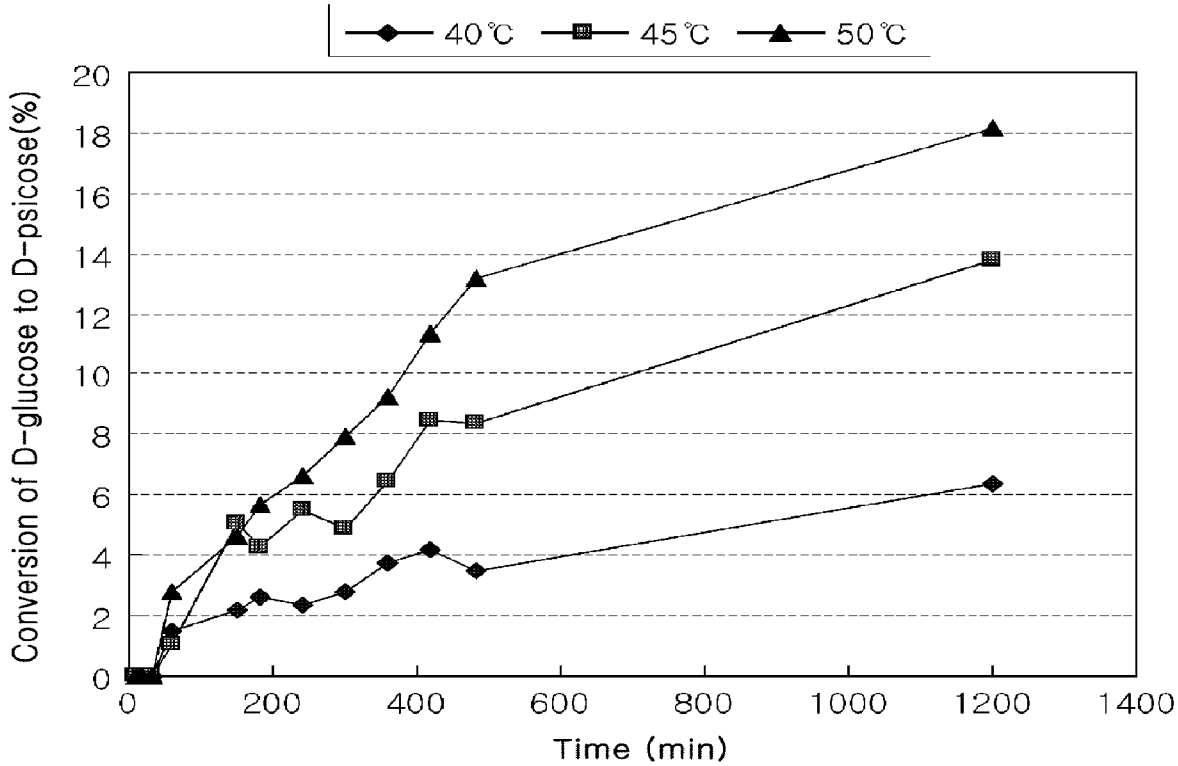
[Fig. 4]


Productivity
Conversion
Productivity (g/ℓ/시)
Conversion(%)
Inflow rate (ml/min)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
0
0.5
1
1.5
2
2.5
3
3.5

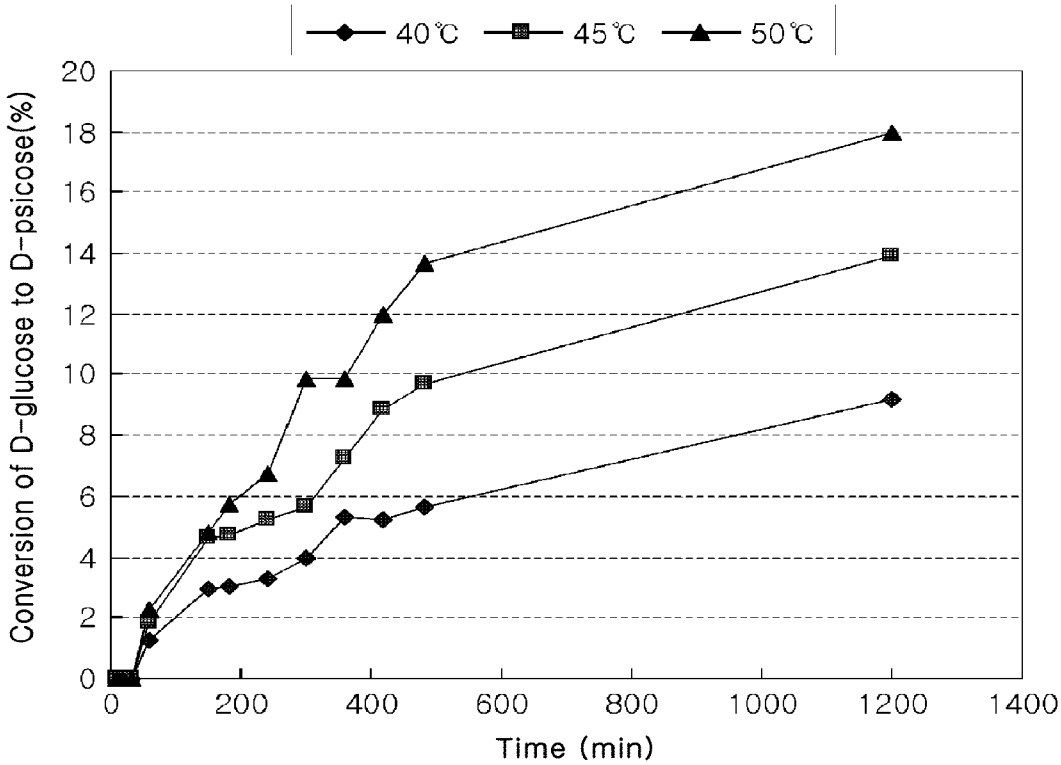
[Fig. 5]


Operational stability at 50℃
Relative activity(%)
Time (h)
0
20
40
60
80
100
120
0
200
400
600
800
1000
1200
1400

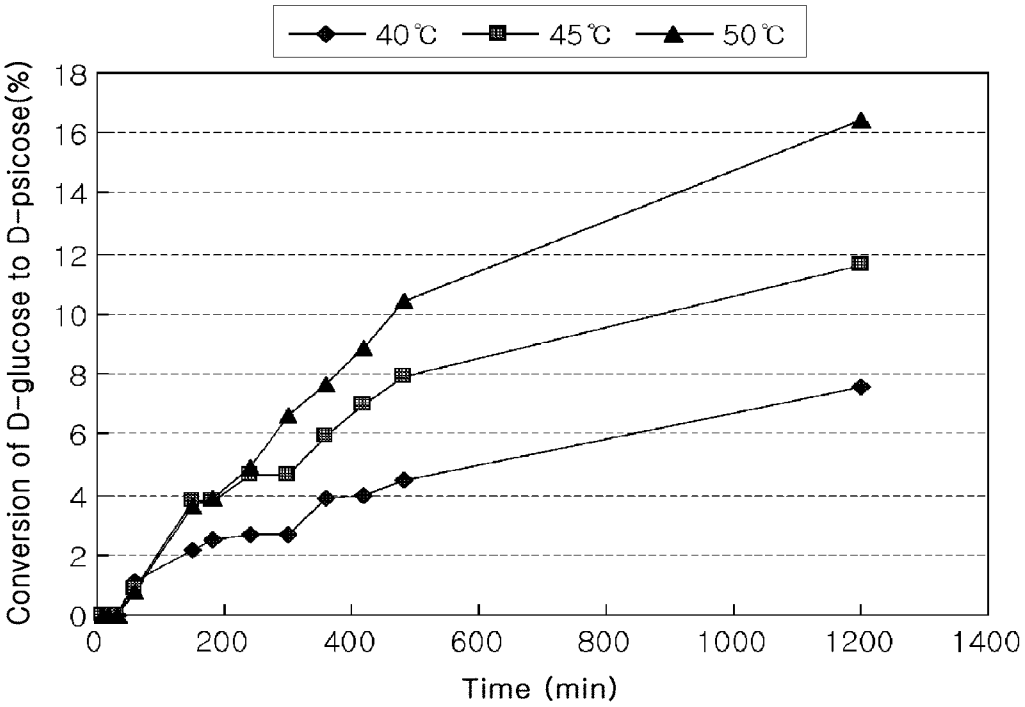
[Fig. 6a]


40℃
45℃
50℃
Conversion of D-glucose to D-psicose(%)
0
2
4
6
8
10
12
14
16
18
20
0
200
400
600
800
1000
1200
1400
Time (min)

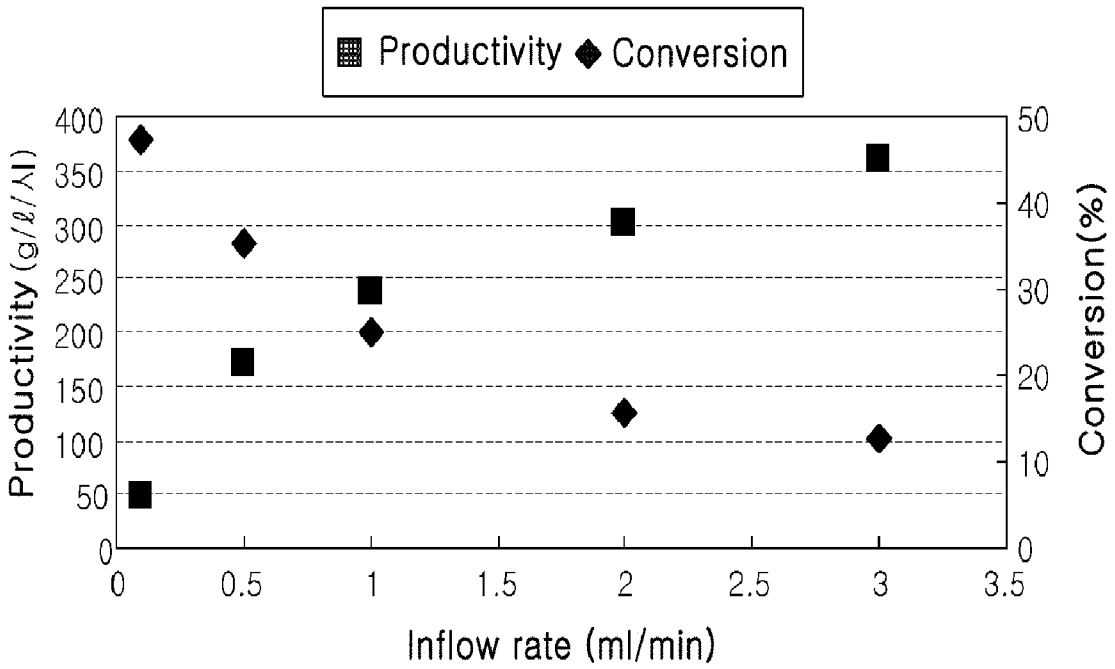
[Fig. 6b]


40℃
45℃
50℃
Conversion of D-glucose to D-psicose(%)
0
2
4
6
8
10
12
14
16
18
20
0
200
400
600
800
1000
1200
1400
Time (min)

[Fig. 6c]


40℃
45℃
50℃
Conversion of D-glucose to D-psicose(%)
0
2
4
6
8
10
12
14
16
18
0
200
400
600
800
1000
1200
1400
Time (min)

[Fig. 7]


Productivity
Conversion
Productivity (g/ℓ/시)
0
50
100
150
200
250
300
350
400
Conversion(%)
0
10
20
30
40
50
0
0.5
1
1.5
2
2.5
3
3.5
Inflow rate (ml/min)