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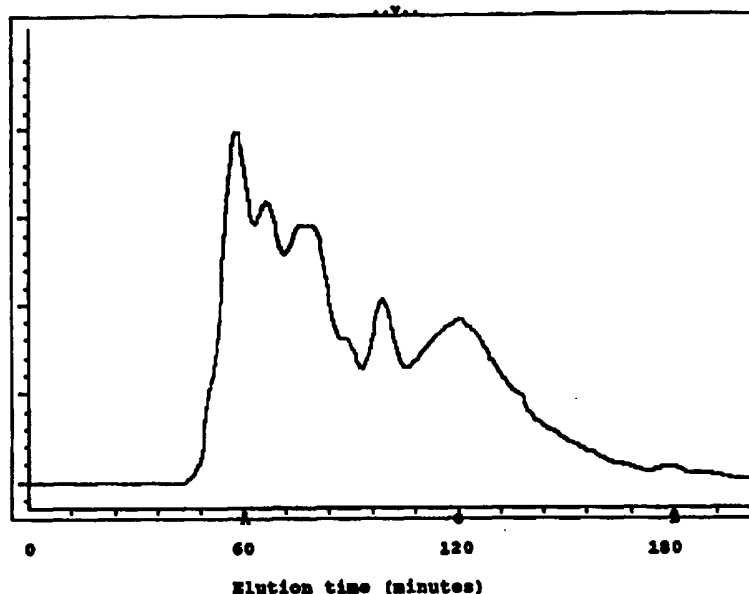
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(54) Title: A METHOD FOR INCREASING MONOSACCHARIDE LEVELS IN THE SACCHARIFICATION OF STARCH AND ENZYMES USEFUL THEREFOR**(57) Abstract**

During the commercial production of dextrose from starch, the disaccharide maltulose is produced which cannot be hydrolysed by the enzymes amyloglucosidase, pullulanase or α -amylase. The present invention discloses an enzyme preparation for the hydrolysis of maltulose. Enzymatic hydrolysis of maltulose provides a new and additional method for improvement of the monosaccharide levels in the saccharification of starch.

Chromatogram of gel filtration of α -galactosidase on Sephacryl S 200 HR. OD (280 nm) versus elution time.



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A METHOD FOR INCREASING MONOSACCHARIDE LEVELS IN THE SACCHARIFICATION OF STARCH AND ENZYMES USEFUL THEREFOR

Field of the Invention

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The present invention relates to the enzymatic degradation of starch. More specifically, the invention provides a method yielding increased monosaccharide levels from liquefied starch.

Background of the Invention

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Native starch is known to contain two types of macromolecules composed of glucose units. One type of starch, amylose, is linear and consists of glucose units exclusively linked with α -1,4 bonds. The second type, amylopectin, is highly branched and contains α -1,6 bonds in addition to α -1,4 bonds. The overall content

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of α -1,6 bonds is generally less than 5%.

Sugars prepared from starch in the form of concentrated dextrose (glucose) syrups are currently produced in a two stage enzyme catalysed process, involving: (1) a liquefaction step (or thinning) involving hydrolysis of starch with α -amylase into dextrins having an average degree of polymerization of about 7-10, and (2)

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saccharification of the resulting liquefied starch (dextrins) with amyloglucosidase, which results in a syrup having a high glucose content (92-96% by weight of total solids). Much of the dextrose syrup produced commercially is enzymatically isomerized to a dextrose/fructose mixture, known as iso-syrup.

The two enzymes currently in use, α -amylase and amyloglucosidase, differ in

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two important aspects. First, α -amylase is an endo-enzyme which attacks the internal linkages of starch molecules at random. Amyloglucosidase, on the other hand, is an exo-enzyme which splits glucose units from the non-reducing ends of dextrin molecules. Secondly, α -amylase almost exclusively attacks α -1,4 bonds whereas amyloglucosidase splits α -1, 6 bonds as well, though at a considerably

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lower rate than α -1,4 bonds.

The recommended name of amyloglucosidase is exo-1,4- α -D-glucosidase (EC3.2.1.3) the systematic name is α -1,4 glucan glucohydrolase. Amyloglucosidase is also called AG or glucoamylase and it should be understood that these terms, as used hereinafter, are synonymous.

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The saccharification stage of commercial dextrose production has long been recognized to be sub-optimal in many respects. In particular, the amyloglucosidases currently available catalyse glucose production reactions as well as reversion

reactions, e.g. conversion of dextrose into isomaltose at a rate dependent on the glucose concentration. The formation of by-products in this way has limited the saccharification of starch hydrolysates into glucose to not more than a DX value of about 95% at 33% d.s. (i.e. in syrups containing at least 33% dry solids by weight).

- 5 The term DX value is defined as the percentage by weight of dextrose on a carbohydrate dry solids basis.

In an effort to increase the DX value it has been proposed to use a debranching enzyme in conjunction with amyloglucosidase to more efficiently hydrolyze the branched oligosaccharides (containing α -1, 6 bonds) present in the
10 liquefied starch. European Patent Application EP-A1-0 063 909, describes a debranching enzyme of the pullulanase type which is produced by a *Bacillus* called *Bacillus acidopullulyticus*. This method appears to result in a 0.4-0.6 DX increase.

In another effort to further increase the DX value it has been proposed to use an amyloglucosidase with an increased acid amylase level (side activity present in
15 AG reparations). This has been described in European Patent EP B1-0 140 410. The method results in a DX increase of 0.5 at 33% d.s.

High temperatures are generally used during the starch liquefaction process. These high temperatures, in combination with the applied pH, stimulate isomerization of the glucose unit present at the reducing end of dextrin molecules into fructose.
20 Hydrolysis of dextrans, including the isomerized glucose unit (fructose) at the reducing end, results in free glucose and a disaccharide, called maltulose (glucose- α -1,4-fructose). The disaccharide maltulose is not hydrolysed by the enzymes AG, pullulanase and acid amylase and, as a consequence, will remain in the dextrose syrup produced. Depending on the conditions used during liquefaction, the
25 concentration of maltulose can be as high as 2% of the total sugar. Hydrolysis of the saccharide maltulose would provide a new and additional method for improvement of the DX value in the saccharification of starch for the production of dextrose syrups and iso-syrups. Because maltulose is not utilized by microorganisms in fermentation processing, conversion of maltulose into a fermentable sugar, such as glucose and
30 fructose, would increase the fermentable yield of starch saccharification processes and facilitate higher yields of primary metabolites such as ethanol.

Summary of the Invention

It is an object of the invention to provide for an enzyme useful in a method of hydrolyzing unfermentable sugars, particularly maltulose, to produce fermentable sugars such as glucose and fructose.

5 It is a further object of the invention to provide for a cost-effective method of increasing the yield of dextrose in the saccharification process of starch.

It is yet a further object of the invention to provide for a cost-effective method of increasing the yield of the primary, metabolite of a fermentation process, for example ethanol.

10 The present invention provides for an enzyme preparation comprising an enzyme capable of the hydrolysis of maltulose. In a preferred embodiment, the enzyme preparation comprises a purified or enriched maltulase enzyme. In a more preferred embodiment the maltulase enzyme is derived from a fungal source, most preferably from *Aspergillus* or *Trichoderma*. In a specific composition embodiment,
15 the maltulase enzyme is derived from *Aspergillus niger* and has a molecular weight of about 132 kD as measured by gel filtration.

In a method embodiment of the invention, a method for the enzymatic hydrolysis of maltulose is provided. The invention also provides method for the saccharification of sugar syrups to produce dextrose syrups with the enzyme
20 preparation of the invention so as to increase the DX value of the produced sugar syrup. The invention further discloses dextrin and dextrose syrups, including iso-syrups, which are produced from starch and which are free of maltulose.

Brief description of the figures

25 Figure 1. Chromatogram of gel filtration of α -galactosidase on Sephacryl 5200 HR OD (at OD 280) vs. elution time.

Description of the invention

The present invention is based on the surprising discovery that maltulose can
30 be hydrolysed into glucose and fructose by an enzyme present in broths obtained through microbial fermentation. Suitable enzymes for the hydrolysis of maltulose may be derived from, for example, commercial enzyme preparations, and preferably fungal cellulase and α -galactosidase preparations. The enzyme with maltulose hydrolysing activity, i.e., having the ability to hydrolyze maltulose to, for example,
35 glucose and fructose, is referred to as maltulase hereinafter. The maltulase can be

purified from fermentation broths or enzyme preparations using standard protein purification methods available to the person skilled in the art (see e.g. R.K. Scopes 1987, "Protein Purification, Principles and Practice" Springer Verlag, New York, 2nd edn).

5 Although maltulase activity was unexpectedly identified, as illustrated herein, in commercial fungal cellulase and α -galactosidase preparations obtained from *Trichoderma* or *Aspergillus* species, respectively, it is very probable that maltulases can be obtained from other fungi or even other classes of microorganisms and that therefore microbial maltulases in general fall within the scope of the present
10 invention. Given the techniques available through the instant disclosure for the identification of maltulose hydrolyzing activity, it will be possible for one of skill in the art to identify and purify maltulase enzyme from any number of commercially available enzymes preparations or fermentation broths produced from incubations of many microorganisms.

15 Preferably, the maltulase is purified to homogeneity, which allows determination of the biochemical, physical and kinetic parameters of the enzyme, such as specific activity, molecular weight, or the partial or full amino acid sequence(s) of the polypeptide(s) with maltulase activity. The obtained partial or full amino acid sequences are used to design oligonucleotide probes which allow the
20 molecular cloning of the gene(s) encoding the maltulase (see e.g. Sambrook et al. 1989, "Molecular Cloning: a laboratory manual" Cold Spring Harbour Laboratories, Cold Spring Harbour, New York). Alternatively, the purified maltulase is used to raise antibodies which allow the cloning of the gene through expression cloning. The cloned maltulase gene(s) is used to construct over-expressing strains of
25 industrial microorganisms such as e.g. *Aspergillus*, *Trichoderma*, *Bacillus*, *Streptomyces*, *Saccharomyces*, or *Kluyveromyces* (see e.g. Bennett and Lasure eds., 1991 "More Gene Manipulations in Fungi" Academic Press Inc., New York). These overproducing strains provide a cost-effective and virtually unlimited source of pure maltulase. Whether through purification or overproducing strains, the obtained
30 maltulase preparation may be used to enrich an existing enzyme preparation or can be used in a substantially purified form by itself on a sugar solution which includes maltulose. A substantially purified maltulase will preferably have insignificant additional enzyme activity in terms of saccharification.

 An "enriched" maltulase preparation according to the present invention is a
35 preparation which is derived from a fermentation broth produced by the fermentation

of a naturally occurring microorganism which produces maltulase and which preparation includes a higher concentration of maltulase than would be found naturally from fermentation of the microorganism. Alternatively, an enriched maltulase preparation can be prepared by purifying the maltulase enzyme from the fermentation broth of a natural or genetically engineered microorganism so as "enrich" the maltulase relative to the removed contaminants. Similarly, the purified maltulase enzyme can be added to a naturally occurring enzyme mixture containing, for example, pullulanase, glucoamylase or acid amylase, in a concentration greater than exists in the naturally occurring fermentation of the organism(s) from which the enzyme mixture is desired. Additionally, an enriched maltulase preparation may be derived from the fermentation of a genetically modified microorganism which has been subject to recombinant techniques so as to amplify expression of maltulase in a fermentation broth.

As stated above, it is believed that maltulase enzymes are found in many different enzyme preparations in small quantities. However, the quantities of maltulase which are produced by microorganisms cultured under conditions to produce commercially available enzyme preparations (i.e., commercially available α -galactosidase) are generally insufficient to achieve practical improvement in starch processing or are in enzyme preparations which are not generally applied to starch substrate at the saccharification stage (i.e., commercially available cellulase). In an especially preferred embodiment of the invention, the maltulase content of an enzyme preparation in which it is employed is enriched to increase the maltulose hydrolyzing activity thereof to a commercially significant level. The added maltulase enzyme should be sufficient to substantially hydrolyze the maltulose in solution. Of course, the amount of added maltulase enzyme according to the invention will depend on the amount of maltulose in the starch or sugar solution. For example, in a 40% dry solids sugar solution containing 2% maltulose, maltulose will be present in a quantity of approximately 8 g/kg of sugar. Thus, where 1 unit equals the hydrolysis of 1 μ mole of maltulose/minute, 6 U/kg of syrup will be needed to hydrolyze the maltulose in solution in 72 hours. Alternatively, 432 U/kg would be necessary to hydrolyze the maltulose in solution in 1 hour. Thus, preferably, the maltulose hydrolyzing activity of the enzyme composition of the invention is greater than about 1 U/kg sugar d.s., more preferably between 50 and 5000 U/kg sugar d.s., and most preferably between 50 and 1000 U/kg sugar d.s.

The use of maltulase for the hydrolysis of maltulose into the monosaccharides glucose and fructose will increase the DX value in the production of dextrose syrups, which syrups can subsequently be used as such or for the production of iso-syrups. The enzymatic hydrolysis of maltulose will result in
5 dextrose syrups or iso-syrups with reduced maltulose levels, preferably less than 0.5 % maltulose (percentage maltulose of total sugar), or most preferably below the detection level of about 0.1%, (i.e. substantially free of maltulose). The hydrolysis of maltulose should preferably take place between a pH of about 3 and 7, and more preferably between a pH of about 4 and 5; at temperatures preferably between
10 about 15°C and 70°C, and more preferably between about 20°C and 60°C.

The method for hydrolyzing maltulose provided by the present invention is preferably performed at the end of the conventional saccharification process. However, because maltulose cannot be hydrolysed by the enzymes AG, pullulanase and acid amylase, a further embodiment the method of the present invention
15 provides for the use of maltulose in combination to the methods that are part of the state of the art. Thus, the use of pullulanase, glucoamylase or acid amylase in combination with maltulase will effect a further increase in the DX value in saccharification processes. The combined use of maltulase, pullulanase and acid amylase allows for the preparation of dextrose syrups with higher DX values than
20 with the combined use of only pullulanase and acid amylase. In fact, the method of the invention can easily improve the DX level of a dextrose syrup by up to 2 units.

In a preferred embodiment, the maltulase enzyme is utilized to hydrolyze maltulose in a liquefied starch solution. Thus, for example, the maltulase enzyme can be added to the liquefied starch produced by jet liquefaction of starch with α -
25 amylase. Alternatively, the maltulase enzyme can be added simultaneously with glucoamylase in the saccharification step. In yet another variation, the maltulase enzyme can be added after the liquefied starch has been treated with glucoamylase to further increase the DX value of the saccharified starch. Finally, isomerized fructose/glucose syrups may be treated with the maltulase enzyme to further
30 increase the concentration of glucose and fructose and reduce maltulose contaminant content. Each of these variations will benefit from the enzyme of the present invention through the increased production of fermentable sugars. However, the choice of which variation to use in a given process will depend on the specific parameters under which the process at hand is operated. While those of skill in the
35 art would be able to easily ascertain which variation is optimal with a given starch

processing method, it is preferred that the maltulose hydrolyzing step occur during or after saccharification or during fermentation as these steps contain the highest concentration of maltulose.

Maltulose hydrolysis will result in a higher yield in fermentation processes in which dextrose syrups are used as a carbon and/or energy source. An example of this is the fermentative production of ethanol from dextrose syrups. Because of the presence of maltulose in glucose syrups obtained through the hydrolysis of starch into glucose, the yield of ethanol based on glucose syrup consumed will increase upon hydrolysis of maltulose. Similar increases in yields of biomass, (primary or secondary) metabolites, drugs (penicillin) or enzymes will also occur in other fermentation processes where maltulose can be hydrolysed into a fermentable sugar. Thus, in a variation of the invention, a dextrose or iso-syrup is used as an energy source or source for primary metabolites in the fermentation of microorganisms.

The following examples are only illustrative of the invention and should not be interpreted as limiting thereof.

EXAMPLES

Example 1

Analysis of commercial dextrose and iso-syrups

Dextrose and glucose/fructose iso-syrups produced from starch contain maltulose. The analysis was performed by subjecting different syrups which were produced by saccharification from commercially available dextrin mixtures. The saccharification was carried out at 60°C and pH 4.2 using amyloglucosidase (Amigase from Gist-brocades) for 48 hours. Samples from the resulting glucose syrups were analyzed for maltulose content by means of HPLC as described below. The results of these analyses are shown in Tables 1 and 2. As shown in Tables 1 and 2, common saccharification procedures applied to commercial dextrin mixtures results in syrups containing maltulose.

Saccharide concentrations can be detected by high performance liquid chromatography. A suitable method utilizes the following conditions:

Column: Bio-Rad HPX 87C.

Eluent: distilled water.

Column temperature: 85°C.

Flow rate: 0.6 ml/min.

Detection: RI (refractive index) detection.

Table 1

Syrup sample	Fructose %	Glucose %	Maltose %	Maltulose %	DP3 %	DP4+ %
Saccharified Maldex 15 from Amylum	0.0	92.51	2.34	0.62	0.58	3.92
Saccharified MD 03 from Roquette	0.0	91.42	2.06	1.75	0.73	4.03
Dormamix 98/70 (Pfeiffer & Langen)	0.1	93.7	2.1	0.5	2.1	1.4
Levudex 42 (IMASA)	41.9	52.5	3.3	0.3	0.7	1.3
Levudex 55 (IMASA)	53.5	41.8	2.7	0.3	0.5	1.1
Dextrose syrup (Barendse)	0.0	87.0	6.7	1.2	2.8	2.3
Dextrose syrup (Cerestar)	0.0	94.3	3.8	0.5	0.8	0.7
Gelastin T58 after saccharification (Cerestar)	0.0	89.4	5.9	0.8	2.1	1.8
Dextrose syrup (Amylum)	0.0	96.6	2.2	0.2	0.4	0.6
Dextrose syrup (Cargill)	0.0	94.7	2.3	1.0	0.6	1.3

Example 2**Maltulase Detection Analysis**

5

(A) Preparation of maltulose

Maltulose is the disaccharide α -D-glucopyranosyl-1,4- α -fructofuranose. This disaccharide can be prepared by alkaline isomerization of the glucose residue at the reducing end of the disaccharide maltose (α -D-glucopyranosyl 1,4- α -glucopyranose) as follows: 2 g of aluminiumoxide are mixed with 100 ml of a 40% (w/v) maltose solution. The pH is adjusted to pH 11.5 using sodium hydroxide. The reaction mixture is kept at 60°C for 24 hours. Next, the pH is adjusted to pH 4.5 and 5 g of baker's yeast are added in order to ferment maltose and other fermentable sugars resulting from the alkaline incubation conditions (maltulose is not fermented by the yeast). Finally, the reaction mixture is filtered to obtain a clear solution, which is concentrated under vacuum to remove the ethanol (resulting from the fermentation) and to obtain a high dry solids solution of maltulose.

(B) Enzymatic hydrolysis of maltulose

Maltulose hydrolysis activity was investigated in a number of commercially available enzyme preparations. The enzyme preparations were mixed with a 5 % maltulose solution in distilled water. For each of the enzymes, 5 mg of enzyme was

added to 5 ml of maltulose solution. The mixtures were incubated under conditions listed in Table 2. The reaction mixtures were analyzed using HPLC as described under Example 1. The results of these analyses are shown in Table 2.

Table 2

Series	Enzyme preparation	Temp. in °C	pH	Incubation time h.	Fructose %	Glucose %	Maltulose %	Others %
1	Starting material				0.0	0.0	87.0	13.0
1	<i>T. reesei</i> cellulase; MAXAZYME CL 2,000 (Gist-brocades)	50	4.5	40	38.0	29.4	24.0	8.6
1	<i>Kluveromyces lactis</i> β - galactosidase; MAXILACT LX 5,000 (Gist-brocades)	37	6.5	40	0.0	0.0	86.9	13.1
1	Yeast Invertase; MAXINVERT L 10,000 (Gist-brocades)	50	4.5	40	0.0	0.0	91.5	8.5
2	Starting material series 2				0.0	0.0	56.2	43.8
2	<i>A. niger</i> α - galactosidase; SUMIZYME AGS (Shin Nihon)	60	4.2	4	15.5	14.6	11.5	58.4

Example 3**The effect of a cellulase preparation on the
monosaccharide level after saccharification with AG**

To demonstrate the effect of a cellulase preparation on monosaccharide level of a sugar solution after saccharification, 20 mg of cellulase were added to 10 ml of dextrose syrup. This dextrose syrup was prepared by saccharification of a 33 % w/w dextrin solution with AG. The AG was inactivated at the maximum DX level by placing the dextrose syrup in a waterbath for 10 minutes at 100°C. After addition of cellulase and incubation during one hour at pH=4.5 and 50°C, 0.1 ml of reaction mixture were diluted with 3 ml of distilled water and the reaction was stopped by placing the diluted mixture in a boiling water bath. Eventually, samples were analyzed by HPLC as described under Example 1. The results are shown in Table 3.

Table 3

Sample	DP1 %	DP2 %	DP3%	DP4%
Starting material (dextrose syrup)	94.5	3.97	0.69	0.84
Treated with <i>T. reesei</i> Maxazyme CL 2000 cellulase; Gist-brocades	95.06	3.61	0.63	0.69

(DP means degree of polymerization; DP4+ means a polymerization degree of 4 or
5 higher)

The results shown in Table 4 demonstrate that addition of the cellulase preparation results in an increase in monosaccharide level with 0.5-0.6 DX.

It should be considered that while the foregoing description and examples are illustrative of the invention, it is the following claims which define the scope of the
10 invention.

Example 4

Partial purification of the maltulose- and residual sugar hydrolysing activity from Sumizyme AGS

15 The α -galactosidase preparation (Sumizyme AGS, Shin Nihon, Japan) was partially purified using gel filtration chromatography. The procedure is described below. Collected fractions were screened for the presence of different activities. Interesting fractions were pooled for determination of the specific activity.

20 **Chromatographic procedure:**

Column: 58 x 2.5 cm.

Support material: Sephacryl S 200 HR.

Elution buffer: 50 mM acetate buffer, pH = 4.5 including 0.02% sodiumazide.

Flowrate: 2 ml/min.

25 Detection: UV 280 nm.

Fraction collection: 2 minute fractions.

Sample: 4 ml of 30 mg/ml Sumizyme AGS (lot 60902-02) in elution buffer.

Activity detection (screening of fractions):

1. α -Galactosidase activity detection.

30 100 μ l 1 mM paranitrophenyl- α -D-galactose in 50 mM acetate buffer pH 5.5 was incubated with 100 ml of collected fraction. After a 3 minute incubation at room temperature 100 μ l of 0.0625 M borax buffer pH 9.7 was added to stop the reaction.

The yellow colour, resulting from paranitrophenol, was a measure for α -galactosidase activity. The result was judged visually.

2. Maltulose hydrolysing activity detection.

The maltulose preparation was diluted 4 times with distilled water. 100 μ l of this solution were mixed with 200 μ l of a fraction having maltulose hydrolyzing activity and 700 μ l of distilled water. This mixture was incubated for 3 hours at 33°C. Next, the mixtures were placed in a boiling water bath in order to inactivate the enzyme. Finally, the mixtures were analyzed on HPLC using the Bio-Rad HPX 87C column. The increase in the fructose peak area was a direct measure for the activity.

3. Residual sugar hydrolysing activity.

50 μ l of a fraction having residual sugar hydrolyzing activity was mixed with 50 μ l beer (from wet milling fuel ethanol production, from Pekin Energy, Pekin, Illinois) and incubated for 16 hours at 33°C. Next, 900 μ l 0.006 N sulphuric acid was added and the reaction mixture was centrifuged in an Eppendorf centrifuge. The supernatant was analyzed by means of HPLC on a BIO-Rad HPX 87H column. The decrease of the residual sugar peak was a direct measure for the activity.

Specific activity assay:

The specific activity is expressed as an activity value per mg of protein per minute of reaction time.

1. Determination of the protein content.

The protein content is determined using the BCA method with bovine serum albumin as standard.

2. The α -galactosidase activity.

A solution is made of 10 mM p-nitrophenol in 50 mM sodium acetate buffer pH 5.5. This solution is diluted to 240-160-80-40 mM. 1 ml of these solutions is added to 2 ml of 0.80 mM p-nitrophenyl- α -D-galactopyranoside in acetate buffer. To this mixture 5 ml 625 mM borax buffer pH 9.7 is added (stop reagent). The OD of these solutions is measured at 405 nm against water (standard curve).

Enzyme incubation: 1 ml of diluted enzyme solution in stead of p-nitrophenol. The mixture is incubated for 15 minutes at 37°C. The reaction is stopped by adding 5 ml borax solution. The OD is measured as mentioned before.

Activity definition: one Unit of α -galactosidase is the amount of enzyme which hydrolyses 1 μ mol of p-NPGal/minute under the standard conditions.

3. The maltulose hydrolysing activity.

100 µl of maltulose solution is mixed with 200 µl of enzyme solution and 700 µl of distilled water. The mixture is incubated at 33°C. Samples were taken at different incubation times and analyzed by HPLC in order to determine the amount of maltulose hydrolysed.

4. The residual sugar hydrolysing activity.

100 µl of beer from wet milling ethanol production is mixed with 200 µl of enzyme solution and incubated at 33°C. 700 µl of 8 mM sulphuric acid was added to samples taken at different incubation times. The samples are analyzed by HPLC in order to determine the amount of residual sugar hydrolysed.

Definitions of specific activity:

1. α-galactosidase: units α-gal per mg protein.

2. Maltulose hydrolysing activity: mg maltulose hydrolysed per mg of protein per minute.

3. Residual sugar hydrolysing activity: µg residual sugars hydrolysed per mg of protein per minute.

Molecular Weight Determination:

Standard commercially available protein mixtures (Bio-Rad) were used as molecular weight markers as follows: Thyroglobulin (MW = 670 kD), gamma-globulin (MW = 158 kD), ovalbumin (MW = 44 kD), myoglobin (MW = 17 kD), vitamin B12 (MW = 13.5 kD). Gel filtration of the markers with subsequent comparison to the maltulase and residual sugars hydrolyzing enzyme resulted in an approximate molecular weight of maltulase of about 132 kD and 120 kD, respectively.

Results:

The chromatogram resulting from gel filtration of the α-galactosidase preparation is presented in Figure 1. The results of assaying of fractions on the presence of α-galactosidase activity, maltulose hydrolysing activity and residual sugar hydrolysing activity are presented in the following Table 4. The results were used to pool fractions. The α-galactosidase pool was contained in fraction 8-10 (elution time = 55-60 minutes). The residuals- and maltulose hydrolysing activity pool was contained in fractions 12-14 (elution time = 63 - 68 minutes). The pooled fractions and starting material were assayed for specific activity. The results are shown in Table 5.

Table 4

Fraction number	Fraction elution time	α -Galactosidase activity (yellow colour)	Maltulose hydrolysing activity	Residual sugar hydrolysing activity (peak reduction)
2	43-44			
4	47-48	+		2
6	51-52	++	3	11
8	55-56	+++	33	37
10	59-60	+++	100	63
12	63-64	++	93	100
14	67-68	+	91	100
16	71-72		67	58
18	75-76		9	14
20	79-80			2
22	83-84			
24	87-88			
26	91-92			
28	95-96			
30	99-100			
32	103-104			
34	107-108			
36	111-112			
38	115-116			
40	119-120			
42	123-124			
44	127-128			
46	131-132			
48	135-136			

Table 5

Material	Specific α -galactosidase activity units/mg	Ratio	Specific maltulose hydrolysing activity mg maltulose /mg.min	Ratio	Specific residual sugar hydrolysing activity mg residuals/mg. min	Ratio
Starting material	8100		4.82		0.52	
Fractions 8-10	184000	22.7	87.0	18.1	12.9	24.8
Fractions 12-14	16900	2.1	163.8	34.0	28.4	54.6

Discussion/conclusions:

- 5 Tables 4 and 5 demonstrate that the maltulose and residual sugar hydrolysing activity are side activities in the α -galactosidase preparation and are not due to the α -galactosidase itself. In addition, it appears that the specific activity of both enzymes can be significantly increased by a single purification step.

10

Example 5**Heat inactivation of the α -galactosidase activity**

A solution of α -galactosidase was heat treated for 30 minutes at 65°C.

- 15 Next, the starting material and the heat treated solution were assayed for specific activity.

The results of this experiment are shown in Table 6.

20

Table 6

Material	Specific α -galactosidase activity units/mg	Ratio	Specific maltulose hydrolysing activity mg maltulose/ mg/min	Ratio	Specific residual sugar hydrolysing activity mg residuals/ mg/min	Ratio
Starting material	8100		4.82		0.52	
Heat treated material	18.5	.002	4.19	0.9	0.42	0.8

The results demonstrate that the α -galactosidase activity and the residuals and maltulose hydrolysing activity are coming from different enzymes.

CLAIMS

1. An enzyme preparation comprising an enzyme capable of the hydrolysis of maltulose, wherein said enzyme preparation is enriched for said enzyme capable of the hydrolysis of maltulose.
2. An enzyme preparation according to claim 1, wherein the enzyme capable of the hydrolysis of maltulose is obtained from a microorganism.
3. An enzyme preparation according to claim 2, wherein the microorganism is a fungus.
4. An enzyme preparation according to claim 3, wherein the fungus is an *Aspergillus* strain or a *Trichoderma* strain.
5. An enzyme preparation according to claim 4, wherein said enzyme preparation comprises a maltulase enzyme derived from *Aspergillus niger* having a molecular weight of about 132 kD as measured by gel filtration.
6. Use of an enzyme preparation for the hydrolysis of maltulose.
7. Use of an enzyme preparation according to claim 1 for the hydrolysis of maltulose.
8. A method for hydrolysis of maltulose characterized by using an enzyme preparation for the hydrolysis of said maltulose.
9. A method for the production of glucose from a solution comprising dextrose, iso-syrup, and/or dextrin including maltulose comprising:
 - (a) preparing an aqueous solution containing dextrose, iso-syrup and/or dextrins;
 - (b) adding to said solution an enzyme preparation comprising an enzyme capable of the hydrolysis of maltulose, wherein said enzyme preparation is enriched for said enzyme capable of the hydrolysis of maltulose; and,

(c) incubating said solution from (b) for a time and under conditions suitable to hydrolyze maltulose.

10. A method according to claim 9, further comprising the use of the enzymes
5 pullulanase, acid amylase, or a combination thereof.

11. A fermentation process comprising the utilization of a dextrose syrup produced according to claim 7.

10 12. A fermentation process according to claim 11 for the fermentative production of ethanol.

13. A dextrose syrup obtainable from starch and substantially free of maltulose.

15 14. An iso-syrup obtainable from starch and substantially free of maltulose.

Chromatogram of gelfiltration of α -galactosidase on Sephacryl S 200 HR.
OD (280 nm) versus elution time.

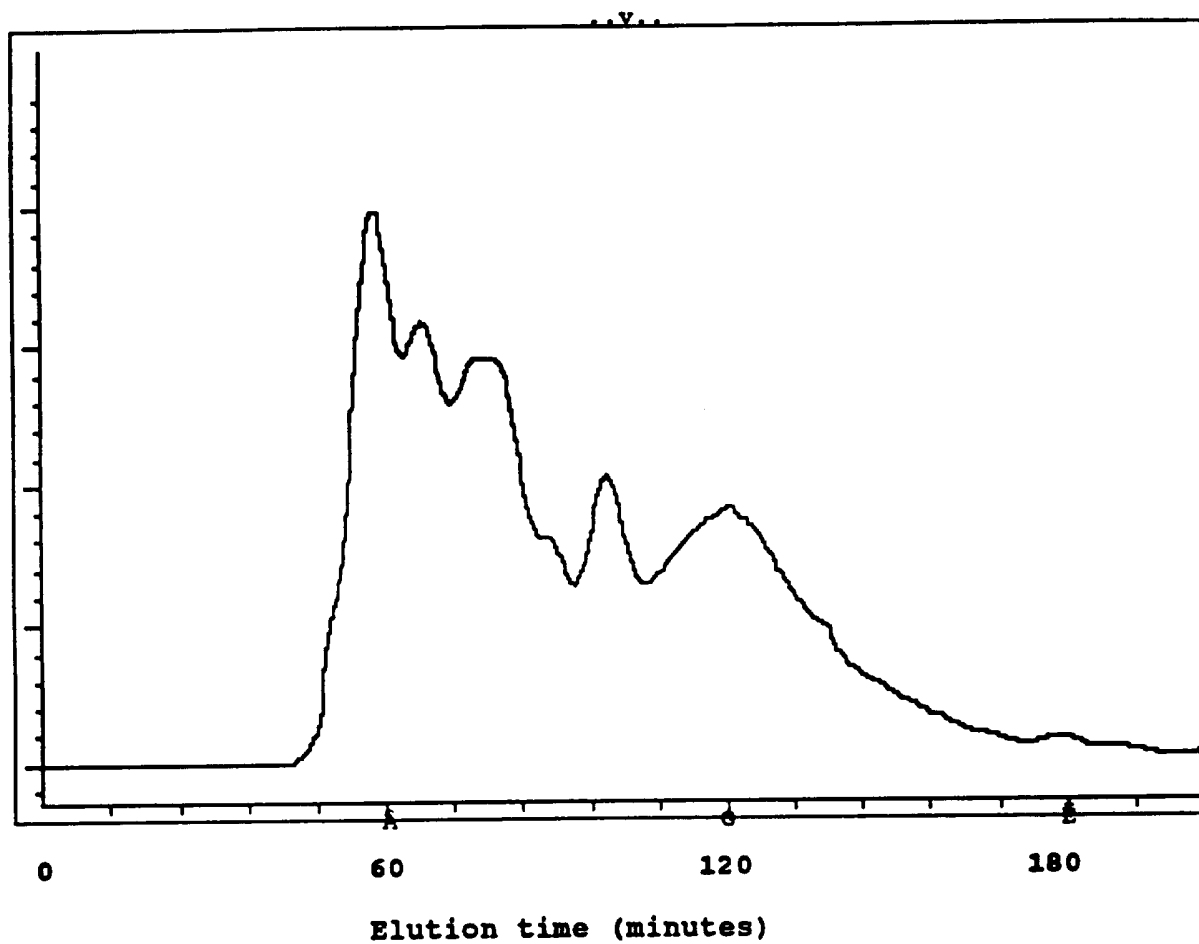


FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/13879

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P19/14 C12P19/20 C12P7/06 C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY (MICROFILMS), vol. 237, no. 4, 1962 MD US, pages 1002-1006, PAZUR J.H. ET AL 'The hydrolysis of alpha-D-glycosides by amyloglucosidase from Aspergillus niger' see table I --- -/--	1-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

19 March 1996

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 95/13879

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	EP,A,0 140 410 (GIST-BROCADES) 1985 see the whole document	8-12
X	EP,A,0 189 838 (MILES LABORATORIES INC) 1986 see page 4, line 19 - page 5, line 1; example IX	13,14
X	EP,A,0 252 730 (NOVO INDUSTRIAS) 1988 see the whole document	13,14
X	CEREAL FOODS WORLD, vol. 33, no. 11, 1988 ST. PAUL, pages 929-934, SHETTY J.K. ET AL 'An acid stable , thermostable alpha-amylase for starch liquefaction' see table I see table III	13,14
X	STARCH, vol. 39, no. 2, 1987 WEINHEIM, pages 64-66, F.F.DIAS ET AL 'maltulose formation during saccharification of starch' see the whole document	13,14
A	WO,A,92 20777 (SOLVAY ENZYMES) 1992 see the whole document	11,12

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Information on patent family members

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PC1/US 95/13879

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