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(54) **ENZYMATIC METHOD OF PRODUCING 4-0-B-D GALACTOPYRANOSYL-D-XYLOSE, 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE OBTAINED USING SAID METHOD, COMPOSITIONS CONTAINING SAME AND THE USE THEREOF IN EVALUATING INTESTINAL LACTASE**

ENZYMATISCHES VERFAHREN ZUR HERSTELLUNG VON 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE, MIT DIESEM VERFAHREN GEWONNENE 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE, DIESE ENTHALTENDE ZUSAMMENSETZUNGEN UND VERWENDUNG DAVON BEIM QUANTIFIIZIEREN INTESTINALER LACTASE

PROCEDE ENZYMATIQUE D'OBTENTION DE 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE, 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE OBTENU SELON CE PROCEDE, COMPOSITIONS CONTENANT DU 4-0-B-D-GALACTOPYRANOSYL-D-XYLOSE ET SON APPLICATION DANS L'EVALUATION DE LA LACTASE INTESTINALE

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Description

[0001] The present invention is comprised in the field of the process to obtain compounds, specifically disaccharides useful in bloodless evaluation methods of intestinal lactase activity.

BACKGROUND OF THE INVENTION

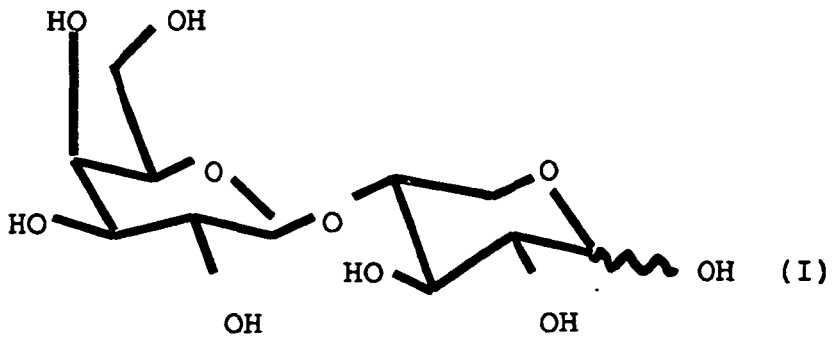
[0002] The deficiency or low intestinal lactase activity that results in insufficient capacity or up to the incapacity to digest lactase, is rare as a congenital metabolic error, but it is a common syndrome in human adults. However, in most mammals there is a noticeable reduction of lactase activity from the moment of weaning. In humans whose ancestors have depended on a substantial consumption of milk or milk products for a long time, this reduction is less frequent. On the other hand, in unweaned babies, deficient or low intestinal lactase activity is rather infrequent.

[0003] The determination of intestinal lactase activity is important in pediatrics and gastroenterology and it can be carried out directly, from a sample of mucous membrane, or indirectly, from the level of sugar in the blood or from exhaled hydrogen, after administering a dose of lactase to the individual.

[0004] Direct determination has the disadvantage of being a complex and expensive method due to the fact that it requires special instruments and very specialized staff in order to remove the sample that should be subjected to analysis afterwards, aside from the fact that it is unpleasant and somewhat dangerous for the individual.

[0005] Other methods to determine intestinal lactase are based on the fact that disaccharides are, based on their affinity to lactase, capable of acting as a lactase substrate and they are converted, by action of the enzyme, into certain monosaccharides that are easily absorbed by the intestine and excreted in urine.

[0006] Spanish patent ES-P-9001680 describes the preparation of 4-O-β-galactopyranosyl-D-xylose disaccharide of formula (I)



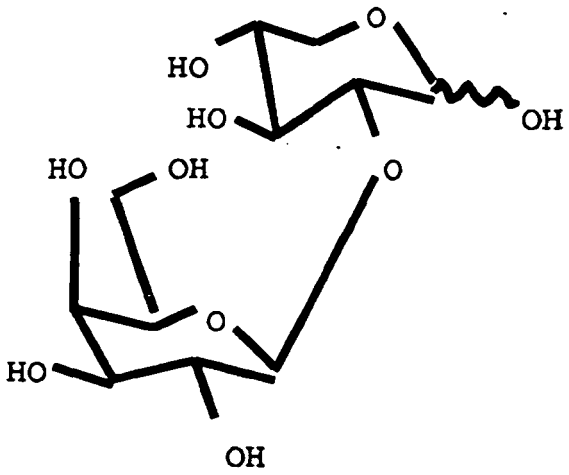
(I)

for the evaluation of intestinal lactase activity. Said disaccharide is administered orally, acts as a substrate of intestinal lactase and therefore it decomposes in the intestinal tract, into xylose and galactose, the xylose being absorbed and excreted in urine, wherein xylose can be evaluated directly by means of a simple colorimetric method.

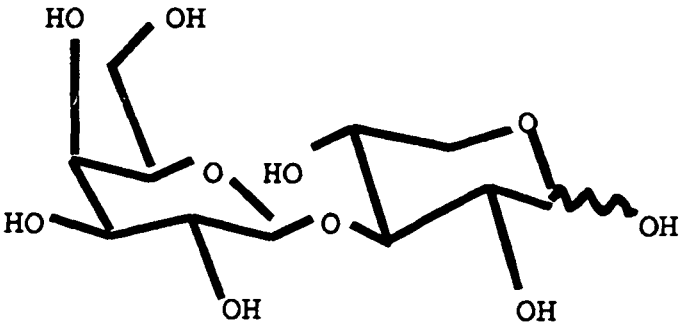
[0007] The amounts of xylose excreted in urine are correlated with the levels of intestinal lactase.

[0008] Spanish patent ES-P-9001680 also describes a method for basically preparing 4-O-β-galactopyranosyl-D-xylose, that comprises synthesis of benzyl ß-D-xylopyranoside and that follows a sequence of operations that implies selective protection, glycosilation and deprotection reactions. The number of steps of the reaction, as well as the use of expensive reagents such as silver triflate in the glycosilation reaction, and the use of chromatography columns in the purification of intermediates and of the final product, produce costs and have difficulties to carry out this process on an industrial scale.

[0009] Spanish patents ES-P-9502185 and ES-P-9701156 describe enzymatic processes for the preparation of mixtures of galactopyranosyl-xylose disaccharides that contain the disaccharide (I) and its regioisomers 2-O-β-D-galactopyranosyl-D-xylose and 3-O-β-D-galactopyranosyl-D-xylose that, respectively, have the following formulae:



(II)



(III)

[0010] The processes described in Spanish patents ES-P-9502185 and ES-P-9701156 make it possible to obtain in a single reaction step and after chromatographic purification, mixtures of 2-, 3- and 4-0-β-D-galactopyranosyl-D-xylose useful as substrates and, therefore, for the determination of the enzymatic activity of intestinal lactase. Said processes, although feasible from accessible substrates and enzymes, have difficulties, from the point of view of industrial synthesis, for the characterization of the most suitable proportions, reproducibility of the preparation in said proportions and the determination of possible impurities.

[0011] On the other hand, Gorin et al. in "The Synthesis of β-Galacto- and β-Gluco-Pyranosyl Disaccharides by *Sporobolomyces Singularis",* Can. J. Chem. 42(1964) 2307-2319, describe the synthesis of a plurality of disaccharides, among them 2-O-β-D-galactopyranosyl-D-xylose and 3-O-β-D-galactopyranosyl-D-xylose, by means of a process using cells. This publication does not describe any use of the different synthesized disaccharides. However, Aragón et al. in "Evaluation of rat intestinal lactase in vivo with 4-galactosylxylose" proposes the use of the aforesaid disaccharide for evaluating intestinal lactase activity.

OBJECT OF THE INVENTION

[0012] The first object of the invention is to overcome the above-cited inconveniences of the prior art.

[0013] Another object of the invention is to provide an improved process that implies any enzymatic reaction between D-xylose and a β-D-galactopyranoside substrate and a subsequent phase of isolation and purification, that makes it possible to increase the proportion of 4-O-β-D-galactopyranosyl-D-xylose in the final mixture of the enzymatic reaction with respect to the 2- and 3-O-β-D-galactopyranosyl-D-xylose, from whose final mixture 4-0-β-D-galactopyranosyl-D-xylose can be isolated by simple operations.

[0014] The 4-O-β-D-galactopyranosyl-D-xylose that can be obtained by means of the above-cited process, as well as the compositions that comprise said 4-O-β-D-galactopyranosyl-D-xylose, constitute subsequent objects of the invention.

[0015] Another object of the invention is to use 4-O-β-D-galactopyranosyl-D-xylose in the preparation of compositions and solutions useful in the in vivo evaluation of intestinal lactase activity.

DESCRIPTION OF THE INVENTION

[0016] The above-cited objects are achieved in accordance with the present invention, by means of an enzymatic process to obtain 4-O-β-D-galactopyranosyl-D-xylose that comprises
a first step of preparation of a first reaction mixture of
2-20% by weight of D-xylose
0.5-5% by weight of a β-D-galactopyranoside substrate
75-97.5% by weight of a reaction medium that comprises buffered water at a pH between 5.0 and 9.0;
adding 10 to 1,000 units of a β-D-galactosidase enzyme, per gram of β-D-galactopyranoside to the first reaction mixture;
and obtaining a second reaction mixture;
a second step wherein the second reaction mixture is subjected to a reaction at a temperature comprised between a temperature higher than the freezing point of the second reaction mixture and 45°C, for 2 to 48 hours, in order to form disaccharides in the second reaction mixture;
a third step wherein the reaction is stopped when the disaccharides have been formed in the desired amount, by means of a treatment chosen between deactivation of ß-D-galactosidase by freezing the second reaction mixture at a temperature between -20°C and -170°C, deactivation of b-D-galactosidase by heating the second reaction mixture at a temperature between 95 and 110°C, and separation of b-D-galactosidase from the second reaction mixture by ultrafiltration; obtaining a third reaction mixture;
a fourth step wherein an aglyconic fragment of the 1-D-galactopyranoside substrate used in the first step is separated from the third reaction mixture by extraction or filtration; obtaining a fourth reaction mixture;
a fifth step comprising isolation of fractions that contain 4-O-b-D-galactopyranosyl-D-xylose, selected among
adding celite to the fourth reaction mixture, followed by solid-liquid extraction with a solvent and elution with a first eluent in a column;
and directly adding active carbon to the fourth reaction mixture followed by filtration and elution with a second eluent,
and a sixth step, wherein the fractions that contain 4-0- b-D-galactopyranosyl-D-xylose are crystallized in a crystallization mixture selected among mixtures of acetone/methanol in a ratio between 5/1 and 20/1 and mixtures of acetone/water in a ratio between 5/1 and 20/1.

[0017] In accordance with the invention, the proportion of D-xylose in the second reaction mixture is preferably 7.5% by weight, the proportion of b-D-galactopyranoside in the second reaction mixture is 1.5% by weight, and 100 units of β-D-galactosidase per gram of β-D-galactopyranoside are added.

[0018] Optionally, the reaction medium may also comprise at least a cosolvent medium selected among dimethylsulfoxide, dimethylformamide, dioxane and mixtures thereof, preferably in a proportion of 20.5 as referred to the reaction medium. In an embodiment of the invention, the reaction medium is buffered to a pH of 7.

[0019] The reaction is conveniently carried out at a constant temperature for the purpose of increasing its reproducibility. In an embodiment of the process of the invention, the reaction temperature is higher than the freezing point of the second reaction mixture and is lower than 40°C. In another embodiment, the reaction is carried out at room temperature, which permits good yields without the need of cooling the second reaction mixture. The reaction may also be carried out at -5°C or at 37°C. The reaction temperature is preferably lower than 0°C but higher than the freezing point of the second reaction mixture.

[0020] In accordance with the invention, the β-D-galactopyranoside substrate is preferably selected among o-nitrophenyl β-D-galactopyranoside (Gal-ONP) and lactose. The ß-galactosidase enzyme can be E. coli ß-galactosidase or *Kluyveramyces lactis* β-galactosidase (such as for example MAXILACT®). When Gal-ONP is used as the substrate on-nitrophenol is formed in the reaction and the same is eliminated by extraction with ethyl acetate in the event that the reaction is stopped by heating, or else it is eliminated by simple filtration in the event that the reaction is stopped by cooling.

[0021] When, in the third step of the process the reaction is stopped by freezing the second reaction mixture, a temperature of -78°C is preferably applied. On the other hand, when in the third step the reaction is stopped by heating the second reaction mixture, a temperature of 100°C is preferably applied.

[0022] In the fifth step, the 4-O-β-D-galactopyranosyl-D-xylose may be isolated from the reaction mixture, by means of several alternative methods.

[0023] According to a first alternative method, water is eliminated from the fourth reaction mixture in order to obtain a reaction residue that contains disaccharides, the reaction residue is subjected to an acetylation treatment in order to obtain a peracetylated 4-O-β-D-galactopyranosyl-D-xylose derivative and to separation of the peracetylated derivative in a silica gel chromatographic column. Acetylation of the reaction residue is preferably carried out with acetic anhydride in pyridine, whereas, deacetlyation of the peracetylated derivative is carried out catalytically with sodium methoxide in methanol.

[0024] According to a second alternative method, the fourth reaction mixture is subjected to elution in a column with a first eluent that may be selected among mixtures of water with methanol, ethanol or isopropanol, preferably a mixture of water/isopropanol with a proportion of isopropanol of 1 to 10% (v/v), preferably 2% (v/v).

[0025] Elution is carried out in a filtration column selected among filtration columns with cross-linked dextrane polymer fillers, such as for example a column with SEPHADEX filler, filtration columns with acrylamide polymer fillers, such as for example, a column with BIOGEL filler, and filtration columns of active carbon or of active carbon-celite in order to obtain fractions that contain 4-O-β-D-galactopyranosyl-D-xylose.

[0026] Preferably, the fourth reaction mixture is concentrated before being subjected to elution in the column. According to a third alternative method, celite is added to the fourth reaction mixture, the mixture thus obtained is concentrated to dryness and the residue is subjected to solid-liquid extraction with an organic solvent in a Soxhlet extractor followed by elution in a column. The solvent preferred for the solid-liquid extraction is ethyl acetate. The column is selected among filtration columns with cross-linked dextrane polymer fillers, such as for example a column with SEPHADEX filler, filtration columns with acrylamide polymer fillers, such as for example a column with BIOGEL filler and filtration columns of active carbon or of active-carbon-celite. Preferably the column is of active carbon-celite, wherein the carbon is deactivated by adding hydrochloric acid.

[0027] This third alternative method offers the advantage of eliminating most of the xylose - above all when it is used in great excess in the reaction - before elution in the column whereby the fillers, as well as the amount of first eluent that is needed for elution is much less. Another advantage of this third alternative method is that the solid-liquid extraction in ethyl acetate is completely selective given that in the liquid phase no presence of disaccharides is observed, but rather only of xylose and galactose.

[0028] According to a fourth alternative method, elution in the fifth step is carried out by adding active carbon to the fourth reaction mixture instead of using a filler column, once the aglyconic fragment has been separated from the substrate in the fourth step, thus achieving that the 4-O-β-D-galactopyranosyl-D-xylose is adsorbed on the active carbon and eluting the 4-O-β-D-galactopyranosyl-D-xylose of the active carbon with a second eluent. Said elution is preferably carried out by means of consecutive washings with water and with diluted isopropanol with a growing proportion in volume of isopropanol in successive steps. The proportion in volume of isopropanol is comprised between 1% and 3% in a first step, between 3% and 5% in a second step, and between 5% and 7% in a third step. The concentration of isopropanol preferred for washing is a 2% isopropanol sequence, followed by elution with 4% isopropanol and followed by elution with 6% isopropanol. Pure 4-O-β-D-galactopyranosyl-D-xylose is obtained from the residue obtained, by concentration crystallizing it in acetone-water.

[0029] Preferably, according to this fourth alternative method o-nitrophenyl β-D-galactopyranoside is used as a substrate for the reaction.

[0030] According to this fourth alternative method, multiple advantages are obtained, such as the fact that it is not necessary to heat the second reaction mixture to 100°C to stop the reaction, nor is it necessary to separate the aglyconic fragment from the substrate in the fourth step by means of extraction. Likewise, the need to concentrate the fourth reaction mixture is avoided, and hence, caramelization thereof is not produced. The amount of active carbon that would be needed for the filler of a column is reduced, the total amount of eluents is also reduced and the use of celite is avoided.

[0031] In accordance with the invention according to the sixth step the fractions that contain 4-O-β-D-galactopyranosyl-D-xylose obtained in a crystallization mixture selected among mixtures of acetone/methanol in a ratio between 5/1 and 20/1 and mixtures of acetone/water in a ratio between 5/1 and 20/1, preferably a ratio of 10/1, are crystallized.

[0032] The invention also refers to 4-0-ß-D-galactopyranosyl-D-xylose obtained by the above-described method, and to compositions and saline or aqueous solutions that comprise a 4-O-β-D-galactopyranosyl-D-xylose obtained by means of said process, as well as use of a 4-O-β-galactopyranosyl-D-xylose in the preparation of compositions and solutions for in vivo evaluation of intestinal lactase in humans.

[0033] In such compositions and solutions, the β-D-galactopyranosyl-D-xylose is combined with pharmaceutically acceptable amounts of at least an additive selected from among pharmaceutically acceptable stabilizers, protecting agents, flavoring agents, lactose, gelling agents, fluidizing agents, and preservatives, which in themselves are conventional.

[0034] The 4-O-β-D-galactopyranosyl-D-xylose or compositions or solutions that contain it are administered orally and lead to the existence of xylose in urine and this xylose that is spectrophotometrically analyzed, is used in a specific, routine, bloodless and simple manner for diagnostic evaluation of deficiencies of lactase activity.

EMBODIMENTS OF THE INVENTION

[0035] The invention will now be described on the basis of some examples that will illustrate with more detail some of the above-cited characteristics.

Example 1: In order to determine the influence of the reaction temperature, the following test was carried out:

[0036] Samples of reaction mixtures comprised of
125 mg (500 mM) of D-xylose

25 mg (50 mM) of o-nitrophenyl β-D-galactopyranoside
1.75 ml of a reaction medium comprised
of an aqueous solution buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1mM $MgCl_2$, 5mM mercaptoethanol),
were prepared and units of *E. coli* β-galactosidase enzyme were added to these samples in terms of the reaction temperatures applied, in accordance with the following table:

reaction temperature (°C)	added units of enzyme (u)
45	1.6
37	1.6
25	1.6
5	10
-5	20

[0037] The increases in the amount of enzyme were necessary in order to compensate the slowing down of the reaction produced by the drop of the reaction temperature. It should be indicated that it is possible to work at temperatures below the freezing point of water thanks to the cryoscopic drop that is produced in the reaction medium thanks to the high concentration of sugar in the samples.

[0038] The ratio between 4-, 2- and 3-O-β-D-galactopyranosyl-D-xylose was determined for each one of the samples and for each step of the process by gas chromatography by a chromatograph equipped with a flame ionization detector and a SE-54 capillary column 15 m long, with an inside diameter of 0.15 mm and a thickness of 0.3 μm. A nitrogen flow of 1 ml/min was used. The temperature program used was

Initial temperature:	160°C
Initial time:	2 min
Temperature increase:	5°C/min
Final temperature:	250°C

[0039] The samples were analyzed after trimethylsililation by means of the following protocol:
An aliquot (10 μl) was frozen at -170°C and lyophilized until a dry residue was obtained, after which pyridine (25μl) that contained as an internal reference benzyl β-D-xylopyranoside (10 mM) and N-trimethylsilimidazole (25 μl)was added to the dry residue and heating at 60°C was continued for 30 minutes. The retaining times of the peaks assignable to the different disaccharides were the following:

Benzyl β-D-xylopyranoside:	12.04 min.
2-O-β-D-galactopyranosyl-D-xylose:	18.46 and 19.50 min.
3-O-β-D-galactopyranosyl-D-xylose:	18.30 min.
4-O-β-D-galactopyranosyl-D-xylose:	20.35 and 20.50 min.

[0040] The following table reflects the proportions taken at the maximum formation of disaccharides, of 4-O-β-D galactopyranosyl-D-xylose (= compound I) with respect to the sum of its regioisomers 2- and 3-O-β-D-galactopyranosyl-D-xylose that were obtained:

Table I

Temperature	Approximate reaction time (minutes)	Ratio of compound I/compounds II+III
45	90	68:32
37	150	71:29
25	180	79:21
5	270	80:20
-5	120	83.17

From the above table it is inferred that as the temperature dropped, there was an increase in the proportion of 4-O-β-D-galactopyranosyl-D-xylose.

Example 2: In order to determine the influence of the pH on the reaction, the following samples were prepared:

[0041]

Gal-ONP (50 mM):	25 mg	
D-xylose (500 mM):	125 mg	
E. coli galactosidase	1.6 u	
Buffered aqueous solution (potassium phosphate 50 mM, 1 mM $MgCl_2$, 5 mM mercaptoethanol) at pH	8.5	1.6 ml
	7	1.6 ml
	5	1.6 ml

and they were reacted at 37°C.

[0042] The progress of the reaction was followed in the same way as described in example 1.

[0043] The following table reflects the proportions of 4-O-ß-D-galactopyranosyl-D-xylose (= compound I) with respect to the sum of its regioisomers 2- and 3-O-β-D-galactopyranosyl-D-xylose that were obtained:

Table 2

PH	Approximate reaction time (minutes)	Ratio of compound I/compounds II+III
8.5	60	68.32
7	150	71:29
5	180	81:19

[0044] From the above table it is inferred that in a basic medium (pH = 8.5), the proportion of compound I was lower than in a neutral medium (pH = 7), detecting the highest proportion of compound I in an acid medium (pH = 5).

[0045] **Example 3**: In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 6 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONF)and 25 g of D-xylose were dissolved in 330 ml of water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 2 mg (640 u) of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 30°C in an orbital stirrer until the Gal-ONP was practically consumed (approximately 4 hours). The follow-up of the reaction was carried out by thin layer chromatography (tlc) with isopropanol/NH_3 (30%)/H_2O = 7.5/0.5/2.5 as eluent and taking as reference the following Rf values:

Rf	(Gal-ONP):	0.58
Rf	(D-xylose):	0.47
Rf	(4-O-β-D-galactopyranosyl-D-xylose):	0.17
Rf	(2-O-ß-D-galactopyranosyl-D-xylose + 3-O-ß-D-galactopyranosyl-D-xylose):	0.26

[0046] The reaction was stopped by heating in a water bath at 100°C for 10 minutes and afterwards the o-nitrophenol formed was extracted with CH_2Cl_2. The aqueous solution was concentrated to dryness and the residue was acetylated in a conventional manner (acetic anhydride/pyridine = 1:1, at room temperature, overnight and with magnetic stirring). Afterwards, the reaction mixture was concentrated and the pyridine and acetic anhydride residues were eliminated by successive additions and evaporations of toluene. The precipitated salts were filtered, the filtrate was concentrated to dryness and the residue was chromatographed in a silica gel column using a gradient of hexane/ethyl acetate in a ratio of 4:1 - 1:1 as the eluent. First acetylated D-xylose was eluted from the column and afterwards the mixture of acetylated disaccharides. Once the fractions that contained the mixture of disaccharides were concentrated, the residue was dissolved in MeOH, a solution of 1M MeONa/MeOH was added and the mixture thus obtained was stirred until deacetylation was complete (follow-up by tlc with isopropanol/NH_3/H_2O). The mixture was neutralized with AMBERLITE IR-120 (H^+) and concentrated to dryness. The mixture of free disaccharides was crystallized twice successively with MeOH/acetone, obtaining 1.07 g of pure 4-O-β-D-galactopyranosyl-D-xylose with a yield of 17% based on the initial Gal-ONP. (Melting point: 171-176°; ^{1}HNMR (D_2O): δ 5.17 and 4.58 (2D, 1h, J. 3.8 and 7.8 Hz, H-1α and H-1β), 4.55 and 4.45 (2d, 1H, J 7.8 Hz, H-1'), 4.05 (dd, 1H, J 5.3 and 11.6 Hz, H-5e), 3.38 (dd, 1H, J 10.6 and 11.6 Hz), 3.25 (dd, 1H, J 7.8 and 9.4 Hz, H-2').

[0047] **Example 4:** A column of active carbon/celite was prepared by dry mixing 200 g of activated carbon (DARCO G-60) and 200 g of celite and water was added until a homogeneous paste was formed. The paste was treated with 150

ml of HCl (35%) in order to deactivate the carbon, as well as to wash the residue of iron and alkaline ashes and afterwards this was washed with water until the washing water was neutral. Once washed, the paste was packed in a 5 cm (ϕ) x 50 cm chromatography column and compacted.

[0048] In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 5 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONP) and 25 g of D-xylose were dissolved in 330 ml of water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 2 mg (640 u) of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer until the Gal-ONP was practically consumed (approximately 2 hours). Following the methodology put forth in example 3, the reaction was stopped by heating at 100°C for 10 minutes and the ortho-nitrophenol formed was extracted with ethyl acetate. The aqueous solution was concentrated up to an approximate volume of 50 ml, filtered through glasswool and passed through the active carbon/celite column. First of all, the excess D-xylose was eluted with water and afterwards, using a fractioned gradient of EtOH/H_2O (2%-10% of EtOH) the mixture of disaccharides was collected. The enriched fractions in the 4-O-β-D-galactopyranosyl-D-xylose regioisomer were combined and concentrated to a reduced volume, after which acetone was added until turbidity appeared and the mixture thus obtained was left to stand cold. The 4-O-β-D-galactopyranosyl-D-xylose was crystallized in a pure form, obtaining 970 mg, namely, a yield of 19% based on the initial Gal-ONP, whose spectral data coincided with those given for the product obtained in accordance with example 3.

[0049] **Example 5:** An active carbon/celite column was prepared by dry mixing 200 g of activated carbon (DARCO G-60) and 200 g of celite and water was added until a homogeneous paste was formed. The paste was treated with 150 ml of HCl (35%) in order to deactivate the carbon and to wash residues of iron and alkaline ashes and afterwards this was washed with water until the washing water was neutral. Once washed, the paste was packed in a 5 cm (ϕ) x 50 cm chromatography column and compacted.

[0050] In order to synthesize 4-O-ß-D-galactopyranosyl-D-xylose, 5 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONP) and 25 g of D-xylose were dissolved in 330 ml of water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 2 mg (640 u) of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer until the Gal-ONP was practically consumed (approximately 2 hours). Following the methodology put forth in example 3, the reaction was stopped by heating at 100°C for 10 minutes and the ortho-nitrophenol formed was extracted with ethyl acetate. The aqueous solution was concentrated up to an approximate volume of 50 ml, filtered through glass wool and passed through the active carbon/celite column.

[0051] In order to crystallize the 4-O-β-D-galactopyranosyl-D-xylose, first of all, the excess D-xylose was eluted with water and afterwards, using a fractioned gradient of EtOH/H_2O (2%-10% of EtOH) the mixture of disaccharides was collected. The enriched fractions in the 4-O-β-D-galactopyranosyl-D-xylose regioisomer were combined and concentrated to a reduced volume and dissolved in the minimum amount of water possible, after which acetone was added drop by drop until turbidity appeared and the mixture thus obtained was left at room temperature for two hours. After two hours, a check was made with a thin layer of the supernatant (transparent) that there was still an amount of uncrystallized 4-O-β-D-galactopyranosyl-D-xylose. Acetone was added again until there was turbidity and same was left to stand for another two hours. Finally, more acetone was added and the sample was stored in a refrigerator overnight and it was observed that the supernatant produced contained only a minimum amount of 4-O-β-D-galactopyranosyl-D-xylose. The crystals of 4-O-β-D-galactopyranosyl-D-xylose were filtered and washed with acetone.

[0052] The 4-O-β-D-galactopyranosyl-D-xylose was obtained in a pure form, obtaining 1,557 mg, in other words, a yield of 30% based on the initial Gal-ONP, whose spectral data coincide with those given regarding the product obtained in accordance with example 3.

[0053] **Example 6:** An active carbon/celite column was prepared by dry mixing 200 g of activated carbon (DARCO G-60) and 200 g of celite and water was added until a homogenous paste was formed. The paste was treated with 150 ml of HCl (35%) in order to deactivate the carbon and wash the residues of iron and alkaline ashes and afterwards it was washed with water until the washing water was neutral. Once washed, the paste was packed in a 5 cm ϕ x 50 cm chromatography column and compacted.

[0054] In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 5 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONP)and 25 g of D-xylose were dissolved in 330 ml of water buffered to a pH of 6.8 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 70 units of *Kluyveramyces lactis (MAXILACT®)* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer until the Gal-ONP was practically consumed (approximately 2 hours). The reaction was followed by thin layer chromatography with isopropanol/NH_3 (30%)/H_2O (7.5/0.5/2.5) resulting in the following Rf values:

Rf	(Gal-ONP):	0.58
Rf	(D-xylose):	0.47
Rf	(4-O-β-D-galactopyranosyl-D-xylose):	0.17
Rf	(2-O-β-D-galactopyranosyl-D-xylose + 3-O-β-D-galactopyranosyl-D-xylose):	0.26

[0055] Following the methodology put forth in example 4, the reaction was stopped by heating at 100°C for 10 minutes and the ortho-nitrophenol formed was extracted with ethyl acetate and was filtered to eliminate the enzyme residues. The aqueous solution was concentrated under a vacuum up to an approximate volume of 45 ml and passed through the active carbon/celite column. First of all, the excess D-xylose was eluted with water and afterwards, using a fractioned gradient of EtOH/H_2O (2%-10% of EtOH) the mixture of disaccharides was collected. The enriched fractions in the 4-O-β-D-galactopyranosyl-D-xylose regioisomer were combined and concentrated to a reduced volume, after which acetone was added until turbidity appeared and the mixture thus obtained was left to stand cold. The crystallized 4-O-β-D-galactopyranosyl-D-xylose, was filtered through a filtering plate, obtaining 817 mg, namely, a yield of 16% based on the initial Gal-ONP.

[0056] **Example 7:** An active carbon/celite column was prepared by dry mixing 200 g of activated carbon (DARCO G-60) and 200 g of celite and water was added until a homogenous paste was formed. The paste was treated with 150 ml of HCl (35%) in order to deactivate the carbon and wash the residues of iron and alkaline ashes and afterwards it was washed with water until the washing water was neutral. Once washed, the paste was packed in a 5 cm ϕ x 50 cm chromatography column and compacted.

[0057] In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 5 g of o-nitrophenyl ß-D-galactopyranoside (Gal-ONP)and 25 g of D-xylose were dissolved in 330 ml of_ water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 80 units of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer for 24 hours. The reaction was followed by thin layer chromatography with isopropanol/NH_3(30%)/H_2O (7.5/0.5/2.5) resulting in the following Rf values:

Rf	(Gal-ONP):	0.58
Rf	(D-xylose):	0.47
Rf	(4-O-β-D-galactopyranosyl-D-xylose):	0.17
Rf	(2-O-β-D-galactopyranosyl-D-xylose + 3-O-β-D-galactopyranosyl-D-xylose):	0.26

[0058] Following the methodology put forth in example 3, the reaction was stopped by heating at 100°C for 10 minutes and the ortho-nitrophenol formed was extracted with ethyl acetate and was filtered to eliminate the enzyme residue. The aqueous solution was concentrated under a vacuum up to an approximate volume of 70 ml and the concentrated solution was eluted through a active carbon/celite column. First of all, it was eluted with isopropanol/water (2%) and 1.3 liters were collected. Afterwards 4% fractions were collected up to 2.6 liters, using a total volume of 3.9 liters.

[0059] The enriched fractions in the 4-O-β-D-galactopyranosyl-D-xylose regioisomer were combined and concentrated to a reduced volume, after which acetone was added until turbidity appeared and the mixture thus obtained was left to stand cold. The crystallized 4-0-β-D-galactopyranosyl-D-xylose was filtered through a filtering plate, obtaining 1,213 mg, namely, a yield of 24% based on the initial Gal-ONP.

[0060] **Example 8:** An active carbon/celite column was prepared by dry mixing 24 g of activated carbon (DARCO G-60) and 24 g of celite and water was added until a homogenous paste was formed. The paste was treated with 18 ml of HCl (35%) in order to deactivate the carbon and wash the residues of iron and alkaline ashes and afterwards it was washed with water until the washing water was neutral. Once washed, the paste was packed in a chromatography column and compacted.

[0061] In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 5 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONP)and 25 g of D-xylose were dissolved in 330 ml of water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 80 units of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer until the Gal-ONP was practically consumed (24 hours). The reaction was followed by thin layer chromatography with isopropanol/NH_3(30%)/H_2O (7.5/0.5/2.5) in a way similar to the one indicated in example 7. Following the methodology put forth in example 3, the reaction was stopped by heating at 100°C for 10 minutes, it was allowed to cool and the ortho-nitrophenol formed was extracted with ethyl acetate. Celite (40 g) was added to the aqueous solution and the mixture was concentrated to dryness. The solid residue was subjected to solid-liquid extraction using a Soxhlet extractor equipped with a cellulose cartridge and using ethyl acetate (500 ml) as the solvent. After 23 hours, the resulting solid was washed with water (3 x 40 ml) and the aqueous solution was eluted through an active carbon-celite column. First of all, it was eluted with isopropanol/water (2%) and afterwards with isopropanol/water (4%), using a total volume of eluent of 400 ml. The enriched fractions in the 4-O-β-D-galactopyranosyl-D-xylose regioisomer were combined and concentrated to dryness. The residue was crystallized from acetone-water in a way similar to the one described in example 7, obtaining 0.44 g of pure crystalline disaccharide.

[0062] **Example 9:** In order to synthesize 4-O-β-D-galactopyranosyl-D-xylose, 4.12 g of o-nitrophenyl β-D-galactopyranoside (Gal-ONP) and 20.6 g of D-xylose were dissolved in 272 ml of water buffered to a pH of 7 (0.05 M KH_2PO_4/K_2HPO_4, 1 mM $MgCl_2$, 5 mM mercaptoethanol), 66 units of *E. coli* β-galactosidase enzyme were added and the solution thus obtained was subjected to incubation at 37°C in an orbital stirrer until the Gal-ONP was practically

consumed (21 hours). The reaction was stopped by cooling to 0°C and the o-nitrophenol was filtered as a solid. 60 g of active carbon were added to the filtrate and the resulting mixture was stirred for 30 min. By means of tlc of the supernatant, the absence of disaccharide in the solution was observed, since same was adsorbed on the active carbon. The mixture was filtered and the active carbon solid was washed with water (400 ml), 2% isopropanol (100 ml), 4% isopropanol (200 ml) and 6% isopropanol (200 ml). The fractions that contained disaccharide 4-O-β-D-galactopyranosyl-D-xylose were concentrated and the residue (2.38 g) was crystallized from acetone-water, obtaining 1.55 g of a solid that was crystallized again from the same mixture of solvents in a way similar to the one used in example 7. 1.32 g of pure disaccharide (32%) were obtained.

Claims

1. An enzymatic process to obtain 4-O- β-D-galactopyranosyl-D-xylose that comprises:

a first step of preparation of a first reaction mixture of
2-20% by weight of D-xylose
0.5-5% by weight of a 13-D-galactopyranoside substrate
75-97.5% by weight of a reaction medium that comprises buffered water at a pH between 5.0 and 9.0;
adding 10 to 1,000 units of a β-D-galactosidase enzyme, per gram of β-D-galactopyranoside, to the first reaction mixture; and obtaining a second reaction mixture
a second step wherein the second reaction mixture is subjected to a reaction at a temperature comprised between a temperature higher than the freezing point of the second reaction mixture and 45°C, for 2 to 48 hours, in order to form disaccharides in the second reaction mixture;
a third step wherein the reaction is stopped when the disaccharides have been formed in the desired amount, by means of a treatment chosen between deactivation of β-D-galactosidase by freezing the second reaction mixture at a temperature between -20°C and -170°C, deactivation of β-D-galactosidase by heating the second reaction mixture at a temperature between 95 and 110°C, and separation of β-D-galactosidase from the second reaction mixture by ultrafiltration; obtaining a third reaction mixture;
a fourth step wherein an aglyconic fragment of the β-D-galactopyranoside substrate used in the first step is separated from the third reaction mixture by extraction or filtration; obtaining a fourth reaction mixture;
a fifth step comprising isolation of fractions that contain 4-0-β-D-galactopyranosyl-D-xylose, **characterized in that**, this isolation step is selected between:

i) addition of celite to the fourth reaction mixture, followed by solid-liquid extraction with a solvent and elution with a first eluent in a column or
ii) addition of active carbon to the fourth reaction mixture followed by filtration and elution with a second eluent

a sixth step, in which the fractions that contain 4-0-β-D-galactopyranosyl-D-xylose, are crystallized in a crystallization mixture selected between:

i) mixtures of acetone/methanol in a ratio between 5/1 to 20/1 or
ii) mixtures of acetone/water in a ratio between 5/1 to 20/1.

2. Process according to claim 1, **characterized in that** the fourth reaction mixture is concentrated before being subjected to elution in the column.

3. Processes according to claim 1, **characterized in that** the mixture of acetone/methanol has a ratio of 10/1.

4. Processes according to claim 1, **characterized in that** the mixture of acetone/water has a ratio of 10/1.

5. Processes according to claim 1, **characterized in that** the first eluent is a mixture of water/isopropanol that contains 1 to 10% (v/v) of isopropanol.

6. Processes according to claim 1, **characterized in that** the mixture of water/ isopropanol contains 2% (v/v) of isopropanol.

7. Process according to claim 1, **characterized in that** the fifth step consiste of adding celite to the fourth reaction mixture and concentrating to dryness, followed by solid-liquid extraction with an organic solvent in a Soxhlet extractor

that has a cartridge made 35 out of a material compatible with said organic solvent, and eluting with a first eluent in a column selected from: filtration columns with cross-linked dextrane polymer fillers, filtration columns with acrylamide polymer fillers, filtration columns of active carbon or active 5 carbon-celite columns.

8. Process according to claim 7, **characterized in that** the organic solvent is ethyl acetate.

9. Process according to claim 7, **characterized in that** the organic solvent is used in an amount comprised between 10 ml and 25 ml per gram of initial xylose.

10. Process according to claim 7, **characterized in** 15 that the celite is used in an amount comprised between 1 g and 2 g per gram of initial xylose.

11. Process according to claim 7, **characterized in that** the column is of active carbon-celite wherein the carbon is deactivated by adding 35% hydrochloric acid.

12. Process according to claim 11, **characterized in that** the celite is used in an amount comprised between 0.5 g and 2 g of celite per gram of initial xylose.

13. Process according to claim 11, **characterized in that** the active carbon is used in an amount comprised between 0.5 g and 2 g of active carbon per gram of initial xylose.

14. Process according to claim 7, **characterized in that** said first eluent is used in an amount comprised between 5 ml and 25 ml per gram of initial xylose.

15. Process according to claim 11, **characterized in that** the hydrochloric acid is used in an amount comprised between 0.5 ml and 1.5 ml per gram of initial xylose.

16. Process according to claim 1, **characterized in that** in the fifth step, the fourth reaction mixture is subjected to direct addition of at least a second eluent on the active carbon wherein the 4-O-β-D-galactopyranosyl-D-xylose is adsorbed and the second eluent is water followed by diluted isopropanol with a growing proportion in volume of isopropanol in successive steps.

17. Process according to claim 16, **characterized in that** the proportion in volume of isopropanol is comprised between 1% and 3% in a first step, between 3% and 5% in a second step and between 5% and 7% in a third step.

18. Process according to claim 16, **characterized in that** the active carbon is used in an amount comprised between 2 g and 4 g of active carbon per gram of initial xylose.

19. Process according to claim 16, **characterized in that** the second eluent is used in a total amount comprised between 30 ml and 50 ml of second eluent per gram of initial xylose.

20. Process according to claim 1 or 16, **characterized in that** the reaction is stopped by cooling the second reaction mixture at 0°C.

21. Process according to claim 1, 16 and 20, 35 **characterized in that** the fourth reaction mixture is obtained by separating the aglyconic fragment from the β-D-galactopyranoside substrate by means of filtration.

22. Process according to claim 1, **characterized in** 5 that the proportion of D-xylose in the second reaction mixture is 7.5% by weight.

23. Process according to claim 1, **characterized in that** the proportion of (β-D-galactopyranoside in the 10 second reaction mixture is 1.5% by weight.

24. Process according to claim 1, **characterized in that** 20 units of β-D-galactosidase per gram of R-D-galactopyranoside are added.

25. Process according to claim 1, **characterized in that** the reaction medium also comprises at least a cosolvent medium selected among dimethylsulfoxide, dimethylformamide, dioxane and mixtures thereof.

26. Process according to claim 25, **characterized in that** the reaction medium comprises 20% by weight of the cosolvent medium.

27. Process according to claim 1, **characterized in that** the reaction is carried out at a constant temperature.

28. Process according to claim 1 or 27, 30 **characterized in that** the reaction temperature is from - 5°C to 40°C.

29. Process according to claim 1 or 27, **characterized in that** the reaction temperature is higher than the freezing temperature of the second mixture and lower than 0°C.

30. Process according to claim 1, 28 or 29, **characterized in that** the reaction temperature is -5°C.

31. Process according to claim 1 or 28, **characterized in that** the reaction temperature is room temperature.

32. Process according to claim 1, 26 or 27, **characterized in that** the reaction medium is buffered to a pH of 7.

33. Process according to claim 1, **characterized in** 15 that, in the third step., the reaction is stopped by freezing the second reaction mixture at a temperature of 78°C.

34. Process according to claim 1, **characterized in that**, in the third step, the reaction is stopped by heating the second reaction mixture up to a temperature of 100°C.

35. Process according to claim 1, **characterized in** 25 that, in the third step, the reaction is stopped by separating the β-D-galactosidase by ultrafiltration.

36. Process according to claim 1, **characterized in that** the β-D-galactopiranoside substrate is selected 30 between o-nitrophenyl (β-D-galactopiranoside and lactose.

37. Process according to claim 1, **characterized in that** the (3-D-galactosidase enzyme is *E. coli* β-D-galactosidase.

38. Process according to claim 1, **characterized in that** the β-D-galactosidase enzyme is *Kluyveramyces lactis* β-D-galactosidase.

Patentansprüche

1. Enzymatisches Verfahren zum Erhalt von 4-O-β-D-Galactopyranosyl-D-xylose, umfassend:

einen ersten Schritt des Herstellens eines ersten Reaktionsgemisches aus
2 bis 20 Gew.-% D-Xylose,
0,5 bis 5 Gew.-% eines 13-D-Galactopyranosid-Substrats,
75 bis 97,5 Gew.-% eines Reaktionsmediums, das gepuffertes Wasser mit einem pH zwischen 5,0 und 9,0 umfasst;
Zugabe von 10 bis 1.000 Einheiten eines β-D-Galactosidase-Enzyms pro Gramm β-D-Galactopyranosid zu dem ersten Reaktionsgemisch; und Erhalt eines zweiten Reaktionsgemisches;
einen zweiten Schritt, in dem das zweite Reaktionsgemisch einer Umsetzung bei einer Temperatur zwischen einer Temperatur, die höher ist als der Gefrierpunkt des zweiten Reaktionsgemisches, und 45°C 2 bis 48 Stunden lang unterzogen wird, um Disaccharide in dem zweiten Reaktionsgemisch zu bilden;
einen dritten Schritt, in dem die Reaktion, nachdem die Disaccharide in der gewünschten Menge gebildet wurden, mit Hilfe einer Behandlung gestoppt wird, die ausgewählt ist aus Desaktivierung der β-D-Galactosidase durch Tiefkühlen des zweiten Reaktionsgemisches bei einer Temperatur zwischen -20°C und -170°C, Desaktivierung der β-D-Galactosidase durch Erwärmen des zweiten Reaktionsgemisches bei einer Temperatur zwischen 95 und 110°C, und Abtrennen der β-D-Galactosidase von dem zweiten Reaktionsgemisch durch Ultrafiltration; Erhalt eines dritten Reaktionsgemisches; einen vierten Schritt, in dem ein Aglycon-Fragment des in dein ersten Schritt verwendeten β-D-Galactopyranosid-Substrats von dem dritten Reaktionsgemisch durch Extraktion oder Filtration abgetrennt wird; Erhalt eines vierten Reaktionsgemisches;
einen fünften Schritt, umfassend die Isolierung von Fraktionen, die 4-O-β-D-Galactopyranosyl-D-xylose enthalten, **dadurch gekennzeichnet, dass** dieser Isolationsschritt ausgewählt ist aus:

i) Zugabe von Celite zu dem vierten Reaktionsgemisch, gefolgt von Fest-Flüssig-Extraktion mit einem Lösungsmittel und Eluieren mit einem ersten Eluierungsmittel in einer Säule oder
ii) Zugabe von Aktivkohle zu dem vierten Reaktionsgemisch, gefolgt von Filtration und Eluieren mit einem zweiten Eluierungsmittel;

einen sechsten Schritt, in dem die Fraktionen, die 4-O-β-D-Galactopyranosyl-D-xylose enthalten, in einem Kristallisationsgemisch kristallisiert werden, das ausgewählt ist aus:

i) Gemischen von Aceton/Methanol in einem Verhältnis zwischen 5/1 bis 20/1 oder
ii) Gemischen von Aceton/Wasser in einem Verhältnis zwischen 5/1 bis 20/1.

2. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das vierte Reaktionsgemisch konzentriert wird, bevor es einer Elution in der Säule unterzogen wird.

3. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das Gemisch von Aceton/Methanol ein Verhältnis von 10/1 aufweist.

4. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das Gemisch von Aceton/Wasser ein Verhältnis von 10/1 aufweist.

5. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das erste Eluierungsmittel ein Gemisch von Wasser/Isopropanol ist, das 1 bis 10% (Vol./Vol.) Isopropanol enthält.

6. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das Gemisch von Wasser/Isopropanol 2% (Vol./Vol.) Isopropanol enthält.

7. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** der fünfte Schritt in der Zugabe von Celite zu dem vierten Reaktionsgemisch und Konzentrieren bis zur Trockne, gefolgt von Fest-Flüssig-Extraktion mit einem organischen Lösungsmittel in einem Soxhlet-Apparat mit einer Patrone aus einem Material, das mit dem organischen Lösungsmittel kompatibel ist, und Eluieren mit einem ersten Eluierungsmittel in einer Säule, ausgewählt aus Filtrationssäulen mit vemetzten Dextranpolymer-Füllstoffen, Filtrationssäulen mit Acrylamidpolymer-Füllstoffen, Filtrationssäulen aus Aktivkohle oder Aktivkohle-Celite-Säulen, besteht.

8. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** das organische Lösungsmittel Ethylacetat ist.

9. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** das organische Lösungsmittel in einer Menge zwischen 10 ml und 25 ml pro Gramm anfänglicher Xylose verwendet wird.

10. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** das Celite in einer Menge zwischen 1 g und 2 g pro Gramm anfänglicher Xylose verwendet wird.

11. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** die Säule aus Aktivkohle-Celite besteht, wobei der Kohlenstoff durch Zugabe von 35% Salzsäure desaktiviert wird.

12. Verfahren nach Anspruch 11, **dadurch gekennzeichnet, dass** das Celite in einer Menge zwischen 0,5 g und 2 g Celite pro Gramm anfänglicher Xylose verwendet wird.

13. Verfahren nach Anspruch 11, **dadurch gekennzeichnet, dass** die Aktivkohle in einer Menge zwischen 0,5 g und 2 g Aktivkohle pro Gramm anfänglicher Xylose verwendet wird.

14. Verfahren nach Anspruch 7, **dadurch gekennzeichnet, dass** das erste Eluierungsmittel in einer Menge zwischen 5 ml und 25 ml pro Gramm anfänglicher Xylose verwendet wird.

15. Verfahren nach Anspruch 11, **dadurch gekennzeichnet, dass** die Salzsäure in einer Menge zwischen 0,5 ml und 1,5 ml pro Gramm anfänglicher Xylose verwendet wird.

16. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** im fünften Schritt das vierte Reaktionsgemisch einer direkten Zugabe mindestens eines zweiten Eluierungsmittels auf die Aktivkohle unterzogen wird, wobei die 4-O-

β-D-Galactopyranosyl-D-xylose adsorbiert wird und das zweite Eluierungsmittel Wasser, gefolgt von verdünntem Isopropanol ist, wobei der Volumenanteil an Isopropanol in aufeinanderfolgenden Schritten zunimmt.

17. Verfahren nach Anspruch 16, **dadurch gekennzeichnet, dass** der Volumenanteil an Isopropanol zwischen 1% und 3% in einem ersten Schritt, zwischen 3% und 5% in einem zweiten Schritt und zwischen 5% und 7% in einem dritten Schritt beträgt.

18. Verfahren nach Anspruch 16, **dadurch gekennzeichnet, dass** die Aktivkohle in einer Menge zwischen 2 g und 4 g Aktivkohle pro Gramm anfänglicher Xylose verwendet wird.

19. Verfahren nach Anspruch 16, **dadurch gekennzeichnet, dass** das zweite Eluierungsmittel in einer Gesamtmenge zwischen 30 ml und 50 ml des zweiten Eluierungsmittels pro Gramm anfänglicher Xylose verwendet wird.

20. Verfahren nach Anspruch 1 oder 16, **dadurch gekennzeichnet, dass** die Reaktion durch Abkühlen des zweiten Reaktionsgemisches auf 0°C gestoppt wird.

21. Verfahren nach Anspruch 1, 16 und 20, **dadurch gekennzeichnet, dass** das vierte Reaktionsgemisch durch Abtrennen des Aglycon-Fragments von dem β-D-Galactopyranosid-Substrat mittels Filtration erhalten wird.

22. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** der Anteil an D-Xylose in dem zweiten Reaktionsgemisch 7,5 Gew.-% beträgt.

23. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** der Anteil an β-D-Galactopyranosid in dem zweiten Reaktionsgemisch 1,5 Gew.-% beträgt.

24. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** 20 Einheiten β-D-Galactosidase pro Gramm R-D-Galactopyranosid zugegeben werden.

25. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das Reaktionsmedium auch mindestens ein Colösungsmittel-Medium, ausgewählt aus Dimethylsulfoxid, Dimethylformamid, Dioxan und Gemischen davon, umfasst.

26. Verfahren nach Anspruch 25, **dadurch gekennzeichnet, dass** das Reaktionsmedium 20 Gew.-% des Colösungsmittel-Mediums umfasst.

27. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Reaktion bei konstanter Temperatur durchgeführt wird.

28. Verfahren nach Anspruch 1 oder 27, **dadurch gekennzeichnet, dass** die Reaktio nstemperatur-5°C bis 40°C beträgt.

29. Verfahren nach Anspruch 1 oder 27, **dadurch gekennzeichnet, dass** die Reaktionstemperatur höher als der Gefrierpunkt des zweiten Gemischs und niedriger als 0°C ist.

30. Verfahren nach Anspruch 1, 28 oder 29, **dadurch gekennzeichnet, dass** die Reaktionstemperatur -5°C beträgt.

31. Verfahren nach Anspruch 1 oder 28, **dadurch gekennzeichnet, dass** die Reaktionstemperatur Raumtemperatur ist.

32. Verfahren nach Anspruch 1, 26 oder 27, **dadurch gekennzeichnet, dass** das Reaktionsmedium auf einen pH von 7 gepuffert ist.

33. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Reaktion im dritten Schritt durch Tiefkühlen des zweiten Reaktionsgemisches bei einer Temperatur von 78°C gestoppt wird.

34. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Reaktion im dritten Schritt durch Erwärmen des zweiten Reaktionsgemisches bis zu einer Temperatur von 100°C gestoppt wird.

35. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** die Reaktion im dritten Schritt durch Abtrennen der β-D-Galactosidase durch Ultrafiltration gestoppt wird.

36. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das β-D-Galactopyranosid-Substrat aus o-Nitrophenyl-β-D-Galactopyranosid und Lactose ausgewählt ist.

37. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das 3-D-Galactosidase-Enzym E. coli-β-D-Galactosidase ist.

38. Verfahren nach Anspruch 1, **dadurch gekennzeichnet, dass** das β-D-Galactosidase-Enzym Kluyveramyces lactis-β-D-Galactosidase ist.

Revendications

1. Procédé enzymatique d'obtention de 4-O-β-D-galacto-pyranosyl-D-xylose, qui comprend :

une première étape de préparation d'un premier mélange réactionnel de :

2 à 20% en poids de D-xylose,
0,5 à 5% en poids d'un substrat de β-D-galactopyranoside,
75 à 97,5% en poids d'un milieu réactionnel qui comprend de l'eau tamponnée à un pH compris entre 5,0 et 9,0 ;
l'addition de 10 à 1.000 unités d'une enzyme β-D-galactosidase par gramme de β-D-galactopyranoside au premier mélange réactionnel ; et l'obtention d'un deuxième mélange réactionnel,

une deuxième étape dans laquelle le deuxième mélange réactionnel est soumis à une réaction à une température comprise entre une température supérieure au point de congélation du deuxième mélange réactionnel et 45°C, pendant 2 à 48 heures, pour former des disaccharides dans le deuxième mélange réactionnel ;
une troisième étape dans laquelle la réaction est stoppée lorsque les disaccharides ont été formés en une quantité désirée, au moyen d'un traitement choisi entre une désactivation de la β-D-galactosidase par congélation du deuxième mélange réactionnel à une température comprise entre -20°C et -170°C, une désactivation de la β-D-galactosidase par chauffage du deuxième mélange réactionnel à une température comprise entre 95 et 110°C, et une séparation de la β-D-galactosidase à partir du deuxième mélange réactionnel par ultrafiltration ; de façon à obtenir un troisième mélange réactionnel ;
une quatrième étape dans laquelle un fragment aglyconique du substrat de β-D-galactopyranoside utilisé dans la première étape est séparé du troisième mélange réactionnel par extraction ou filtration; de façon à obtenir un quatrième mélange réactionnel ;
une cinquième étape comprenant l'isolement des fractions qui contiennent du 4-O-β-D-galactopyranosyl-D-xylose, **caractérisé en ce que** cette étape d'isolement est choisie entre :

i) l'addition de célite au quatrième mélange réactionnel, suivie d'une extraction solide-liquide avec un solvant et d'une élution avec un premier éluant dans une colonne, ou
ii) l'addition de charbon actif au quatrième mélange réactionnel suivie d'une filtration et d'une élution avec un second éluant ;

une sixième étape dans laquelle les fractions qui contiennent du 4-O-β-D-galactopyranosyl-D-xylose sont cristallisées dans un mélange de cristallisation choisi entre :

i) des mélanges d'acétone et de méthanol selon un rapport compris entre 5/1 et 20/1, ou
ii) des mélanges d'acétone et d'eau selon un rapport compris entre 5/1 et 20/1.

2. Procédé selon la revendication 1, **caractérisé en ce que** le quatrième mélange réactionnel est concentré avant d'être soumis à une élution dans la colonne.

3. Procédé selon la revendication 1, **caractérisé en ce que** le mélange d'acétone et de méthanol a un rapport de 10/1.

4. Procédé selon la revendication 1, **caractérisé en ce que** le mélange d'acétone et d'eau a un rapport de 10/ 1.

5. Procédé selon la revendication 1, **caractérisé en ce que** le premier éluant est un mélange d'eau et d'isopropanol qui contient 1 à 10% (en volume/volume) d'isopropanol.

6. Procédé selon la revendication 1, **caractérisé en ce que** le mélange d'eau et d'isopropanol contient 2% (en volume/volume) d'isopropanol.

7. Procédé selon la revendication 1, **caractérisé en ce que** la cinquième étape consiste en une addition de célite au quatrième mélange réactionnel et en une concentration à siccité, suivies d'une extraction solide-liquide avec un solvant organique dans un extracteur Soxhlet qui a une cartouche faite d'un matériau compatible avec ledit solvant organique, et une élution avec un premier éluant dans une colonne choisie parmi des colonnes de filtration avec des charges de polymère de dextrane réticulé, des colonnes de filtration avec des charges de polymère acrylamide, des colonnes de filtration de charbon actif ou des colonnes de célite-charbon actif

8. Procédé selon la revendication 7, **caractérisé en ce que** le solvant organique est l'acétate d'éthyle.

9. Procédé selon la revendication 7, **caractérisé en ce que** le solvant organique est utilisé en une quantité comprise entre 10 ml et 25 ml par gramme de xylose initial.

10. Procédé selon la revendication 7, **caractérisé en ce que** la célite est utilisée en une quantité comprise entre 1 g et 2 g par gramme de xylose initial.

11. Procédé selon la revendication 7, **caractérisé en ce que** la colonne est une colonne de célite-charbon actif dans laquelle le charbon est désactivé par l'addition d'acide chlorhydrique à 35%.

12. Procédé selon la revendication 11, **caractérisé en ce que** la célite est utilisée en une quantité comprise entre 0,5 g et 2 g de célite par gramme de xylose initial.

13. Procédé selon la revendication 11, **caractérisé en ce que** le charbon actif est utilisé en une quantité comprise entre 0,5 g et 2 g de charbon actif par gramme de xylose initial.

14. Procédé selon la revendication 7, **caractérisé en ce que** ledit premier solvant est utilisé en une quantité comprise entre 5 ml et 25 ml par gramme de xylose initial.

15. Procédé selon la revendication 11, **caractérisé en ce que** l'acide chlorhydrique est utilisé en une quantité comprise entre 0,5 ml et 1,5 ml par gramme de xylose initial.

16. Procédé selon la revendication 1, **caractérisé en ce que** dans la cinquième étape, le quatrième mélange réactionnel est soumis à l'addition directe d'au moins un deuxième éluant sur le charbon actif dans lequel le 4-O-β-D-galacto-pyranosyl-D-xylose est adsorbé et le deuxième éluant est de l'eau, puis de l'isopropanol dilué avec une proportion croissante en volume d'isopropanol dans des étapes successives.

17. Procédé selon la revendication 16, **caractérisé en ce que** la proportion en volume d'isopropanol est comprise entre 1 % et 3% dans une première étape, entre 3% et 5% dans une deuxième étape et entre 5% et 7% dans une troisième étape.

18. Procédé selon la revendication 16, **caractérisé en ce que** le charbon actif est utilisé en une quantité comprise entre 2 g et 4 g de charbon actif par gramme de xylose initial.

19. Procédé selon la revendication 16, **caractérisé en ce que** le deuxième éluant est utilisé en une quantité totale comprise entre 30 ml et 50 ml du deuxième éluant par gramme de xylose initial.

20. Procédé selon la revendication 1 ou 16, **caractérisé en ce que** la réaction est stoppée par refroidissement du deuxième mélange réactionnel à 0°C.

21. Procédé selon les revendications 1, 16 et 20, **caractérisé en ce que** le quatrième mélange réactionnel est obtenu par séparation du fragment aglyconique du substrat de β-D-galactopyranoside au moyen d'une filtration.

22. Procédé selon la revendication 1, **caractérisé en ce que** la proportion de D-xylose dans le deuxième mélange réactionnel est de 7,5% en poids.

23. Procédé selon la revendication 1, **caractérisé en ce que** la proportion de β-D-galactopyranoside dans le deuxième

mélange réactionnel est de 1,5% en poids.

24. Procédé selon la revendication 1, **caractérisé en ce que** 20 unités de β-D-galactosidase par gramme de β-D-galactopyranoside sont ajoutées.

25. Procédé selon la revendication 1, **caractérisé en ce que** le milieu réactionnel comprend aussi au moins un milieu cosolvant choisi parmi le diméthyl-sulfoxyde, le diméthylformamide, le dioxane et leurs mélanges.

26. Procédé selon la revendication 25, **caractérisé en ce que** le milieu réactionnel comprend 20% en poids du milieu cosolvant.

27. Procédé selon la revendication 1, **caractérisé en ce que** la réaction est conduite à une température constante.

28. Procédé selon la revendication 1 ou 27, **caractérisé en ce que** la température de réaction est comprise entre -5°C et 40°C.

29. Procédé selon la revendication 1 ou 27, **caractérisé en ce que** la température de réaction est supérieure à la température de congélation du deuxième mélange et inférieure à 0°C.

30. Procédé selon les revendications 1, 28 ou 29, **caractérisé en ce que** la température de réaction est de -5°C.

31. Procédé selon la revendication 1 ou 28, **caractérisé en ce que** la température de réaction est la température ambiante.

32. Procédé selon la revendication 1, 26 ou 27, **caractérisé en ce que** le milieu réactionnel est tamponné à un pH de 7.

33. Procédé selon la revendication 1, **caractérisé en ce que** dans la troisième étape, la réaction est stoppée par congélation du deuxième mélange réactionnel à une température de -78°C.

34. Procédé selon la revendication 1, **caractérisé en ce que** dans la troisième étape, la réaction est stoppée par chauffage du deuxième mélange réactionnel jusqu'à une température de 100°C.

35. Procédé selon la revendication 1, **caractérisé en ce que** dans la troisième étape, la réaction est stoppée par séparation de la β-D-galactosidase par ultrafiltration.

36. Procédé selon la revendication 1, **caractérisé en ce que** le substrat de β-D-galactopyranoside est choisi entre l'o-nitrophényl β-D-galactopyranoside et le lactose.

37. Procédé selon la revendication 1, **caractérisé en ce que** l'enzyme 3-D-galactosidase est la β-D-galactosidase de *E. coli.*

38. Procédé selon la revendication 1, **caractérisé en ce que** l'enzyme β-D-galactosidase est la β-D-galactosidase de *Kluyveramyces lactis.*