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(54) Title: ENZYME CATALYZED DE-ACYLATION OF CHLORINATED SUGAR DERIVATIVES

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A process of production of trichlorogalactosucrose is described in which deacylation of sucrose-6-ester is achieved by subjecting the reaction mixture after chlorination, neutralization and adjustment of pH to 6.5 to 7 to deacylation by using a lipase or a protease, in a free or in an immobilized form.

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(54) Title: ENZYME CATALYZED DE-ACYLATION OF CHLORINATED SUGAR DERIVATIVES

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ENZYME CATALYZED DE-ACYLATION OF CHLORINATED SUGAR DERIVATIVES

TECHNICAL FIELD

The present invention relates to a novel process and a novel strategy for production of 1-6-Dichloro-1-6-DIDEOXY-β-Fructofuranasyl-4-chloro-4-deoxy-galactopyranoside (TGS) involving enzymatic deacylation of the 6-O-protected TGS obtained after the chlorination reaction.

BACKGROUND OF THE INVENTION

Strategies of prior art methods of production of 4,1', 6' trichlorogalactosucrose (TGS) predominantly involve chlorination of sucrose-6-ester by use of Vilsmeier Haack reagent derived from to chlorinate Sucrose-6-ester, to form 6 acetyl 4,1', 6'trichlorogalactosucrose, using various chlorinating agents such as phosphorus oxychloride, oxalyl chloride, phosphorus pentachloride etc, and a tertiary amide such as dimethylformamide (DMF). After the said chlorination reaction, the reaction mass is neutralized to pH 7.0 –7.5 using appropriate alkali hydroxides of calcium, sodium, etc. The pH of the neutralized mass is then further raised to 9.5 or above to deesterify / deacetylate the 6 acetyl 4,1', 6'trichlorogalactosucrose to form 4,1', 6' trichlorogalactosucrose using alkali hydroxides of calcium, sodium, potassium, etc. This alkaline de-esterification / deacylation involves exposure of the reactants to harsh pH in alkaline range which leads to destruction of a significant quantity of DMF, which is an expensive input, adversely affecting its recovery after the reaction.

In prior art process, the reaction mixture is also exposed during the process of deacylation to harsh temperatures which lead to destruction of the product TGS itself.

Hence, there is a need for a method of deacylation which shall not expose DMF to destruction.

A method has been developed to achieve deacylation enzymatically at a pH which does not expose DMF to destruction.

DETAILED DESCRIPTION OF THE INVENTION

Enzymatic deacylation has been reported by Palmer et al (1995) in a US patent no. 5445951 for the preparation of partially acylated derivatives of sucrose by the enzyme catalyzed deacylation of sucrose esters from a sucrose ester selected from the group consisting of sucrose octaacylate, sucrose heptaacylate, and sucrose hexaacylate in an anhydrous organic medium, with an enzyme or combination of enzymes capable of catalyzing the deacylation of said sucrose ester to produce a partially deacylated sucrose derivative having free hydroxyl group(s) in pre-selected position(s), and recovering the resulting partially deacylated sucrose derivative.

No other report is known on enzymatic deacylation of a sucrose ester or its derivative / a precursor.

This invention relates to the enzymatic deacylation of the 6-O-protected TGS obtained after the chlorination reaction during the preparation of the artificial sweetener, TGS. Embodiments of chlorination reaction mixture which can be subjected to the process described in this invention includes, without being limited to, a process stream obtained after mixing sucrose-6-

ester with a chlorinating agent described in Mufti et al. (1983) US patent no 4380476, Walkup et al. (1990 No.4980463), Jenner et al. (1982) US patent no. 4,362,869, Tulley et al. (1989) US pat no. 4,801,700, Rathbone et al. (1989) US pat no. 4,826,962, Bornemann et al. (1992) US pat no. 5,141,860, Navia et al. (1996) US Pat no. 5,498,709, Simpson (1989) US Pat no. 4,889,928, Navia (1990) US Pat no. 4,950,746, Neiditch et al. (1991) US Pat no. 5,023,329, Walkup et al. (1992) 5,089,608, Dordick et al. (1992) US pat no. 5,128,248, Khan et al. (1995) US Pat no. 5,440,026, Palmer et al. (1995) US Pat no. 5,445,951, Sankey et al. (1995) US Pat no. 5,449,772, Sankey et al. (1995) US Pat no. 5,470,969, Navia et al. (1996) US Pat no. 5,498,709, Navia et al.(1996) US Pat no. 5,530,106

The enzymatic deacylation is carried out on the process stream obtained in a way as mentioned above after neutralization of the chlorinated reaction mass after or without intermediate isolation of the 6-O-protected TGS. The solvent, tertiary amide present in the neutralized reaction mass doesn't get decomposed due to the enzymatic reaction and therefore results in enhanced recovery of the said solvent.

In this invention, the chlorinated reaction mass, after chlorination reaction is neutralized with a suitable base. When the pH is controlled during neutralization below 6.0, the compound TGS formed still has the protected group intact at the 6^{th} position. The deblocking of the 6^{th} position is carried out either with or without the isolation of the said compound. Further various references also point out that the deblocking can be carried out with or without the tertiary amide and other solvent and aqueous conditions.

The present invention describes the deacylation at the 6th position using an enzymatic process where in the enzyme selectively removes the protected group in the presence or absence of the tertiary amide, including DMF which is used in the chlorination reaction.

The process of this invention also works well for deacylation of embodiments which are not result of a chlorination reaction, such as a simple solution of pure TGS-6-ester.

Enzyme catalyzed deacylation is well known and the lipase and proteolytic enzymes carry out deacylation and acylation reactions under benign reaction conditions and is widely reported by Soedjak HS, Spradlin JE (1994). Biocatalysis 11: 241–248; Therisod M, Klibanov AM (1986) J. Am. Chem. Soc. 108: 5638 – 5640; B. Cambou and A.M. Klibanov, J. Am. Chem. SOC., 106,2687(1984); Kirpal S Bisht,Pure & Appl. Cbem., Vol. 68, No. 3, pp. 749-752, 1996; F.J. Plou1;_, M.A. Cruces1, Biotechnology Letters 21: 635–639, 1999.

In this invention, after the neutralization of the reaction mass, the pH is adjusted to 6.5 using an appropriate base. The lipase enzyme is then added to the reaction mass slowly under stirring at room temperature. The quantity of enzyme added to the reaction mass varies from 10% to 40% w/v depending on the enzyme activity and reaction conditions. The tertiary amide content in the neutralized reaction mass is around 10% to 40%. The reaction mixture is stirred continuously for a period of 10 to 60 hours, more preferably 16 to 20 hours. The conversion of the 6-O-protected TGS to TGS is monitored by TLC. After the complete deacylation, the reaction mixture is

taken for TGS isolation by affinity chromatography. The isolated TGS is then crystallized by suitable methods.

The use of the lipase or proteolytic enzymes for deacylation of 6-O-protected TGS to TGS can be in its native form or in immobilized form. When the immobilized enzyme is used, the enzyme is filtered off after the completion of the deacylation. This recovered enzyme can be further re-used. Also the immobilized enzyme can be packed in a column and the reaction mass can be passed through the column and *in situ* deacylation of the 6-O-protected TGS can be carried out. These enzymes can be immobilized in or on synthetic polymeric supports such as, but not limited to polyacrylic, or polystyrene or polyacrylamide, nylon based supports; or semisynthetic or natural organic supports like those based on polysaccharides such as, but not limited to cellulose, starch, dextran, agarose, chitosan, chitin, etc.; or inorganic supports like those based on carbon, silica, zirconia, alumina, zirconium phosphate, etc.

The source of the enzyme lipase can be of animal, plant or microbial origin, more preferably microbial or bacterial origin such as *Bacillus thermocatenulatusis*, *Pseudomonas aeruginosa,* etc., fungal origin such as *Penicillium Roquefortii, Asperigillus niger, Asperigillus oryzae, Rhizopus niveus, Candida rugosa, Rhizomucor miheii, Candida antartctica, etc.*

During the process of the said invention, the TGS product is not exposed to any harsh pH or temperature conditions as in the case of the conventional deacylation processes using acid, alkali. The product loss is the most minimal compared any other form of deacylation process.

During the process of the said invention, the tertiary amide is not exposed to any harsh pH or temperature conditions as in the case of the conventional deacylation processes using acid, alkali. Hence the decomposition of the tertiary amide doesn't take place at all. Therefore the efficiency of the recovery of the tertiary amide enhances to a very large extent.

Described in the following are examples, which illustrate working of this invention without limiting the scope of this invention in any manner. Reactants, proportion of reactants used, range of reaction conditions described, enzymes used and the like are only illustrative and the scope extends to their analogous reactants, reaction conditions and reactions of analogous generic nature. In general, any equivalent alternative which is obvious to a person skilled in art of clorinated sucrose production is covered within the scope of this specification. Thus, mention of an acetate covers any equivalent ester group which can perform the same function in the contest of this invention, and use of an enzyme shall cover any alternative capable of providing the action or analogous action of the enzyme described herein under analogous reaction conditions. Several other adaptations of the embodiments will be easily anticipated by those skilled in this art and they are also included within the scope of this specification. Mention in singular is construed to cover its plural also, unless the context does not permit so, viz: use of "an organic solvent" for extraction covers use of one or more of an organic solvent in succession or in a combination as a mixture.

EXAMPLE 1

Chlorination of sucrose-6-acetate

In a 5L reaction flask1280ml of Dimethylformamide was added and cooled to 0 – 5°C. This was followed by addition of 635g of Phosphorous pentachloride (5.4 moles) slowly under stirring, maintaining the temperature of the reaction mass below 30°C. The mass is further cooled to below 0°C and the sucose-6-acetate in DMF is added slowly at 0 –5°C. Then the reaction mass is heated to 80°C and held for 1 hour, further heated to 100°C and held for 6 hours and finally at 110 –115°C and held for 2 – 3 hours. The progress of the reaction is monitored by HPLC analysis.

Then the reaction mixture is cooled to -5 to - 8°C and a 20% solution of Sodium hydroxide is slowly added so as to bring the pH of the mass to 5.5 – 6.5. The yield obtained by this method was 55.4%of the sucrose input.

EXAMPLE 2

Enzymatic deacetylation of 6-O-acetyl TGS by lipase enzyme

The Reaction mass, 1.5 L, containing 15g of 6-O-acetylated TGS and prepared as described in Example 1 was neutralized using 50% calcium hydroxide slurry up to pH 7.5. The neutralized reaction mass was diluted to 6L using water. The DMF content was 33% in the neutralized mass. 84g of lipase enzyme isolated from *Aspergillus oryzae* ATCC 26850; NCIM 1212 was added to the reaction mixture with continuous stirring at ambient temperature. The reaction was continued for several hours and formation of TGS and disappearance of 6-O-acetylated TGS was monitored by TLC. At the end of 42 hours, deacylation upto 98.4 % was achieved.

After the deacylation, the mass was taken for isolation of TGS by suitable methods.

EXAMPLE 3

Enzymatic deacetylation of 6-O-acetyl TGS by immobilized lipase enzyme on Eudragit RL100

In an experiment, 2.5 L of reaction mass containing 80g of 6-O-acetylated TGS was neutralized using 50% calcium hydroxide slurry up to pH 5.5. The neutralized reaction mass was diluted to 6L using water. The DMF content was 33% in the neutralized mass. 120g of immobilized lipase on Eudragit RL100 was added to the reaction mixture with continuous stirring at 25°C - 30°C temperature, which is usually the ambient temperature. The reaction was continued for several hours and formation of TGS and disappearance of 6-O-acetylated TGS was monitored by TLC. At the end of 24 hours, deacylation up to 98.3% was achieved.

The mass was then filtered and taken for TGS isolation. The enzyme obtained in filter cake was washed with water and stored for reuse

EXAMPLE 4

Enzymatic deacetylation of 6-O-acetyl TGS by lipase enzyme immobilized on Eudragit RL 100, packed in a column

In an experiment, 12g of immobilized enzyme was packed on to a 2cm diameter and height 8cm glass column. The column inlet was connected to the delivery of a peristaltic pump and the outlet was connected to a flask containing 500 ml of neutralized mass which had 5 g of 6-O-acetyl-. The inlet of the peristaltic pump was also connected to the neutralized mass. The neutralized mass was circulated at 5 ml/min flow rate through the bed of immobilized lipase, for 6 hours.

The TLC was carried out every one hour to see the extent of deacetylation taking place in the flask. After 6 hours, deacetylation above 98% was observed.

After completion of deacetylation of 6-O-acetyl-TGS to TGS, the bed of immobilized enzyme was washed with de-ionized water and was stored under 10% Acetone in water until further use.

EXAMPLE 5

Enzymatic deacetylation of 6-O-acetyl TGS by Alcalase – a proteolytic enzyme

1.0 L of neutralized mass after chlorination, containing 10g of 6-O-acetylated TGS was taken up for the enzymatic reaction. The neutralized reaction mass was diluted to 3.0L using water. 200 ml of Alclalase 2.4L enzyme obtained commercially from Novozymes derived from B. lichenformis was added to the reaction mixture with continuous stirring at 25-30°C temperature. The reaction was continued for several hours and formation of TGS and disappearance of 6-O-acetylated TGS was monitored by TLC. At the end of 36 hours, deacylation upto 96.4 % was achieved. After the deacylation, the mass was taken for isolation of TGS by suitable methods.

AMENDED CLAIMS
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1. A process of production of a chlorinated sucrose compound in which a chlorinated derivative of a 6-O-protected sucrose in a solution is de-protected by use of an action of an enzyme capable of removing the protecting group.

2. A process of claim 1, wherein:

   a. the said 6-O-protected sucrose comprises one or more of a sucrose-6-acetate, sucrose-6-benzoate, sucrose-6-propionate, sucrose-6-laurate, sucrose-6-glutarate, Sucrose 6 palmitate and the like,

   b. the said solution includes a solution of pure chlorinated 6-O-protected sucrose, or (ii) a process stream obtained in a process of production of a chlorinated sucrose compound.

3. A process of claim 2 wherein:

   a. the said process stream comprises one or more of a process for production of a chlorinated sucrose including a process of chlorination of sucrose or a process of chlorination of a 6-O-protected sucrose , and

   b. the said chlorinated sucrose compound includes one or more of a chlorinated sucrose including a trichlorogalactosucrose, a dichlorogalactosucrose, a tetrachlorogalactosucrose and the like.

4. A process of claim 3 wherein the said process of chlorination comprises reacting a sucrose derivative with one or more of a chlorinating reagent by one or more of a process including:

a. a reaction of 6-O-protected sucrose dissolved in pyridine with sulphuryl chloride, or

b. a reaction of 6-O-protected sucrose with thionyl chloride in presence of triphenyl phosphine and 1,1,2-trichloroethane, or

c. a reaction of a 6-O-proetected sucrose, including a sucrose-6-ester, with a Vilsmeier reagent of a general formula [HClC=N.sup.+ R.sub.2]Cl.sup.-, or [HPOCl.sub.2.O.C.sup+=N.sup+.R.sub.2] Cl.sup.-, where R represents an alkyl group preferably a methyl or ethyl group.

5. A process of claim 4 wherein an action of an enzyme capable of removing the protecting group is derived from an enzyme lipase or a protease.

6. A process of claim 5 wherein the said lipase or protease is a free or immobilized enzyme.

7. A process of claim 6 comprising steps of:

a. chlorinating sucrose 6-acetate contained in (i) a solution or (ii) a process stream obtained in a process of production of chlorinated sucrose with a chlorinating reagent selected from the group consisting of a Vilsmeier reagent, sulphuryl chloride or thionyl chloride,

b. adjusting the pH of process stream of step (a.) of this claim to about pH 6.5 to 7,

c. deacylating 6-O-protected TGS formed in the process stream of step (a.) either (i) by bringing the same in contact with a free or immobilized lipase or a free or immobilized

protease, preferably accompanies by shaking in a reaction container or by recirculating through a bed of enzyme packed in a column, preferably at ambient temperature of around 25 to 30 degrees celcius for a period of time sufficient to achieve maximum possible above 95% deacylation,

d. separating TGS from one or more of an undesired component of the process stream of step (c.) of this claim.