Technical Note

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Biosynthesis of glucose-D-$^{13}C_6$ from *Hansenula polymorpha* and methanol-^{13}C

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A simple and effective method for synthesis of glucose-D-$^{13}C_6$ by fermentation using the methylotrophic yeast *Hansenula polymorpha* with 99% abundance methanol-^{13}C is described. Using methanol-^{13}C as a sole source of carbon, *H. polymorpha* can accumulate large amounts of α,α-trehalose-$^{13}C_{12}$ under unfavourable growth conditions; the trehalose can then be hydrolysed to give glucose-D-$^{13}C_6$ with 98.5% abundance ^{13}C.

Keywords: glucose-D-$^{13}C_6$; biosynthesis; methanol-^{13}C; trehalose-$^{13}C_{12}$

Introduction

Isotope-labelled D-glucose has been produced commercially by biosynthesis for many years and has especially been used for studying the *in vivo* metabolism of glucose.[1,2] The first labelled forms of D-glucose to be produced by biosynthesis contained the radioactive isotopes ^{11}C and ^{14}C. The pioneering work in this area was accomplished by Lifton and Welch, who prepared glucose-^{11}C using Swiss chard leaves and ^{11}C-labelled carbon dioxide through photosynthesis.[3] Ishiwata *et al*. have modified the Lifton and Welch method to achieve automation of the whole procedure.[4] The use of broad bean or algae has also been reported.[5,6] Enzymatic synthesis is an alternative approach that has been used for preparing isotope-labelled forms of D-glucose; for example, Sturani prepared 3-^{14}C-labelled D-glucose and 4-^{14}C-labelled D-glucose by the action of ribulose diphosphate carboxylase on ribulose-1,5-diphosphate and ^{14}C-labelled carbon dioxide to give 3-phosphoglyceric acid-1-^{14}C followed by its conversion to D-glucose by reversing glycolytic enzyme reactions.[7] A number of biosynthetic methods have been used to prepare ^{13}C-labelled forms of D-glucose. Li *et al*. developed a method for preparing glucose-D-$^{13}C_6$ by growth of microalgae in a photobiological reactor with ^{13}C-labelled carbon dioxide as the carbon source.[8] Luthra *et al*. prepared ^{13}C-labelled D-glucose (60 atom %) by photosynthesis using algae and ^{13}C-labelled carbonate (90 atom %) as carbon source.[9] Baxter *et al*. synthesized D-glucose-3, 4-$^{13}C_2$ (60 atom % $^{13}C_2$) *via* α,α-trehalose-^{13}C that was produced from sodium acetate-1-^{13}C (98 atom %) by sporulating yeast.[10] A drawback in many of the published methods is that the label becomes diluted and those that utilize photosynthesis require complex apparatus to avoid dilution or loss of the labelled carbon dioxide, and it is very difficult to reproduce for scaling up the process. Following work by Jones and Bellion, who investigated the major metabolic pathways of methanol utilisation in the methylotrophic yeast *Hansenula polymorpha* (also known as *Pichia angusta*) by ^{13}C NMR, [11] we recently reported how fermentation with this organism using ^{13}C-labelled methanol as carbon source can be used to produce ^{13}C- labelled α,α-trehalose.[12] In the current work, we describe the full method for preparing and isolating α,α-trehalose-$^{13}C_{12}$ followed by its chemical hydrolysis to give glucose-D-$^{13}C_6$ with 98.5% abundance ^{13}C.

Experimental

General

Methanol-^{13}C (99 atom %) was prepared at the Shanghai Engineering Research Center of Stable Isotope. *H. polymorpha* (ATCC26012) was purchased from ATCC (Manassas, USA). The labelled glucose and trehalose were analysed using a Waters Quattro Premier XE VAB430 LCMS/MS (Waters Corporation, Milford, MA, USA) with a Pinnacle II amino column and refractive index detector. The mobile phase was a 30:70 v/v mixture of water and acetonitrile at a flow rate of 1 ml/min. The ^{13}C abundance was measured using MAT 271 mass spectrometer (Pacific Northwest National Laboratory, Richland, WA, USA). Fourier Transform InfraRed spectra were obtained using a Nicolet 6700 instrument (Thermo Scientific, Rochester, NY, USA). NMR spectra were recorded on a Bruker Avance 500 (Bruker BioSpin Corporation, Billerica, MA, USA) instrument operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR.

Fermentation conditions for ^{13}C-labelling

Hansenula polymorpha was inoculated into the seed medium containing (per litre): methanol-^{13}C (10 ml), $(NH_4)_2SO_4$ (10 g), yeast nitrogen base without amino acids and ammonium sulphate (20 g) and biotin (0.4 mg) in 1 M K_2HPO_4-KH_2PO_4 buffer (pH 6.0). The cultures were grown at 37 °C and 150 rpm for 18 h, then 5 ml was inoculated into 50 ml of the fermentation medium containing (per litre): methanol-^{13}C (10 ml), H_3PO_4 (30 ml), $CaSO_4$ (0.06 g), K_2SO_4 (0.96 g), $MgSO_4$ (3.26 g) and KOH (0.03 g) and trace elements (2 ml) in deionized water with the pH adjusted to 5.0. The fermentation was performed at 37 °C and 150 rpm for 24 h; the temperature was then raised to 42 °C and held constant for 3 h.

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Isolation of trehalose-$^{13}C_{12}$

Cells were harvested from 100 ml broth by centrifugation (4000 × g, 20 min, 0 °C), washed twice with distilled water (20 ml) and extracted with 40% (v/v) ethanol (20 ml). After 30 min at 90 °C, the material was centrifuged (4000 × g, 20 min, 0 °C), 10% $ZnSO_4$ (1 ml) and saturated BaOH solution (2 ml) was added to the supernatant liquor, protein was removed by centrifugation (4000 × g, 20 min, 0 °C), the soluble fraction being adjusted to pH 6.0 with 1 M NaOH and passed through a mixed column of D201 and D101 resin (2:1) eluted with water. The eluate was concentrated under vacuum on a rotary evaporator, and the residue was crystallised from aqueous alcohol affording the dihydrate of α,α-trehalose-$^{13}C_{12}$ (85 mg, 0.22mmol). The conversion ratio of the ^{13}C atoms from the labelled methanol into trehalose is around 11%. The ^{13}C atoms in the thalli and unreacted methanol-^{13}C were recycled; the recovery rate of the ^{13}C atoms was 75%. Mp 95–97 °C (Lit. 97.0 °C);[13] IR (KBr): 3416, 2933, 1637, 1458, 1355, 1241, 1149, 1100, 1030, 996, 955, 910, 811, 803, 537; MS(m/z): 353, 185, 167, 131.

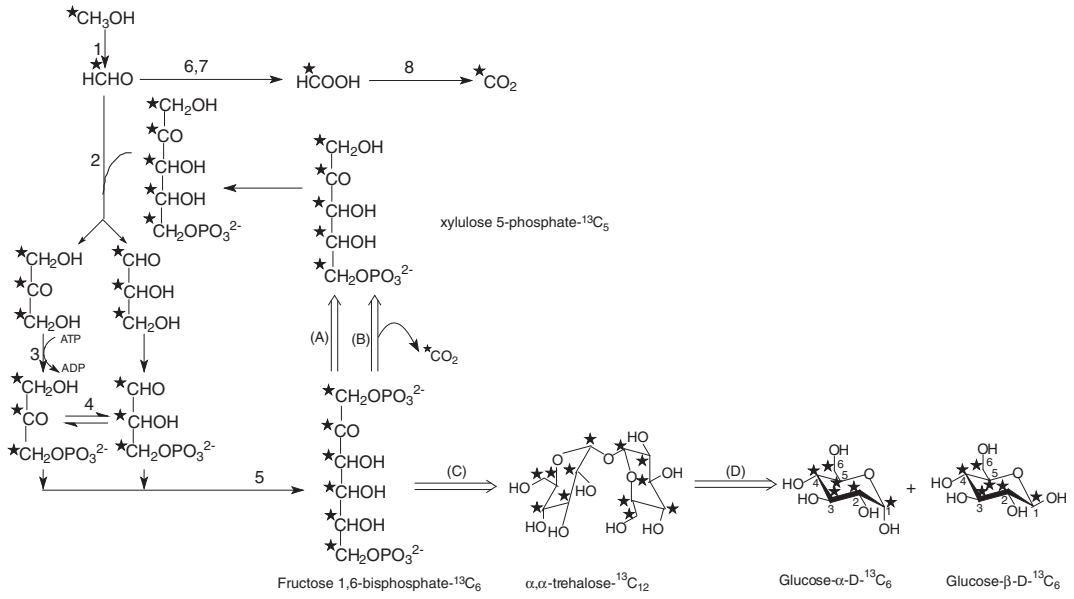
Synthesis of glucose-D-$^{13}C_6$

^{13}C-enriched α,α-trehalose dihydrate (50 mg, 0.13mmol) was dissolved in aqueous 2 M H_2SO_4 (2 ml) and heated in a sealed tube at 100 °C for 6 h. The solution was neutralised with saturated aqueous $BaCO_3$ and filtered. The pH of the filtrate was adjusted to 6.0 with 1 M NaOH and passed through a column of SK1B resin (H^+), and the effluent was subsequently passed through a column of WA30 resin (OH^-) and eluted with water. The eluate was concentrated under vacuum on a rotary evaporator affording glucose-D-$^{13}C_6$ (42 mg, 0.23mmol, 89%) with 98.5% abundance ^{13}C. Mp 149–152 °C (Lit. 150–152 °C [14]); IR (KBr): 3415, 2932, 1637, 1369, 1120, 1083, 1053, 1008, 894, 824, 627; MS (m/z): 185, 154, 123, 92; ^1H NMR (500 MHz, D_2O) δ (ppm): 5.16 (d, J = 170 Hz, H1α), 4.55 (d, J = 152 Hz, H1β), 4.0–3.0 (6H, m, H2α, β; H3α, β; H4α, β; H5α, β; H6α, β). ^{13}C NMR (125 MHz, D_2O) δ (ppm): 95.4 (d, J = 46 Hz, C1β), 91.6 (d, J = 40 Hz, C1α), 75.4 (m, C5β), 75.3 (m, C3β), 73.6 (ddd, J = 45 Hz, 39 Hz and 2.5 Hz, C2β), 72.3 (dd, J = 38 Hz and 38 Hz, C3α), 70.9 (m, C2α, C5α), 69.1 (m, C4α, C4β), 60.3 (d, J = 43 Hz, C6β), 60.0 (d, J = 43 Hz, C6α).

Results and discussion

The methylotrophic yeast *H. polymorpha* uses methanol as a sole source of carbon and energy; α,α-trehalose can be accumulated at high levels when the cells are exposed to unfavourable growth conditions such as high temperature, high osmolarity and nutrient limitations.[15] The work by Jones and Bellion[11] showed that dilution of ^{13}C-labelled α,α-trehalose is obtained when the cells are cultivated in fermentation medium with methanol-^{13}C as the source of carbon, and our recent work has shown how this can be used to synthesize and isolate almost no dilution of ^{13}C-labelled α,α-trehalose on a hundreds of milligrammes scale when the cells are cultivated in the seed and fermentation medium with methanol-^{13}C as the sole source of carbon. [12] In natural substrates, α,α-trehalose is hydrolysed by the enzyme trehalase to give equimolar quantities of α-D-glucose and β-D-glucose;[16] we therefore developed a method for producing ^{13}C-labelled D-glucose by combining the biosynthesis of α,α-trehalose-$^{13}C_{12}$ from *H. polymorpha* and methanol-^{13}C followed by its hydrolysis.

Scheme 1 shows the biosynthetic pathway to ^{13}C-labelled α,α-trehalose from methanol-^{13}C in *H. polymorpha* and its subsequent hydrolysis to glucose-D-$^{13}C_6$, which we chose to perform using a chemical method. From the cultures of *H. polymorpha* grown with methanol-^{13}C as the sole carbon source, we isolated 85 mg of the dihydrate of α,α-trehalose-$^{13}C_{12}$ from a cell volume of 100 ml. Eighty-five milligrammes of the dihydrate of α,α-trehalose-$^{13}C_{12}$ needed 86 mg of the labelled methanol in theory. The conversion ratio of the ^{13}C atoms from the labelled methanol into trehalose is around 11%. We also recycled ^{13}C atoms from the thalli and unreacted methanol-^{13}C with a recovery rate of 75%. Chemical



Scheme 1. Biosynthetic route to α,α-trehalose-$^{13}C_{12}$ from methanol-^{13}C in *Hansenula polymorpha* and its chemical hydrolysis to glucose-D-$^{13}C_6$. Bold asterisks indicate ^{13}C-labelling sites. Key to enzymes: 1, alcohol oxidase; 2, dihydroxyacetone synthase; 3, dihydroxyacetone kinase; 4, triose-phosphate isomerase; 5, aldolase; 6, formaldehyde dehydrogenase; 7, formylglutathione hydrolase; 8, formate dehydrogenase. Major metabolic routes depicted are: (A) re-arrangements via transaldolase and transketolase; (B) pentose phosphate pathway; (C) α,α-trehalose-$^{13}C_{12}$ synthesis; (D) chemical hydrolysis.

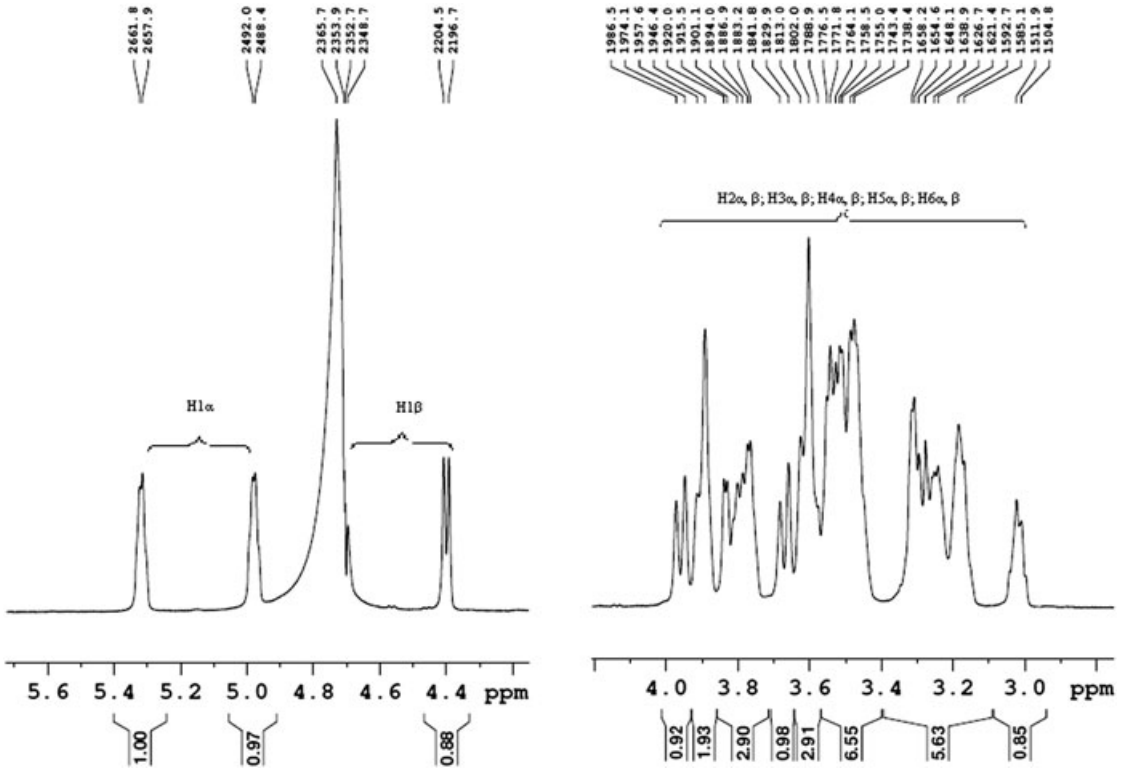


Figure 1. ^{1}H NMR spectrum of glucose-$^{13}C_6$.

hydrolysis of 50 mg α,α-trehalose-$^{13}C_{12}$ led to isolation of 42 mg glucose-D-$^{13}C_6$ with a yield of 89% and 98.5% abundance ^{13}C. The yield for conversion of ^{13}C atoms from methanol-^{13}C into glucose-D-$^{13}C_6$ is therefore 10%. ^{1}H and ^{13}C NMR spectra for the isolated glucose-D-$^{13}C_6$ are shown in Figures 1 and 2, respectively; both have the expected splitting patterns for the uniformly ^{13}C-labelled compound. Figure 3 shows HPLC spectra of glucose-D-$^{13}C_6$. By combining the biosynthesis of α,α-trehalose-$^{13}C_{12}$ by *H. polymorpha* with methanol-^{13}C followed by its chemical hydrolysis using the methods described here, approximately 0.7 g of highly pure

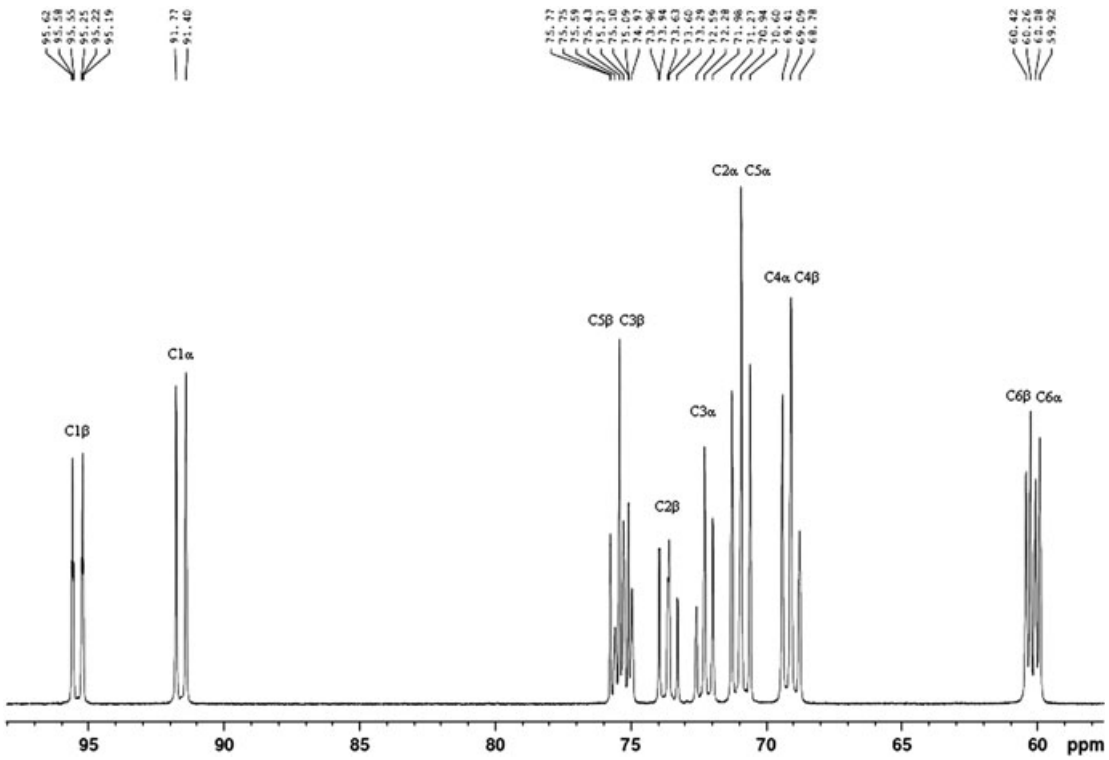


Figure 2. ^{13}C NMR spectrum of glucose-$^{13}C_6$.

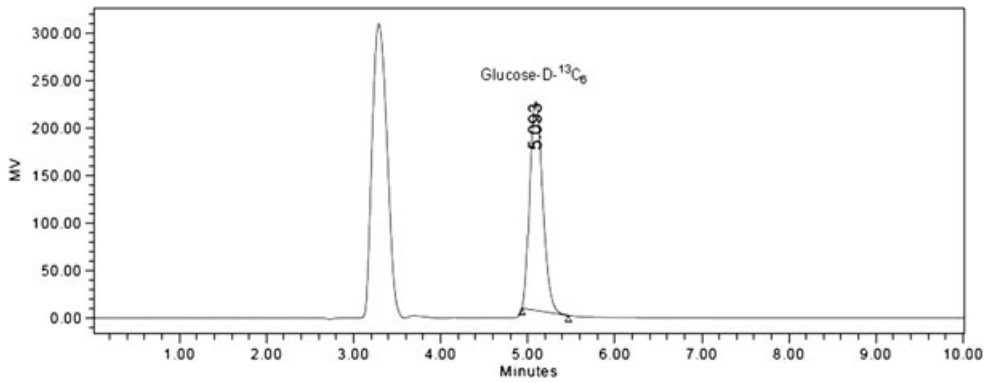


Figure 3. HPLC spectrum of glucose-D-$^{13}C_6$.

glucose-D-$^{13}C_6$ with high abundance ^{13}C is obtainable per litre of cell culture.

Acknowledgements

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