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An Efficient Production of Oligosaccharides by a Reaction using Whole Mammalian Cells as Biocatalysts

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Abstract: Problem statement: Mammalian cells were used for the production of oligosaccharides by saccharide primer method. However, because cells in culture are used, productivity of oligosaccharides is low. **Approach:** In saccharide primer method employing cells under culture, glycosylation was performed only by mixing the collected cells in reaction mixtures. Saccharide primer, 12-azidododecyl β-lactoside that mimics lactosylceramide (LacCer), was incubated with various mammalian cells under stirring or static conditions. The glycosylated primers were generated by adding and agitating cells in reaction mixtures like in the case where cells in culture were treated with saccharide primer. **Results:** In the case of African green-monkey kidney (Vero) cells, the amount of generated GM3-type oligosaccharide increased approximately five times by the reaction under agitating condition as compared with the reaction with cells in culture. GM3-type oligosaccharide was also synthesized by mouse melanoma B16 cells under both agitating or standing conditions. Moreover, the amount of GM3-type and GM1-type oligosaccharides produced by using African green-monkey kidney COS7 cells only by mixing the collected cells was greater than the general saccharide primer method. **Conclusions/Recommendations:** We demonstrated that the suspension mixture of the adhesive cells can be used as a catalyst for the synthesis of oligosaccharides in saccharide primer method. Moreover, suspended cells could produce more amount of oligosaccharides than normally cultured cells.

Key words: Oligosaccharide, saccharide primer, glycolipid, mammalian cells, biocatalyst

INTRODUCTION

Oligosaccharides (sugar chains) are recognized as the third important molecular chain in biological systems. Auto-sequencers and synthesizing equipment for nucleic acids and proteins have been developed rapidly hence, researches of DNA and proteins have proceeded actively. However, such equipment is not available for the analysis or synthesis of oligosaccharides. Recently, researches revealed that aberrations of oligosaccharides are involved with pathogenesis of many diseases, such as cancer, diabetes, hepatitis, emphysema, bacterial and virus infection, cranial and muscular neurological diseases[1-5]. Moreover, the valuable contribution of glycobiology in the field of medicine such as sugar chain medicine, sugar chain vaccine, and so on which is preceeded by treatment of influenza is rapidly growing[6-8].

Since the structures of oligosaccharides are complicated and the oligosaccharide as a molecule cannot be amplified, studies to establish the structure and function of oligosaccharides lag behind. In order to study the role of complex sugar chains on the surface of cells, different oligosaccharide synthetic methods have been developed. Extraction from natural organic resources[9-10], chemical synthetic method[11-13] and a series of enzymatic reactions[14-17] are among the well known methods of preparing oligosaccharides. Recently, glycoengineered *Pichia pastoris* strains capable of producing humanized glycoproteins have been developed[18-20]. In the early 1990's, the saccharide primer method was developed as a novel strategy for the synthesis of oligosaccharides. By this method, oligosaccharides are synthesized artificially utilizing the cell's own oligosaccharide synthetic ability. Saccharide primers, which are used as precursors of the

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biosynthetic carbohydrate in cells, are put into a medium while culturing cells[21-23]. 12-azidododecyl β-lactoside was glycosylated into GM3-type oligosaccharide in mouse melanoma B16 cells[24]. Aside from GM3-type oligosaccharide, the 12-azidododecyl β-lactoside primer gave Gb3-type and Gb4-type oligosaccharides in African green-monkey kidney Vero cells[25], while dodecyl β-lactoside primer gave GM2-type, GM1-type and GD1a-type oligosaccharides in African green-monkey kidney COS7[26]. The oligosaccharides, which are glycosylated products in the cells, are secreted out of cells into the medium. Since oligosaccharide products are in a medium, purification of products is therefore easy and free from contamination by other cell components. Recently, an efficient purification method by using Centrifugal Partition Chromatography (CPC) has been reported[27-28].

In this study, we attempted to synthesize oligosaccharides more efficiently than the usual conditions based on the principle of the saccharide primer method. We examined the efficient production of oligosaccharides only by mixing cells in reaction mixtures or by agitating without the use of cells in culture.

MATERIALS AND METHODS

Mouse melanoma B16 cells, African green-monkey kidney Vero cells and African green-monkey kidney COS7 cells were obtained from Riken Cell Bank (Tsukuba, Japan). High performance thin-layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). D-MEM and Ham's F12 (1:1) (DMEM/F12) and antibioyic-antimycotic (100×), insulin-transferrin-selenium-x supplement (100×), trypsin/EDTA (10×) were purchased from Invitrogen (Tokyo, Japan). Fetal Bovine Serum (FBS) was purchased from Nichirei Bioscience (Tokyo, Japan). Sep-Pak C18 was from Waters (Milford, MA, USA). All organic solvents used for extraction of lipids were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of saccharide primer: Saccharide primer, 12-azidododecyl β-lactoside, was prepared as described previously[29]. A dichloroethane soln. of 12-azido-1-dodecanol (1.34 g, 5.89 mmol), lactose octaacetate (2.0 g, 2.87 mmol) and 4-Å molecular sieves were stirred at 50°C for 1.5 h. Then, $BF_3{\cdot}Et_2O$ (0.5 mL) was added and the soln. was stirred at 50°C. After 3 h, the mixture was diluted with chloroform and the chloroform soln. was neutralized with aq. $NaHCO_3$, washed with H_2O, dried with anhydrous Na_2SO_4 and concentrated. Purification was performed by column chromatography (silica gel 60, 70-230 mesh, Merck, Darmstadt, Germany; hexane/AcOEt 2:1, v/v).

Cell culture: Mouse melanoma B16 cells, African green-monkey kidney Vero cells and African green-monkey kidney COS7 cells were cultured in DMEM/F12 supplemented with 10% FBS and 1% antibiotic-antimycotic and detached through application of 0.25% trypsin/EDTA, passaged every 3-4 days and maintained in humidified atmosphere of 5% CO_2 at 37°C.

Oligosaccharide synthesis by cells in culture (usual method): 2×10^6 mouse melanoma B16 cells, African green-monkey kidney Vero cells or African green-monkey kidney COS7 cells were seeded into 100-mm culture dishes containing 7 ml of medium and incubated for 48 h. Then, the cells were washed with TI-DF (D-MEM/F12 containing 1% Insulin-Transferrin-Selenium-X Supplement containing 1% antibiotic-antimycotic) and cells were incubated with 50 μM of 12-azidododecyl β-lactoside (the primer was dissolve in DMSO to an initial concentration of 50 mM) at 37°C for 48 h. Prior to the incubation of cells with primer, it was essential to remove the FBS from the growth medium by washing with TI-DF, since serum has previously been observed to interfere with ganglioside accumulation by the cells[30].

Oligosaccharide synthesis by cells (modified method): 2×10^6 (×1), 1×10^7 (×5), 4×10^7 (×20) and 1×10^8 (×50) mouse melanoma B16 cells, African green-monkey kidney Vero cells or African green-monkey kidney COS7 cells were added into 7 mL of reaction solution containing D-MEM/F12 with 1% Insulin-Transferrin-Selenium-X Supplement, 1% antibiotic-antimycotic and 50 μM of 12-azidododecyl β-lactoside (the primer was dissolve in DMSO to an initial concentration of 50 mM). Under stirring condition, the reaction solutions were poured into 20 mL vials, the spouts of vials were sealed with Air pore tape sheets (QIAGEN, Hilden, Germany) and then, they were stirred at the rate of 75 rpm in humidified atmosphere of 5% CO_2 at 37°C for 48 h. On the other hand, under static conditions, the reaction solutions were poured into 100 mm culture dishes and incubated in humidified atmosphere of 5% CO_2 at 37°C for 48 h.

Extraction of oligosaccharides from medium: After incubation, culture media or reaction solution were collected and cells were removed by centrifugation and washed with PBS (-). Oligosaccharides in the culture

media and PBS (-) were collected and purified using a Sep-Pak C18 column. After elution from the column, the solution was evaporated and dissolved in 50 µl of chloroform/methanol (1:2, v/v) for analysis by HPTLC.

Purification of acidic glycolipids by anion exchanger cartridges: Acidic glycolipids were separated from total oligosaccharides extracted from culture medium by InertSep SAX, strong anion exchanger cartridges (GL science, Tokyo, Japan), as described in literature[31]. InertSep SAX (500 mg) were placed in the extraction manifold apparatus (Waters, Milford, MA, USA) and washed under vacuum (under 10 kPa) with 10 ml of methanol and 10 ml of distilled water. The anion exchange resin was conditioned by washing with 10 ml of 0.8 M ammonium acetate solution to convert to acetate ion form and then washed with 10 ml of distilled water. After equilibration with 10 ml of chloroform/methanol/H_2O (5:10:1, v/v), the total oligosaccharides extracted from culture medium were diluted with chloroform/methanol/H_2O (5:10:1, v/v) and applied to the cartridge column under vacuum. Then 10 ml of chloroform/methanol/H_2O (5:10:1, v/v) was pulled through for washing. After setting a collection rack with receiving tubes in the extraction manifold apparatus, the cartridge column was eluted with 10 ml of chloroform/methanol/4.0 M ammonium acetate (5:10:1, v/v). Eluates were evaporated by centrifugal concentrator.

HPTLC analysis: Lipids from the cell homogenate and culture medium fraction were analyzed by HPTLC with chloroform/methanol/0.25 % aqueous KCl (5:4:1, v/v) as developing solvent. The HPTLC plates were sprayed with resorcinol reagent and heated at 120°C to detected the separated glycolipids[24]. Densitogram was obtained from HPTLC results using Scion Image Software (scion corporation, http://www.scioncorp.com) to quantify glycolipids.

RESULTS

Mouse melanoma B16 cells[24], African green-monkey kidney Vero cells[25] and African green-monkey kidney COS7 cells[26,32] have been used for oligosaccharide production by saccharide primer method. In this regard, mouse melanoma B16 cells sialylated 12-azidododecyl β-lactoside into GM3-type oligosaccharide by the action of cellular enzymes (Fig. 1a). In the same way, African green-monkey kidney Vero cells produced Gb3-type, Gb4-type and GM3-type oligosaccharides (Fig. 1b), while African green-monkey kidney COS7 produced GM3-type, GM2-type, GM1-type and GD1a-type oligosaccharides (Fig. 1c).

We attempted to synthesize oligosaccharides more efficiently than the usual conditions based on the principle of the saccharide primer method. Initially, mouse melanoma B16 cells and African green-monkey kidney Vero cells were used to prepare oligosaccharides by saccharide primer method under two different conditions. In one condition, cells were suspended with reaction solution and incubated in 100 mm culture dishes under static conditions. In the other condition, the reaction solutions with suspended cells were stirred at the rate of 75 rpm in 20 mL vial. In both conditions, the reaction solutions were incubated in humidified atmosphere of 5% CO_2 at 37°C for 48 h. The glycosylated products in the reaction solution of mouse melanoma B16 cells were extracted and analyzed by HPTLC. The amount of GM3-type oligosaccharide product increased with increasing number of cells for both conditions. Under usual condition using cells in culture, the amount of GM3-type oligosaccharides produced by using mouse melanoma B16 cells and African green-monkey kidney Vero cells were 22.6 and 8.4 nmol/dish, respectively. As shown in Fig. 2a and 3a, when mouse melanoma B16 cells were used, the static reaction condition was better than the stirred reaction condition, but the amount of product in either condition did not surpass the amount produced under usual condition using cells in culture. On the other hand, the amount of GM3-type oligosaccharide produced by using African green-monkey kidney Vero cells markedly increased in both conditions in comparison with usual condition (Fig. 2b, 3b). Interestingly, when the African green-monkey kidney Vero cells were used, the stirred reaction condition was suitable for the production of GM3-type oligosaccharide differently from when the mouse melanoma B16 cells were used. It was possible to increase the amount of production of GM3-type oligosaccharide five times at a maximum (Fig. 2b, 3b). It may be inferred that the property of the cells (for example, viability in floating condition or the control of oligosaccharides synthesis) is associated with conditions suitable for oligosaccharides synthesis. As shown in Fig. 3b, the amounts of Gb3-type and Gb4-type oligosaccharide produced by reaction with various numbers of cells were nearly constant and the ratio of the produced oligosaccharides changed according to the reaction condition.

Fig. 1: Synthesis of oligosaccharides by saccharide primer method using (a): Mouse melanoma B16 cells, (b): African green-monkey kidney vero cells and (c): African green-monkey kidney COS7 cells

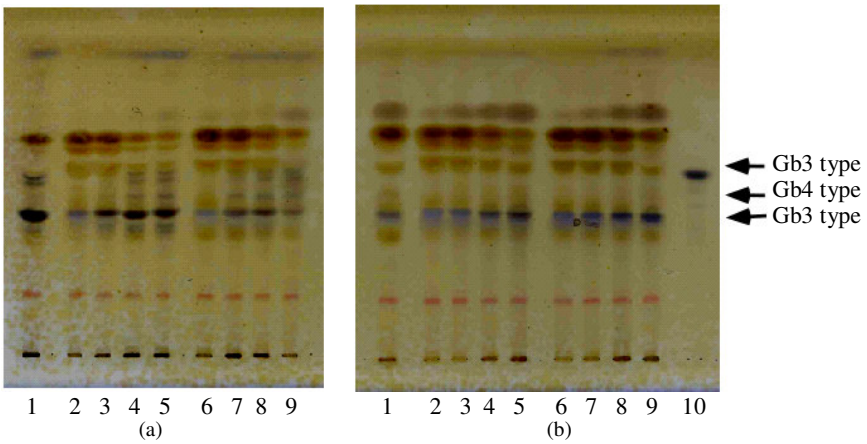

Fig. 2: Oligosaccharide production in (a): Mouse melanoma B16 cells and (b): African green-monkey kidney (Vero) cells. 1: Control (usual method); 2-5: Static reaction condition; 6-9: Stirred reaction condition; 10: Standard endogenous GM3 (2 µg). 2,6: 2×10^6 cells; 3,7: 1×10^7 cells; 4,8: 4×10^7 cells; 5,9: 1×10^8 cells

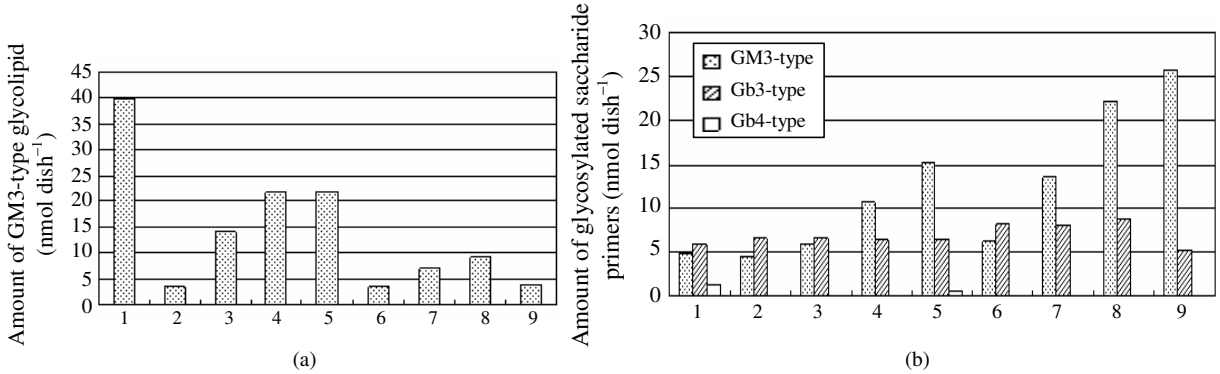

Fig. 3: Amount of oligosaccharides produced in (a) mouse melanoma B16 cells and (b) African green-monkey kidney (Vero) cells. 1: Control (usual method); 2-5: Static reaction condition; 6-9: Stirred reaction condition; 10: standard GM3 (2 µg). 2,6: 2×10^6 cells; 3,7: 1×10^7 cells; 4,8: 4×10^7 cells; 5,9: 1×10^8 cells

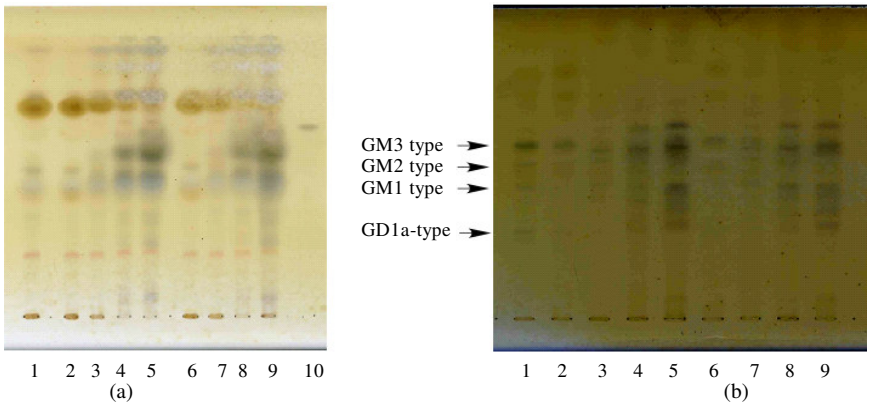

Fig. 4: HPTLC analysis of oligosaccharides produced in African green-monkey kidney COS7 cells after purification by using (a): Sep-Pak C18 and (b): Sep-Pak C18 followed by InertSep SAX. 1: Control (usual method); 2-5: Static reaction condition; 6-9: Stirred reaction condition; 10: Standard endogenous GM3 (1 µg)

When African green-monkey kidney COS7 cells were used for oligosaccharide production by saccharide primer method, GM3-type, GM2-type, GM1-type and GD1a-type oligosaccharides were obtained as described in the literature[26,32]. However, the yield of these products were too low to quantify by HPTLC precisely (Fig. 4a). Thereupon, acidic oligosaccharides were separated from the total oligosaccharides, which were extracted from reaction solutions, by InertSep SAX. As shown in Fig. 4b, the bands of acidic oligosaccharides could be detected clearly by using anion exchange resin cartridge. For both static and usual reaction conditions, the amounts of GM3-type and GM1-type oligosaccharides increased approximately 1.5-3 times when 1×10^8 African green-monkey kidney COS7 cells were used, but the amounts of GM2-type and GD1a-type oligosaccharides decreased. When 1×10^8 African green-monkey kidney COS7 cells were used for reaction under static condition, the amounts of GM3-type and GM1-type oligosaccharides produced were maximum and increased 1.8 and 7.2 times, respectively, compared with the amount of oligosaccharides produced under usual condition using the cells in culture (Fig. 4b).

DISCUSSION

Saccharide primer method is more convenient than chemical synthesis and is useful for oligosaccharides synthesis. The cells are cultured in serum-free medium supplemented with the saccharide primer for 1-2 days. However, there is still a serious problem i.e., productivity is for application to industrial production. We wondered if the harvested cells could be used for glycosylation of the saccharide primer as biocatalyst instead of using cells in culture. In this method, various

cells which were obtained by cultivation or tissue cell dispersion may be used for oligosaccharide production directly without any further process step for oligosaccharide production.

In the case of African green-monkey kidney (Vero) cells, the amount of generated GM3-type oligosaccharide increased approximately five times by the reaction under agitating condition as compared with the reaction with cells in culture. GM3-type oligosaccharide was also synthesized by mouse melanoma B16 cells under both agitating or standing conditions. Moreover, the amount of GM3-type and GM1-type oligosaccharides produced by using African green-monkey kidney COS7 cells only by mixing the collected cells was greater than the general saccharide primer method. Interestingly, in the case of African green-monkey kidney (Vero) cells, the amount of generated GM3-type oligosaccharide increased approximately five times, but the amounts of generated Gb3 and Gb4 type oligosaccharides nearly constant as shown in Fig. 3b. These results suggested that the reaction condition influenced the composition of oligosaccharides produced from saccharide primers. When African green-monkey kidney Vero cells were used for oligosaccharide production by saccharide primer method, each kind of glycosyltransferase in the cell might act differently under certain reaction conditions. It is generally accepted that the saccharide primer is glycosylated by the same enzyme as in the synthesis of natural glycosphingolipids[32]. In the suspended condition the saccharide primers added in the reaction solution tended to be glycosylated by CMP-sialic acid:LacCer α2-3 sialyltransferase in the African green-monkey kidney Vero cells more easily than UDP-Gal:LacCer α1-4 galactosyltransferase.

CONCLUSION

In this study, we demonstrated that the suspension mixture of the adhesive cells can be used as a catalyst for the synthesis of oligosaccharides in saccharide primer method. Moreover, this study reports that the cell strain and the reaction condition influence the amounts of oligosaccharides produced. Suspended cells produced more amount of oligosaccharides than normally cultured cells. Significantly, the culture process can be skipped and the amount of medium required is minimized by suspending cells in reaction mixture.

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REFERENCES

1. Hakomori, S. and R. Kannagi, 1983. Glycosphingolipids as tumor-associated and differentiation markers. J. Natl. Cancer Inst., 71: 231-251. http://www.ncbi.nlm.nih.gov/pubmed/6576183.
2. Faissner, A., A. Clement, A. Lochter, A. Streit, C. Mandl and M. Schachner, 1994. Isolation of a neural chondroitin sulfate proteoglycan with neurite outgrowth promoting properties. J. Cell Biol., 126: 783-799. http://jcb.rupress.org/cgi/content/abstract/126/3/783.
3. Chu, V.C. and G.R. Whittaker, 2004. Influenza virus entry and infection require host cell N-linked glycoprotein. Proc. Natl. Acad. Sci. USA., 101: 18153-18158. http://vivo.cornell.edu/individual/vivo/individual11411.
4. Kawakubo, M., Y. Ito, Y. Okimura, M. Kobayashi, K. Sakura, S. Kasama, M.N. Fukuda, M. Fukuda, T. Katsuyama and J. Nakayama, 2004. Natural antibiotic function of a human gastric mucin against Helicobacter pylori infection. Science, 305: 1003-1006. http://www.sciencemag.org/cgi/content/abstract/305/5686/1003.
5. Ohtsubo, K. and J.D. Marth, 2006. Glycosylation in cellular mechanisms of health and disease. Cell, 126: 855-867. http://www.ncbi.nlm.nih.gov/pubmed/16959566.
6. Rogers, G.N. and J.C. Paulson, 1983. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology, 127: 361-373. http://www.ncbi.nlm.nih.gov/pubmed/6868370.
7. Matrosovich, M., 2000. Early alterations of the receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol., 74: 8502-8512. http://www.ncbi.nlm.nih.gov/pubmed/10954551.
8. Suzuki, Y., 2001. Host mediated variation and receptor binding specificity of influenza viruses. Adv. Exp. Med. Biol., 491: 445-451. http://www.ncbi.nlm.nih.gov/pubmed/14533814?dopt=Abstract.
9. Gazzotti, G., S. Sonnino and R. Ghidoni, 1985. Normal-phase high-performance liquid chromatographic separation of non-derivatized ganglioside mixtures. J. Chromatogr., 348: 371-378. http://www.ncbi.nlm.nih.gov/pubmed/4077945.

10. Ariga, T., R.V. Tao, B.C. Lee, M. Yamawaki, H. Yoshino, N.J. Scarsdale, T. Kasama, Y. Kushi and R.K. Yu, 1994. Glycolipid composition of human cataractous lenses. Characterization of Lewis X glycolipids. J. Biol. Chem., 269: 2667-2675. http://cat.inist.fr/?aModele=afficheN&cpsidt=4008098.
11. Sugimoto, M. and T. Ogawa, 1985. Synthesis of a hematoside (G_{M3}-ganglioside) and a stereoisomer. Glycoconj. J., 1: 5-9. http://www.springerlink.com/content/ww381452716614p1/.
12. Zhang, Y., T. Toyokuni, F. Ruan and S. Hakomori, 2001. A one pot synthesis of mono- and di-lactosyl sphingosines. Glycoconj. J., 18: 557-563. http://www.springerlink.com/content/rlqg3m1739772152/.
13. Miermont, A., Y. Zeng, Y. Jing, X.S. Ye and X. Huang, 2007. Syntheses of lewis(x) and dimeric lewis(x): Construction of branched oligosaccharides by a combination of preactivation and reactivity based chemoselective one-pot glycosylations. J. Org. Chem., 72: 8958-8961. http://pubs.acs.org/doi/abs/10.1021/jo701694k.
14. Nilsson, K.G.I., 1989. Enzymic synthesis of di- and tri-saccharide glycosides, using glycosidases and β-galactoside 3-α-sialyl-transferase. Carbohydr. Res., 188: 9-17. http://www.ncbi.nlm.nih.gov/pubmed/2550126.
15. Sinnott, M.L., 1990. Catalytic mechanism of enzymic glycosyl transfer. Chem. Rev., 90: 1171-1202. http://pubs.acs.org/doi/abs/10.1021/cr00105a006.
16. Zehavi, U., M. Herchman, R.R. Schmidt and T. Bär, 1990. Enzymic glycosphingolipid synthesis on polymer supports. Glycoconj. J., 7: 229-234. http://www.springerlink.com/content/kq57n22206822120/.
17. Flitsch, S.L., D.M. Goodridge, B. Guilbert, L. Revers, M.C. Webberley and I.B. Wilson, 1994. The chemoenzymatic synthesis of neoglycolipids and lipid-linked oligosaccharides using glycosyltransferases. Bioorg. Med. Chem., 2: 1243-1250. http://www.ncbi.nlm.nih.gov/pubmed/7757420.
18. Hamilton, S.R., P. Bobrowicz, B. Bobrowicz, R.C. Davidson, H. Li, T. Mitchell, J.H. Nett, S. Rausch, T.A. Stadheim, H. Wischnewski, S. Wildt and T.U. Gerngross, 2003. Production of complex human glycoproteins in yeast. Science, 301: 1244-1246. http://www.sciencemag.org/cgi/content/abstract/301/5637/1244.
19. Hamilton, S.R., R.C. Davidson, N. Sethuraman, J.H. Nett, Y. Jiang, S. Rios, P. Bobrowicz, T.A. Stadheim, H. Li, B.K. Choi, D. Hopkins, H. Wischnewski, J. Roser, T. Mitchell, R.R. Strawbridge, J. Hoopes, S. Wildt and T.U. Gerngross, 2006. Humanization of yeast to produce complex terminally sialylated glycoproteins. Science, 313: 1441-1443. http://www.sciencemag.org/cgi/content/abstract/313/5792/1441.
20. Hamilton, S.R. and T.U. Gerngross, 2007. Glycosylation engineering in yeast: The advent of fully humanized yeast. Curr. Opin. Biotechnol., 18: 387-392. http://www.ncbi.nlm.nih.gov/pubmed/17951046.
21. Miura, Y., T. Arai and T. Yamagata, 1996. Synthesis of amphiphilic lactosides that possess a lactosylceramide-mimicking N-acyl structure: Alternative universal substrates for endo-type glycosylceramidases. Carbohydr. Res., 289: 193-199. http://www.ncbi.nlm.nih.gov/pubmed/8805779.
22. Miura, Y. and T. Yamagata, 1997. Glycosylation of lactosylceramide analogs in animal cells: amphipathic disaccharide primers for glycosphingolipid synthesis. Biochem. Biophys. Res. Commun., 241: 698-703. DOI: 10.1006/bbrc.1997.7876 .
23. Nakajima, H., Y. Miura and T. Yamagata, 1998. Glycosylation of amphipathic lactoside primers with consequent inhibition of endogenous glycosphingolipid synthesis. J. Biochem., 124: 148-156. http://jb.oxfordjournals.org/cgi/content/abstract/124/1/148.
24. Kasuya, M.C.Z., L.X. Wang, Y.C. Lee, M. Mitsuki, H. Nakajima, Y. Miura, T. Sato, K. Hatanaka, S. Yamagata and T. Yamagata, 2000. Azido glycoside primer: a versatile building block for the biocombinatorial synthesis of glycosphingolipid analogues. Carbohydr. Res., 329: 755-763. http://www.ncbi.nlm.nih.gov/pubmed/11125817.
25. Miyagawa, A., M.C.Z. Kasuya and K. Hatanaka, 2007. Alternative methods of globotrioside production using Vero cells: A microcarrier system procedure. Chem. Cent. J., 1: 26-32. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2213644.
26. Sato, T. and E. Sano, 2002. Process for producing oligosaccharide chain. http://www.wipo.int/pctdb/en/wo.jsp?wo=2002081723.
27. Kato, T., A. Miyagawa, M.C.Z. Kasuya, A. Ito and K. Hatanaka, 2008b. Purification by centrifugal partition chromatography of amphiphilic compounds, glycolipids and pseudo-glycolipids synthesized by using cells. J. Chromatogr., 1178: 154-159. http://cat.inist.fr/?aModele=afficheN&cpsidt=19981558.

28. Kato, T. and K. Hatanaka, 2008c. Purification of gangliosides by liquid-liquid partition chromatography. J. Lipid Res., 49: 2474-2478. http://www.jlr.org/cgi/content/abstract/49/11/2474.
29. Murozuka, Y., M.C.Z. Kasuya, M. Kobayashi, Y. Watanabe, T. Sato and K. Hatanaka, 2005. Efficient Sialylation on Azidododecyl Lactosides by Using B16 Melanoma Cells. Chem. Biodivers., 2: 1063-1078. http://www3.interscience.wiley.com/journal/111082084/abstract?CRETRY=1&SRETRY=0.
30. Schwarzmann, G., P. Hoffmann-Bleihauer, J. Schubert, K. Sandhoff and D. Marsh, 1983. Incorporation of Ganglioside Analogues into Fibroblast Cell Membranes. A Spin-Label Study. Biochemistry, 22: 5041-5048. http://www.ncbi.nlm.nih.gov/pubmed/6315058.
31. Kato, T., M.C.Z. Kasuya and K. Hatanaka, 2008. Rapid separation of gangliosides using strong anion exchanger cartridges. J. Oleo Sci., 57: 397-400. http://www.jstage.jst.go.jp/article/jos/57/7/57_397/_article.
32. Sato, T., K. Hatanaka, H. Hashimoto and T. Yamagata, 2007. Syntheses of oligosaccharides using cell function. Trends Glycosci. Glycotechnol., 105: 1-17. http://www.gak.co.jp/TIGG/105PDF/Sato.pdf.