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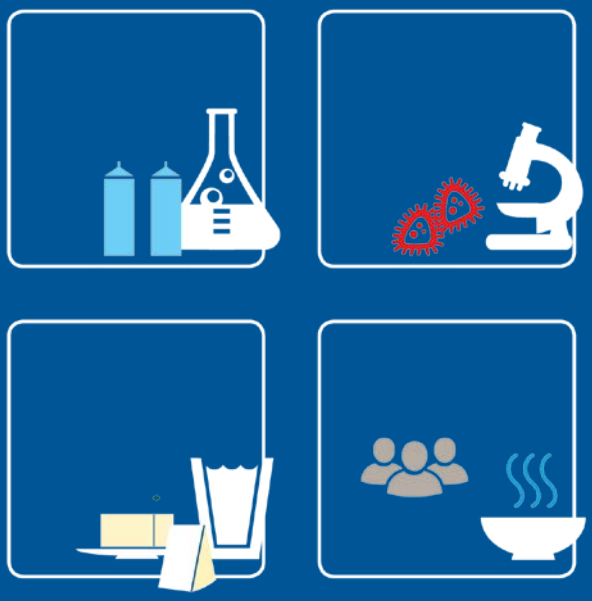
Morgendagens skræddersyede fødevarer



Mejeribrugets
ForskningsFond

OKTOBER 2015

Slutrapport 2015
for samarbejdsprojekter under MFF

1. Projektets titel "*Tailoring the Food of Tomorrow"*

2. Projektleder Professor Jørn Dalgaard Mikkelsen, Chemical & Biochemical Engineering, The Technical University of Denmark, Sølftofts Plads, Building 229, 2800 Lyngby; +4545252938; +4560856300; jdm@kt.dtu.dk

3. Øvrige medarbejdere

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4. Projektperiode Projektstart og -slut, juli 2012 – juli 2015

5. Projektresume

Sialylated glycans such as lactose, LNT and galactooligosaccharides (GOS) represent potential infant formula ingredients and is expected to be substitutet to genuine HMO molecules, These HMO or HMO-like products are anti-infective, immuno-modulating, and take part in infant brain development. Some of the Sialylated glycans such as GOS do not exist in natural milk, but can be produced from cheap and abundantly available food grade sialyl donors such as κ-casein glycomacropeptide (CGMP) by sialidase-catalyzed trans-sialylation. Using a rationally designed mutant of the sialidase from *Trypanosoma rangeli*, Tr13, with superior trans-sialylation activity and low sialidase activity, six different GOS preparations with a varying degree of polymerization (DP) were effectively sialylated with molar yields of 20-30% on the CGMP sialyl in batch reactions. The rate of sialylation of the individual DPs was largely dependent on the DP distribution in each GOS preparation, and Tr13 catalysis did not discriminate against large GOS molecules. Using CGMP, GOS, and Tr13, the production of gram-scale quantities of sialyl-GOS was achieved in 20 liter volume reactions. Using another trans-sialidase from *Pasteurella multicoda* we also produced both 3´- and 6´-Sialylated Glycans, whereas Tr13 only gave rise to the 3´-siallylated Glycans.

6. Projektets formål

- To develop a novel enzymatic concept for production of superior prebiotics molecules, such as sialyl-lactose and sialyl-galactooligosaccharides, based on side-streams from the dairy industry.
- To design new enzymes and a biocatalysis process for a future large scale production.
- To generate proof of concept by demonstrating production of sialyl-lactose and sialyl-galactooligosaccharides by the new enzymatic route and evaluate the structure and functional properties by NMR, Maldi-Tof and prebiotic studies.

7. Projektets delaktiviteter i hele projektperioden

A. Examination of a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities for production of both 3α´-siallyl-lactose, 6´α-siallyl-lactose, 3α´-siallyl-GOS and 6´α-siallyl-GOS.

B.* Quantitative small scale enzymatic production of sialylated galactooligosaccharides with an engineered sialidase from *Trypanosoma rangeli:

C. Gram-scale production of sialylated GOS

D. Production and membrane purification of glycans with negative charges, e.g. Sialylated Glycans:

8. Projektets resultater/faglige forløb i perioden

[Redegørelse for resultater indenfor de enkelte delaktiviteter, så det giver et godt overblik over projektets faglige fremdrift. Brug gerne figurer og tabeller med forklaring]

A. Examination of a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities for production of both 3α´-siallyl-lactose, 6´α-siallyl-lactose, 3α´-siallyl-GOS and 6´α-siallyl-GOS.

This study examined a recombinant Pasteurella multocida sialyltransferase exhibiting dual trans-sialidase activities. The enzyme catalyzed trans-sialylation using either 2-O-(p-nitrophenyl)-α-d-N-acetylneuraminic acid or casein glycomacropeptide (whey protein) as the sialyl donor and lactose as the acceptor, resulting in production of both 3 α-sialyllactose and 6α-sialyllactose. This is the first study reporting α-2,6-trans-sialidase activity of this sialyltransferase (EC 2.4.99.1 and 2.4.99.4). A response surface design was used to evaluate the effects of three reaction parameters (pH, temperature, and lactose concentration) on enzymatic production of 3α_- and 6α-sialyllactoses using 5% (w/v) casein glyco-macropeptide (equivalent to 9 mM bound sialic acid) as the donor. The maximum yield of 3α-sialyllactose(2.75 ± 0.35 mM) was achieved at a reaction condition with pH 6.4, 40◦C, 100 mM lactose after 6 h; and the largest concentration of 6α_-sialyllactose (3.33 ± 0.38 mM) was achieved under a condition with pH 5.4, 40◦C, 100 mM lactose after 8 h. 6α-sialyllactose was presumably formed from α-2,3 bound sialic acidin the casein glycomacropeptide as well as from 3α-sialyllactose produced in the reaction. The kcat/Km value for the enzyme using 3α-sialyllactose as the donor for 6α-sialyllactose synthesis at pH 5.4 and 40◦C was determined to be 23.22 ± 0.7 $M^{-1}s^{-1}$. Moreover, the enzyme was capable of catalyzing the synthesis of both 3α- and 6α-sialylated galactooligosaccharides, when galactooligosaccharides served as acceptors as well as from 3´-sialyllactose produced in the reaction.

B. Quantitative small scale enzymatic production of sialylated galactooligosaccharides with an engineered sialidase from *Trypanosoma rangeli:*

Sialylated galactooligosaccharides (GOS) represent a potential infant formula ingredient, which is believed to contribute with a combination of the beneficial properties of the prebiotic GOS as well as of sialylated human milk oligosaccharides, which are anti-infective, immuno-modulating, and take part in infant brain development. Sialylated GOS do not exist in natural milk, but can be produced from cheap and abundantly available food grade sialyl donors such κ-casein glycomacropeptide (CGMP) by sialidase-catalyzed transsialylation. Using a rationally designed mutant of the sialidase from *Trypanosoma rangeli*, Tr13, with enhanced transsialylation activity, six different GOS preparations with a varying degree of polymerization (DP, Table 1, below) were effectively sialylated with molar yields of 20-30% on the CGMP sialyl in batch reactions. The rate of sialylation of the individual DPs was largely dependent on the DP distribution in each GOS preparation, and Tr13 catalysis did not discriminate against large GOS molecules. Compared to the benchmark transsialidase from pathogenic *Trypanosoma cruzi*, the Tr13 was significantly more thermostable. By employing an enzymatic membrane reactor, Tr13 could be recycled and after seven consecutive 1-hour reaction cycles, the biocatalytic productivity of the enzyme was increased 7-fold compared to the batch reaction. Assuming that the enzyme may be specific for α-2,3-bound sialyl moieties only, and that only 50% of sialyl linkages in CGMP are α-2,3-linked, the molar yield of sialyl-GOS on the available α-2,3-bound sialyl moieties in CGMP reached 80% in the enzymatic membrane reactor system.

C. Gram-scale production of sialylated GOS

Using Cup Oligo P (GOS, see Table 1 below) as acceptor and Whey protein (cGMP) as the donor, the batch process was scaled up to 20 L. The resulting amount of sialyl-GOS was 7.2 g, corresponding to a yield of 6%. Although by far the highest amount reported in literature (Table 1), this yield is 6 times lower than that obtained in the small-scale reaction. The major part of this loss took place during sample handling and anion exchange purification. Approximately 4% of the product was lost in the nanofiltration. Thus, using large-scale equipment suited for the process is expected to significantly increase the yield. Furthermore, a simpler process and a higher yield can be obtained if sialyl-GOS is to be added to infant formula along with unreacted, neutral GOS, which is already added to infant formula today, i.e. as an enrichment of the GOS preparation rather than as a pure compound. In that case, the anion exchange step could be omitted and a simple membrane process could be used to remove CGMP and enzyme from the reaction mixture. Consequently, yields closer to those obtained in the small-scale reactions would be expected in such a process (Table 1).

D. Production and membrane purification of glycans with negative charges, e.g. Sialylated Glycans:

The production of 3'-sialyllactose using cGMP as a donor and the Tr13 or PmST enzymes are evaluated with respect to up-scaling to 20-50 litres. The PmTS enzyme, however, can only be produced in small quantities, and is therefore not a feasible route. A series of time studies have been carried out with Tr13 to determine the optimal reaction conditions and the Department pilot plant at DTU has been taken into use. The reactor is a 50 l reactor, fitted with a pump and a spiral membrane for ultrafiltration. Donor pretreatment, the enzymatic reaction and the first downstream filtration can be done in this ultrafiltration unit. An integrated membrane system was investigated for the production of 3´-sialyllactose by an engineered sialidase using casein glycomacropeptide (CGMP) and lactose as substrates. CGMP was purified by ultrafiltration (UF) to remove any small molecules present and then an enzymatic membrane reactor (EMR) was used to separate the product and reuse the enzyme. A PLCC regenerated cellulose membrane was found to be the most suitable for both the UF purification and EMR. Subsequently, nanofiltration (NF) was conducted to increase the purity of the 3´-sialyllactose by removing the excess lactose present. The NTR7450 membrane outperformed others in NF due to its high retention of 3´-sialyllactose (98%) and relatively low rejection of lactose (40%). The lactose in the permeate could be concentrated by the NF45 membrane and recycled into the EMR. The described integrated membrane system enables a more economic and efficient enzymatic production of 3`-sialyllactose.

Table 1. Degree of polymerization (DP) of GOS preparations used in the current work, DP of the corresponding sialylated GOS, and molar yield of total sialyl-GOS. For sialylated GOS, the sialic acid moiety is not included in the DP number.

Name	DP of GOS[a]	DP of GOS sialylated[b]	Total sialyl-GOS yield[c]
Cup Oligo P	2-10	2-8	30%
Vivinal GOS 10	2-9	2-6	20%
Vivinal GOS	2-9	2-5	n.q.
GOS-570-P	2-24	2-12	23%
GOS-700-P	2-10	2-8	28%
Bimuno Powder	2-14	2-10	22%

[a]Measured by MALDI-TOF. [b]The DP refers to the GOS, i.e. the number of Gal and Glc residues, excluding the Sia moiety from the number. Analyses were carried out by MALDI-TOF and HPAEC-PAD. [c]Molar yield on the limiting donor substrate, i.e. sialic acid in CGMP, in the batch reaction not accounting for the fact that only approx. half of the sialic acid is available to Tr13; n.q.: not quantified.

9. Afvigelser

9.1 Fagligt: No

9.2 Økonomisk: Submitted, No comments

9.3 Tidsplan: Targeted all milestones

10. Planer for næste halvår: NO

[kort angivelse for de enkelte delaktiviteter]

11. Formidling og vidensdeling vedr. projektet

a. **Guo**, Y., C. Jers, A.S. Meyer, H. Li, F. Kirpekar, J.D.Mikkelsen; Modulating the regioselectivity of Pasteurella multocida sialyltransferase for biocatalytic production of 3'- and 6'-sialyllactose; Enzyme and Microbial Technology 78 (2015) 54–62
b. **Zeuner**, B., J. Luo, C. Nyffenegger, V. Aumala, J. D. Mikkelsen, A.S. Meyer; Optimizing the biocatalytic productivity of an engineered sialidasefrom Trypanosoma rangeli for 3_-sialyllactose production; Enzyme and Microbial Technology 55, 85– 93, 2014
c. **Guo** Y., C. Jers, A.S. Meyer, J.D. Mikkelsen: A Pasteurella multocida sialyltransferase displaying dual trans-sialidase activities for production of 3'-sialyl and 6'-sialyl glycans; Journal of Biotechnology;170, 60– 67, 2014
d. Luo J., **R T Nordvang**, ST Morthensen, **B Zeuner**, AS Meyer, JD Mikkelsen, M Pinelo: An integrated membrane system for the biocatalytic production of 3´-sialyllactose from dairy by-products; Bioresource Technology 166 (2014) 9–16

e. **Nordvang Rune,T**., Jianquan Luo, **Birgitte Zeuner**, Rasmus Prior, Mads F. Andersen, Jørn D. Mikkelsen, Anne S. Meyer, Manuel Pinelo; Separation of 3`-sialyllactose and lactose by nanofiltration: A trade-off between charge repulsion and pore swelling induced by high pH: Separation and Purification Technology 138 (2014) 77–83

f. Villumsen N.S., H.B. Jensen, T. T. T. Le, H. S. Moller, **R.T. Nordvang**, L. R Nielsen, S. B. Nielsen, J. Sorensen, M. Hammershoj, L. B. Larsen: Self-assembly of caseinomacropeptide as a potential key mechanism in the formation of visible storage induced aggregates in acidic whey protein isolate dispersions (In Press)

g. Articles a to e are included in the email.

12. Nye kontakter NO

9.3 Tidsplan: Targeted all milestones

10. Planer for næste halvår: NO

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