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Use of Transformed Whole Yeast Cells Expressing β-1,4-Galactosyltransferase for the Synthesis of *N*-Acetyllactosamine

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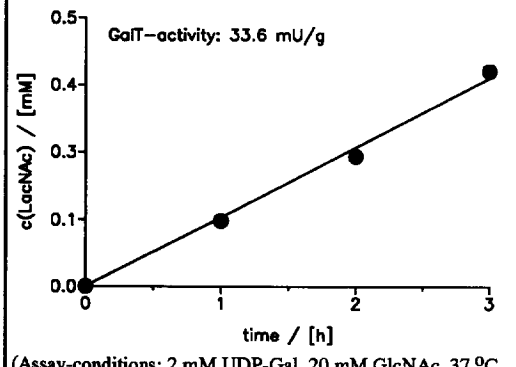
Abstract: We report the use of transformed whole yeast cells for the synthesis of *N*-acetyllactosamine (LacNAc), which is the core-disaccharide of the anti-inflammatory agent Sialyl-Lewis^X. Transformed whole yeast cells, which carry plasmids for the heterologous expression of a soluble human b-1,4-galactosyltransferase (GalT), proved to be a suitable catalyst for the synthesis of LacNAc by employing multi-enzyme-systems. This convenient use of the recombinant enzyme, taken together with the high availability of the transformed yeast through fermentation technology, will provide sufficient amounts of glycosyltransferase for chemoenzymatic synthesis of oligosaccharides.

The development of pharmaceuticals based on carbohydrates such as Sialyl-Lewis^X [1] requires new methods suitable for large scale synthesis of oligosaccharides *in vitro*.[2] Besides chemical synthesis[3] the enzymic synthesis of oligosacharides[4] is becoming more attractive. However, the restricted availability of glycosyltransferases is a major drawback for employing these catalysts in organic synthesis.[5] Recently, a soluble form (stem + catalytic domain, cloned from HeLa-cells) of the human *N*-acetylglucosaminide b-1,4-galactosyltransferase [GalT, E.C. 2.4.1.38] was successfully expressed in *Saccharomyces cerevisiae* with an enzyme-concentration of up to 0.7 U/L in a 500 U scale.[6] GalT catalyzes the transfer of Gal from UDP-Gal to GlcNAc thus forming LacNAc.[7] The enzyme was produced in an industrial scale[6c] and exhibited similar kinetic properties as soluble human milk GalT[6b]. However, the purification of GalT turned out to be tedious.[6c]

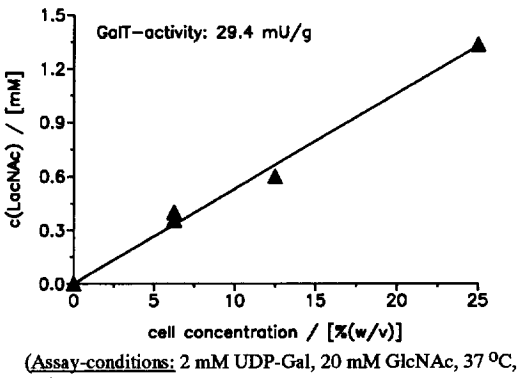
We discovered, that transformed whole yeast cells exhibited 31.5 mU/(g wet cells) GalT-activity as described in **Fig. 1a** and **Fig. 1b**.

Fig. 1a: Synthesis of LacNAc with Whole Yeast Cells



(Assay-conditions: 2 mM UDP-Gal, 20 mM GlcNAc, 37 °C, 6.25 % (w/v) yeast cells)

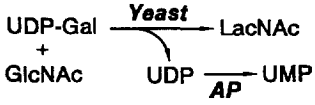
Fig. 1b: Synthesis of LacNAc with Whole Yeast Cells



(Assay-conditions: 2 mM UDP-Gal, 20 mM GlcNAc, 37 °C, 3 h)

For comparison, after disintegration of the cells soluble GalT-activity was 68.1 mU/(g wet cells) as confirmed by an assay based on measurement of incorporation of [^{14}C]Gal into GlcNAc.[6a] We proposed, that transformed whole yeast cells might be a suitable catalyst for the synthesis of LacNAc in a preparative scale (**Scheme 1**).

Scheme 1: Synthesis of LacNAc by Employing Transformed Whole Yeast Cells Starting from UDP-Gal



(Abbreviations: *AP*: alcaline phosphatase from bovine intestinal mucosa, [E. C. 3.1.3.1]; UDP-Gal: uridine-5'-diphospho-galactose, GlcNAc: *N*-acetyl-glucosamine)

We therefore investigated the use of whole yeast cells under different substrate concentrations (**Table 1**).

Table 1: Synthesis of LacNAc by Employing Different Substrate and Yeast Concentrations

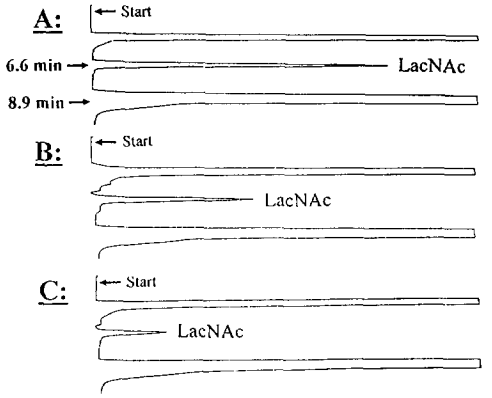
# [-]	yeast [% (w/v)]	UDP-Gal [mM]	GlcNAc [mM]	LacNAc [mM]	Yield [%]
1	25	10	30	5.2	52.5
2	25	20	60	8.7	43.3
3	25	25	75	13.4	53.6
4	12.5	10	30	3.7	36.7
5	25	10	30	5.3	52.5
6	50	10	30	6.7	67.1

(Reaction conditions: 37°C, reaction time: 24 h, 9 U/mL *AP*, 25 % (w/v) yeast contains 7.8 mU/mL GalT; yields were determined by HPLC-analysis)

LacNAc was obtained in moderate to good yields starting from UDP-Gal and GlcNAc (Tab. 1, # 1 - 3) and the yield was increased with higher concentrations of the transformed yeast cells (Tab. 1, # 4 - 6). The results from the assay were thereby confirmed with higher substrate concentrations. The reaction was subsequently scaled up and the preparative synthesis yielded 72 % LacNAc (see experimental procedure). In order to ascertain that transformed yeast cells expressing GalT specifically form β-1,4 linkages, LacNAc from the preparative synthesis was purified and the structure was confirmed by ^{1}H-NMR spectroscopy. The structural reporter groups matched those of reference LacNAc.[2e,8]

Figure 2: Employing Different Preparations of GalT for the Synthesis of LacNAc

A: purified GalT[6c], **B:** crude lyophilized yeast cell extract[6b], **C:** transformed whole yeast cells[6b,c]

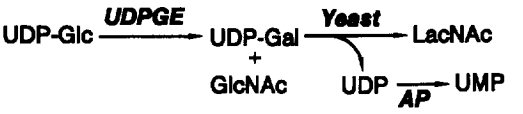


(Retention-times of the HPLC-chromatograms: 6.6 min: LacNAc, 8.9 min: GlcNAc)

Figure 2 demonstrates by a comparison of HPLC-chromatograms that LacNAc of the same purity is obtained (i.e. without any detectable traces of other disaccharides formed in the course of the reaction) by employing either purified GalT (**A**)[6d] or a crude lyophilized yeast-cell extract (**B**)[6b] or transformed whole yeast cells (**C**)[6b,c]. 1 g wet yeast-cells yielded 0.14 g dry weight after lyophilisation. This dry extract is storable for at least one year at -20°C without any detectable loss of GalT-activity thus offering an alternative and convenient preparation of recombinant GalT for use in organic synthesis. **Scheme 2** outlines the synthesis of LacNAc with

transformed whole yeast cells in an multi-enzyme-system starting from UDP-Glc, which is converted to UDP-Gal by means of UDP-galactose 4'-epimerase.

Scheme 2: Synthesis of LacNAc by Employing Transformed Whole Yeast Cells Starting from UDP-Glc



(Abbreviations: *UDPGE*: UDP-galactose 4'-epimerase from yeast, [E.C. 5.1.3.2]; UDP-Glc: uridine-5'-diphospho-glucose)

Table 2 shows concentrations and yields of LacNAc for different substrate- and UDP-galactose 4'-epimerase concentrations.

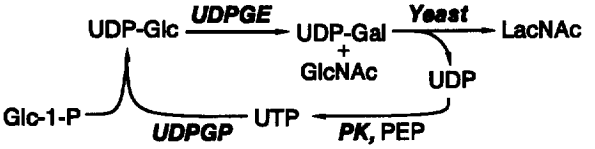
Table 2: Synthesis of LacNAc by Employing Different Substrate and Yeast Concentrations

#	UDPGE	UDP-Glc	GlcNAc	LacNAc	Yield
[-]	[U/mL]	[mM]	[mM]	[mM]	[%]
1	0.625	5	15	1.5	29.1
2	0.625	10	30	2.6	25.8
3	0.625	20	60	3.4	17.1
4	0.625	40	120	4.5	11.3
5	0.312	20	60	3.3	16.6
6	0.625	20	60	3.3	16.9
7	1.25	20	60	3.8	19.2

(Reaction conditions: 37°C, reaction time: 25 h, 4.5 U/mL *AP*, 25 % (w/v) transformed yeast cells, yield determined by HPLC-analysis)

The yields of LacNAc decrease with higher UDP-Glc concentrations (Tab. 2, # 1 - 4), which points to an uncomplete enzymic epimerisation of UDP-Glc to UDP-Gal. However, yields were increased with a higher concentration of UDP-galactose 4'-epimerase (Tab. 2, # 5 - 7). Finally, we synthezised LacNAc by employing transformed whole yeast cells with regeneration of UDP-Gal *in situ* (**Scheme 3**).[7a]

Scheme 3: Synthesis of LacNAc by Employing Transformed Whole Yeast with regeneration of UDP-Gal



(Abbreviations: *UDPGE*: UDP-galactose 4'-epimerase; *PK*: pyruvate kinase from rabbit muscle, [E.C. 2.7.1.40]; *UDPGP*: UDP-glucose-pyrophosphorylase from germinated barley, [E.C. 2.7.7.9]; Glc-1-P: a-D-glucose-1-phosphate; PEP: phosphoenol-pyruvate)

The synthesis started from Glc-1-P and an UDP-glucose-pyrophosphorylase from germinated barley[9] was used for the formation of UDP-Glc. LacNAc was obtained in 13.8 % yield in this way after a reaction time of 45 h.

In summary, we demonstrated that transformed whole yeast cells can conveniently be used as catalysts for galactosylation reactions in multi-enzyme systems. This approach circumvents the tedious procedure of enzyme purification. Furthermore organic chemists will obtain sufficient amounts of glycosyltransferase for large scale synthesis of oligosaccharides by means of fermentation technology.[6c] For laboratory experiments the enzyme is easily accessible by expression in shaking flasks.[6b] Thereby this technique overcomes the hitherto existing bottleneck in the availability of glycosyltransferases and will provide sufficient amounts of catalysts for the large scale synthesis of oligosaccharides[2]. These results extend recent data of the Wong-group, who demonstrated that transformed whole *E. coli* cells can be used for mannosylation-reactions.[10]

Work is in progress to adapt this methodology for other recombinant glycosyltransferases.

ACKNOWLEDMENT

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EXPERIMENTAL PROCEDURE

Yeast strain, culture conditions and expression vector. The protease deficient *Saccharomyces cerevisiae* strain BT150 (MATα, *his4, leu2, ura3, pra1, prb1, prc1, cps1*) was chosen for the heterologous expression of GalT.[6] For shaking flasks experiments *S. cerevisiae* BT150 was grown in the following medium: 6.7 g/L bacto-yeast nitrogen base without amino acids, 20 g/L glucose, 20 mg/L adenine sulfate, 20 mg/L tryptophane, 40 mg/L histidine, 20 mg/L arginine, 30 mg/L tyrosine, 60 mg/L leucine, 30 mg/L isoleucine, 30 mg/L lysine, 50 mg/L phenylalanine and 150 mg/L valine. The flasks were shaken at 30°C and 150 rpm. The large scale production of the human GalT was described in detail elsewhere.[6c] Stock cultures of *S. cerevisiae* BT150 were kept as cell suspension at -70°C in glycerol solution and at 4°C on agar on a petri dish. The vector used to transform yeasts for production of soluble human GalT has recently been described .[6b]

hsgalT-Assay. The assay for GalT was performed using GlcNAc as acceptor substrate; the resulting product was identified by HPLC. The enzyme was recovered from low-speed pellets obtained from aliquots of the yeast containing medium. The pellet was washed once with 10 mM Tris-buffer and then resuspended in 10 mM Tris-buffer containing 10 mM $MnCl_2$. Aliquots were used for the assay (2 mM UDP-Gal, 20 mM GlcNAc, 37°C, 1 - 4 h). Formed LacNAc was detected by means of HPLC (column: Aminex HPX-87H (Bio-Rad) at 65°C, eluent: 6 mM H_2SO_4, 0.7 mL/min, UV-detection (205 nm), retention time LacNAc: 6.6 min, see Figure 2). One unit of GalT was defined as 1 μmol LacNAc formed in one minute. For rapid assays, incorporation of [^{14}C]Gal from UDP-Gal into GlcNAc was measured as described previously.[6a,b]

Synthesis of LacNAc with transformed whole yeast cells. Yeast cells were harvested from shaking flasks after 3 days of incubation (OD = 2 - 3, GalT = 200 mU/L). The cells were washed once with 10 mM Tris-buffer and then resuspended in 10 mM Tris-buffer (pH 7.4) containing 10 mM $MnCl_2$. Storage of the cells as a 50 % (w/v) suspension in buffer at -20°C did not affect enzyme activity over a period of several months. UDP-glucose-pyrophosphorylase was isolated from germinated barley as described elsewhere.[9] All other enzymes and chemicals were from Sigma. Reactions were carried out in 10 mM Tris (pH 7.4) containing 10 mM $MnCl_2$ and 10 mM $MgCl_2$. Substrate and enzyme concentration were as given in table 1 and 2. LacNAc was detected by means of HPLC. For ^{1}H-NMR-analysis, the reaction was done in a 2 mL scale (10 mM UDP-Gal, 30 mM GlcNAc, 3.6 U AP, 25 % (w/v) yeast), and LacNAc was isolated after separation of the cells and precipitation of the proteins with MeOH by means of gelchromatography (Biogel-P2, 120 x 5 cm, 0.3 mL/min, LacNAc was eluted after 270 mL, 5.65 mg, 72 %). The synthesis of LacNAc with regeneration of UDP-Gal *in situ* (Scheme 3) was carried out under the following conditions: 20 mM Glc-1-P, 2 mM UTP, 60 mM GlcNAc, 25 mM PEP, 1 U/mL UDPGE, 0.37 U/mL UDPGP[9], 5 U/mL PK, 2 U/mL inorganic pyrophosphatase from yeast [E.C. 3.6.1.1], 0.1 mg/mL BSA, 45 % (w/v) yeast in reaction buffer at 37°C.

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