# Development of a Chemically Defined Fermentation Medium for the Production of a New Recombinant Fructosyltransferase

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Abstract—The industrial-scale production of fructooligosaccharides from sucrose requires large quantities of the enzyme fructosyltransferase. An Aspergillus terreus fructosyltransferase was therefore expressed in Kluyveromyces lactis GG799 and secreted into the medium. K. lactis was cultivated in shaking flasks at 30 °C and 250 rpm using either rich or chemically defined fermentation media. In order to limit the accumulation of unwanted side products that tend to form in rich media such as veast extract peptone dextrose, a chemically defined FM22 medium was optimized in a two stages, focusing on biomass accumulation and enzyme production, respectively. A design-of-experiments strategy was used to screen for essential vitamins. A two-level fractional factorial design revealed that only biotin, nicotinic acid, pyridoxine and D-pantothenic acid were necessary for biomass accumulation, and an additional D-optimal design was used to optimize the concentration of inorganic salts (MgSO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, CaSO<sub>4</sub>, FeSO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub>) and the fermentation temperature. For enzyme production, the integrated LAC4 promoter was induced with galactose, which was provided in addition to glucose as the carbon source in the adapted FM22 medium.

*Index Terms*—fructo-oligosaccharide, FOS, fructosyltransferase, *Kluyveromyces lactis*, FM22, 1-kestose, prebiotic

#### I. INTRODUCTION

Fructo-oligosaccharides (FOS) are low-calorie sweeteners that occur naturally as long-chain carbohydrates in plants and as exopolysaccharides in microorganisms [1]. FOS cannot be digested by humans, but they can stimulate the growth of beneficial gut bacteria and thus have a prebiotic effect [2], [3]. FOS can be produced industrially by the enzymatic elongation of sucrose or the hydrolysis of polysaccharides such as inulin. Hydrolysis is catalyzed by endo-inulinases (EC 3.2.1.7), whereas the elongation reaction is catalyzed by fructosyltransferases (EC 2.4.1.9), such as those found in commercial enzyme mixes like Pectinex Ultra SP-L, with sucrose acting as both donor and acceptor [4]-[7]. Fructose released from the donor sucrose is bound via a  $\beta(2-1)$  linkage to the fructose moiety of an acceptor sucrose, vielding 1-kestose (GF<sub>2</sub>). Further transfructosylation extends the linear chain of  $\beta(2-1)$ linked fructose moieties yielding nystose (GF<sub>3</sub>) and 1<sup>F</sup>fructofranosylnystose (GF<sub>4</sub>). In addition to the  $\beta$ (2-1)linked FOS type, two related trisaccharides (GF<sub>2</sub>) can be produced by fructosylation: 6-kestose via a  $\beta$ (2-6) linkage to the fructose moiety and neokestose via a  $\beta$ (2-6) linkage to the glucose moiety of the acceptor sucrose [8], [4], [9].

Kluyveromyces lactis has long been used for the industrial-scale production of native  $\beta$ -galactosidase, which is used to manufacture dairy products for people with lactose intolerance. For  $\beta$ -galactosidase production, K. lactis is classified "generally recognized as safe" (GRAS) by the US Food and Drug Administration (FDA) [10]. In a dried and inactivated form, K. lactis is used as a protein additive for food and feed [11]. K. lactis was one of the first fully sequenced yeasts, and one of the first yeasts successfully used for heterologous protein expression [12]. K. lactis is therefore used for the commercial production of bovine chymosin, which coagulates milk in the calf stomach. In the food industry, chymosin and prochymosin are used during cheese production [13]. An emerging use of K. lactis, due to its capacity for post-translational modification [10], is the production of complex therapeutic proteins such as interleukin 1 $\beta$ , interferon  $\alpha$ , lysozyme, serum albumin, and preproinsulin [14]-[18].

In terms of process development, rich complex media generally promote better growth and higher productivity than chemically defined media due to the presence of precursors that are used by the microorganisms in their core metabolic processes [19]. However, rich fermentation media are more expensive than chemically

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defined media and the concentrations of various indigents are inconsistent [20]. For the manufacture of low-margin products such as enzymes in the food industry, low-cost substrates are necessary for economic feasibility and chemically defined media also increase process robustness. Here, we adapted the chemically defined medium FM22 to achieve *K. lactis* cell growth and fructosyltransferase yields comparable to those realized in a complex medium, namely yeast extract peptone dextrose (YPD).

## II. MATERIALS AND METHODS

## A. Strain and Materials

We used a recently described Kluvveromyces lactis GG799 strain from New England Biolabs GmbH (Germany) expressing an integrated fructosyltransferase gene from Aspergillus terreus NIH2624 and secreting the enzyme to the medium [21]. All chemical reagents, if not otherwise stated, were purchased from Merck KGaA (Germany). Ammonium sulfate, trisodium citrate and LC-MS grade triethylamine were purchased from VWR International GmbH (Germany). Biotin, galactose, tryptone and sucrose were purchased from Carl Roth GmbH & Co KG (Germany). We obtained D-pantothenic acid, inositol, nicotinic acid, pyridoxine and analytical grade glucose, fructose and sucrose for HPLC analysis from Sigma-Aldrich (Germany). Iron(II) sulfate and 4-amino-benzoic acid were purchased from AppliChem GmbH (Germany) and cobalt(II)-chloride was purchased from Alfa Aesar Haverhill (USA). We purchased a FOS standard set comprising 1-kestose, nystose and 1<sup>F</sup>-fructofranosylnystose from Wako Pure Chemical Industries Ltd (Japan). LC-MS grade acetonitrile was purchased from Altmann Analytik GmbH & Co. KG (Germany).

## B. Small-Scale Shake Flask Conditions and Media

Cultures were grown in 500-mL shake flasks with a working volume of 50 mL, and were incubated at 250 rpm in an INFORS HT Multitron (Infors AG, Switzerland). FM22 medium [22], [23] was used at 30 °C and pH 5. If not otherwise stated, the medium comprised 5 g  $L^{-1}$  (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g  $L^{-1}$  CaSO<sub>4</sub>·H<sub>2</sub>O, 1.75 g  $L^{-1}$ K<sub>2</sub>HPO<sub>4</sub>, 8.6 g L<sup>-1</sup> K<sub>2</sub>SO<sub>4</sub>, 20 g L<sup>-1</sup> glucose, 10 g L<sup>-1</sup> inositol, 25.7 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 16.4 g L<sup>-1</sup> MgSO<sub>4</sub>·H<sub>2</sub>O, 15.6 g  $L^{-1}$  Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>, 160 mg  $L^{-1}$  biotin, 410 mg  $L^{-1}$  thiamin, 410 mg  $L^{-1}$  D-pantothenic acid, 410 mg  $L^{-1}$  nicotinic acid, 410 mg L<sup>-1</sup> pyridoxine, 80 mg L<sup>-1</sup> 4-aminobenzoic acid, 410 mg L<sup>-1</sup> riboflavin, 2.4 mg L<sup>-1</sup> CuSO<sub>4</sub>, 0.01 mg L<sup>-1</sup> NaI, 3.6 mg L<sup>-1</sup> MnSO<sub>4</sub> · H<sub>2</sub>O, 0.24 mg L<sup>-1</sup> Na2MoO4·2H2O, 0.02 mg L<sup>-1</sup> H3BO3, 1 mg L<sup>-1</sup> CoCl2, 14 mg  $L^{-1}$  ZnCl<sub>2</sub> and 26.4 mg  $L^{-1}$  FeSO<sub>4</sub>. The vitamins and a trace elements were prepared as individual filter sterilized stock solutions before mixing.

Stock solutions containing the carbon source, the potassium phosphate buffer, and the remaining salts were autoclaved at  $121 \,^{\circ}$ C for 15 min to avoid phosphorus salt precipitation and caramelization reactions. Cultures were

inoculated from frozen glycerol stocks stored at  $-80 \text{ }^{\circ}\text{C}$  to an optical density of  $\Delta OD_{600} = 0.2$  and were cultivated until they reached the stationary phase.

## C. Reactor-Scale Expression Conditions

Small cultures were transferred to MiniBio 500 (Applikon Biotechnology, Netherlands) reactors containing 300 mL medium, including 30 g L<sup>-1</sup> glucose and 7.5 g  $L^{-1}$  galactose as carbon sources. For production of the recombinant fructosyltransferase, the integrated LAC4 promoter was induced with galactose. The K. lactis dry cell weight and protein yields were compared in the adapted FM22 medium and YPD medium at pH 5 and 6 in duplicate fermentations. The pH was regulated using a PID controller by adding 25% concentrated ammonia hydroxide as a base when necessary. The reactor vessel was aerated at 1 vvm and with 0-0.27 vvm oxygen to maintain the dissolved oxygen (DO) saturation above 50%. We used 42 µL J673A solution as an anti-foaming agent (Schill + Seilacher "Struktol" GmbH, Germany). The YPD medium contained 20 g L<sup>-1</sup> peptone ex casein and 10 g  $L^{-1}$  yeast extract in addition to the carbon source.

## D. Cell Dry Weight

The empty weight of a 2-mL tube was measured on a precision balance after drying the tube for at least 24 h at 70 °C. We then added 2 mL fermentation broth, centrifuged for 3 min (4 °C, 16,100 x g), and the supernatant was removed. In addition, to remove disruptive medium components, the pellet was dissolved in physiological sodium chloride solution and treated as described above. This washing step was repeated. The open tube containing the biomass was then incubated in the drying oven for 24h at 70 °C, cooled to room temperature in a desiccator and then re-weighed on the precision balance. The difference between the final weight and the initial weight of the empty tube was reported as the cell dry weight.

## E. HPLC Analytics and Enzyme Assay

Samples were analyzed on a Dionex UltiMate 3000 HPLC system coupled with a Corona Veo Charged Aerosol Detector (Thermo Fisher Scientific, USA). Samples were fractionated in an XBridge Amide,  $3.5 \mu m$ ,  $4.6 \times 150 mm$  column (Waters GmbH, Germany). The mobile phase was 70:30 (v/v) acetonitrile/water. The water fraction was supplemented with 0.2% (v/v) trimethylamine before analysis.

To determine the enzyme activity, 100  $\mu$ L cell-free fermentation broth was incubated with 900  $\mu$ L substrate solution at 60 °C for 20 min, agitated at 1000 rpm in a Thermomixer Comfort (Eppendorf AG, Germany). The substrate solution consisted of 600 g L<sup>-1</sup> sucrose in 50 mM potassium phosphate (pH 6). After incubation, the reaction was interrupted by heating the sample to 95 °C for 20 min. The samples were diluted 1:50 with 50:50 (v/v) acetonitrile/water and clarified by passing through a 0.45- $\mu$ m nylon membrane filter. Enzyme activity was determined by measuring the amount of released glucose minus the amount of free fructose [24].

#### F. Design of Experiments

Design Expert v10 (Stat-Ease, USA) was used to identify the essential vitamins and to adjust the salt concentrations and temperature. The concentration of the vitamins was varied as follows, defining the -1/1 values: biotin (A), 0.0-0.41 g L<sup>-1</sup>; thiamin (B), 0.0-0.41 g L<sup>-1</sup>; inositol (C), 0.0–25.0 g L<sup>-1</sup>; D-pantothenic acid (D), 0.0-0.41 g L<sup>-1</sup>; nicotinic acid (E), 0.0-0.41 g L<sup>-1</sup>; pyridoxine (F), 0.0-0.41 g L<sup>-1</sup>; 4-aminobenzoic acid (G), 0.0-0.08 g L<sup>-1</sup>; riboflavin (H), 0.0-0.41 g L<sup>-1</sup>. Optimized salt concentrations and the fermentation temperature were determined using a D-optimal design with the following–1/1 values: KH<sub>2</sub>PO<sub>4</sub> (A), 1.0-18.9 g L<sup>-1</sup>; MgSO<sub>4</sub>·H<sub>2</sub>O (B), 0.85-16.4 g L<sup>-1</sup>; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (C), 0.5-10.0 g L<sup>-1</sup>; CaSO<sub>4</sub>·H<sub>2</sub>O (D), 0.004-1.007 g L<sup>-1</sup>; FeSO<sub>4</sub> (E). 0.002-0.19 g L<sup>-1</sup>; and the temperature (F) 20-30 °C.

#### G. Polyacrylamide Gel Electrophoresis (SDS PAGE)

Protein samples from aliquots of cell-free fermentation broth from the shake flask and reactor scale cultures were analyzed by SDS-PAGE using Criterion TGX Stain-Free Protein 4-20% Gels from Bio-Rad (Germany). For each sample, 31  $\mu$ L of the protein solution was incubated with 11.7  $\mu$ L of 4x Laemmli Sample Buffer (Bio-Rad) and 1.3  $\mu$ L 2-mercaptoethanol (Bio-Rad) at 95 °C for 5 min. We used 5  $\mu$ L Precision Plus Protein Unstained Protein Standards (Bio-Rad) as size markers. The gel were run for 25 min at 250 V, then activated with the Chemidoc MP Imaging System (Bio-Rad).

#### III. RESULTS AND DISCUSSION

#### A. Vitamin Screening

A two-level fractional factorial design was used to determine which vitamins are essential for the growth of *K. lactis.* The responses in terms of cell dry weight are summarized in Table I.

Factors Response CDW С D Е Run A В F G Η  $(g L^{-1})$ -11 -1  $0.30 \pm 0.04$ 1 -11 1 \_1 -1  $^{-1}$ -1  $^{-1}$ 0.08±0.01 1 1 1 3 1 -11  $^{-1}$ -11  $^{-1}$ 1 0.07 + 0.014  $^{-1}$ -1 $^{-1}$ -1-1-1 -1-1 $0.07 \pm 0.01$ 0 0 0 0 5 0 0 0 0  $4.52 \pm 0.08$ 6 1 1 -1 1  $^{-1}$ -1  $0.34 \pm 0.01$ 1  $^{-1}$ 7 1 -1 1 -1 -1  $0.06 \pm 0.00$ 8  $0.04\pm0.00$ -1-1-11 1  $^{-1}$ 1 1 9 4.20±0.18 1 1 1 1 1 1 1 1 10 -1  $^{-1}$ -1 1 1 -1 0.23 ±0.01 1 1 11  $^{-1}$ 1  $^{-1}$  $^{-1}$ 1 1 -11  $0.25 \pm 0.04$ 12  $0.24 \pm 0.05$ 1  $^{-1}$  $^{-1}$  $^{-1}$ 1  $^{-1}$ 1 1 13  $0.04 \pm 0.00$ 1 -1 -1 -1 1 -1 14 -1  $^{-1}$ -1 1 -1 1 1  $0.07 \pm 0.01$ 1 0 0 15 0 0 0 0 0 0 4.93±0.13 0 0 0 0 16 0 0 0 0 5.30±0.01 17  $^{-1}$ 1  $^{-1}$ 1 1  $^{-1}$ 1 -10.75±0.07 18  $^{-1}$ 1 -1 $^{-1}$ -1  $0.07\pm0.01$ 1 19 -1 1 -1 -1 5 08 + 0 07 1 -11 1

 TABLE I.
 VITAMIN SCREENING

Concentration in coded format for the factors: A = biotin; B = thiamin; C = inositol; D = D-pantothenic acid; E = nicotinic acid; F = pyridoxine; G = 4-aminobenzoic acid, and H = riboflavin. CDW = cell dry weight.

These results show which vitamins must be added to the medium because they cannot be synthesized by the cells. Table I shows that despite the three centerpoints (Runs 5, 15 and 16) and the high concentrations of all vitamins (Run 9), only Run 19 achieved a cell dry weight (5.08 g L<sup>-1</sup>) comparable to the nonadapted FM22 medium (4.40 g L<sup>-1</sup>).



Figure 1. Pareto chart of the two-level fractional factorial vitamin screening. The chart shows effects above the Bonferroni limit indicating factors with a significant impact on *K. lactis* growth in the model [25]. Orange bars define positive effects and blue bars define negative effects. DE, AE, AD and AF indicate two-factor interactions.

The vitamins necessary for the growth of K. lactis are biotin, D-pantothenic acid, nicotinic acid and pyridoxine (Fig. 1). To confirm the model, Run 19 was repeated and subsequent experiments were carried out lacking individual vitamins, resuling in the absence of measurable cell dry weights. These results confirmed that all four vitamins must be present in the medium. The entire model, as well as the factors biotin, D-pantothenic acid and nicotinic acid, have p-values < 0.0001. Pyridoxine and the interactions of the other factors have p-values < 0.001 and are thus also clearly in the significant range. This means the probability that the effect is caused by noise in the process model is < 0.1%. In further experiments, only the essential vitamins nicotinic acid, biotin, D-pantothenic acid and pyridoxine were included in the vitamin adapted FM22 medium.

#### B. D-opitmal Optimization of Salt Cocnentrations

Next we investigated the impact on cell dry weight of different salt concentrations in the vitamin adapted FM22 medium and different fermentation temperatures. Each factor was examined in individual ranges based on data available in the literature. A two-factor interaction model was used, as suggested by Design Expert, and square root data transformation, derived from the Box Cox diagram, was therefore applied to evaluate the model. The evaluation of the process model confirmed significance with a p-value < 0.0001. Furthermore, significant model terms included potassium phosphate, ammonium sulfate,

the temperature and the two-level interactions between potassium phosphate and magnesium sulfate, potassium phosphate and iron(II) sulfate, magnesium sulfate and iron(II) sulfate, and ammonium sulfate and iron(II) sulfate.

TABLE II. D-OPTIMAL DESIGN

Run         A         B         C         D         E         F         CDW           1         -0.3         -1.0         1.0         -0.3         -1.0         -1.0         5.87±0.13           2         1.0         -0.8         -1.0         -1.0         1.0         0.83±0.03           3         1.0         1.0         0.5         -1.0         0.9         1.0         6.83±0.03           4         -1.0         -0.2         1.0         -0.5         1.0         1.0         0.47±0.08           6         -1.0         -1.0         -1.0         1.0         -1.0         0.47±0.03           7         0.3         0.3         -1.0         0.5         0.5         0.0         0.83±0.03           8         0.0         0.0         0.0         -0.2         0.0         0.0         2.98±0.03           10         -1.0         1.0         -1.0         1.0		Factors						Response
1 $-0.3$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $1.0$ $0.83 \pm 0.03$ 2 $1.0$ $-0.8$ $-1.0$ $0.9$ $1.0$ $0.83 \pm 0.03$ 3 $1.0$ $-0.2$ $1.0$ $-0.5$ $1.0$ $1.0$ $5.38 \pm 0.28$ 5 $1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.47 \pm 0.03$ 6 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.47 \pm 0.03$ 7 $0.3$ $0.3$ $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.47 \pm 0.03$ 8 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $3.00 \pm 0.03$ 9 $-1.0$ $1.0$ $-1.0$ $0.0$ $0.0$ $2.98 \pm 0.03$ 11 $0.0$ $0.0$ $0.0$ $0.0$ $0.0$ $2.98 \pm 0.03$ 12 $1.0$ $1.0$ $1.0$ $1.0$ $1.0$ $1.0$ $1.0$ 13 $0.3$	Run	Α	В	С	D	E	F	CDW
2         1.0         -0.8         -1.0         -1.0         1.0         0.83 $\pm$ 0.03           3         1.0         1.0         0.5         -1.0         0.9         1.0         6.83 $\pm$ 0.03           4         -1.0         -0.2         1.0         -0.5         1.0         1.0         5.38 $\pm$ 0.28           5         1.0         -1.0         -1.0         -1.0         -1.0         0.47 $\pm$ 0.08           6         -1.0         -1.0         -1.0         1.0         -1.0         0.47 $\pm$ 0.03           7         0.3         0.3         -1.0         0.5         0.5         0.0         0.83 $\pm$ 0.03           8         0.0         0.0         0.0         -0.2         0.0         0.0         3.00 $\pm$ 0.2           10         -1.0         1.0         -1.0         1.0         1.0         1.05 $\pm$ 0.13         1.0         1.05 $\pm$ 0.05         1.0           11         0.0         0.0         0.0         -0.2         0.0         0.0         2.98 $\pm$ 0.03           12         1.0         1.0         -1.0         -1.0         1.0         5.12 $\pm$ 0.6           14         1.0         -1.0         1.0         -1.0 <td>1</td> <td>-0.3</td> <td>-1.0</td> <td>1.0</td> <td>-0.3</td> <td>-1.0</td> <td>-1.0</td> <td>5.87±0.13</td>	1	-0.3	-1.0	1.0	-0.3	-1.0	-1.0	5.87±0.13
3         1.0         1.0         0.5         -1.0         0.9         1.0         6.83 $\pm$ 0.03           4         -1.0         -0.2         1.0         -0.5         1.0         1.0         5.38 $\pm$ 0.28           5         1.0         -1.0         -1.0         -1.0         -1.0         0.47 $\pm$ 0.08           6         -1.0         -1.0         -1.0         1.0         -1.0         0.47 $\pm$ 0.03           7         0.3         0.3         -1.0         0.5         0.5         0.0         0.47 $\pm$ 0.03           8         0.0         0.0         0.0         -0.2         0.0         0.0         3.00 $\pm$ 0.0           9         -1.0         1.0         -1.0         0.5         1.0         -1.0         1.05 $\pm$ 0.13           10         -1.0         1.0         -1.0         1.0         1.0         1.05 $\pm$ 0.13           10         -1.0         -1.0         -1.0         1.0         1.0         1.05 $\pm$ 0.13           11         0.0         0.0         0.0         -0.2         0.0         0.0         2.98 $\pm$ 0.3           12         1.0         1.0         1.0         -1.0         -1.0         1.0	2	1.0	-0.8	-1.0	-1.0	1.0	1.0	0.83±0.03
4-1.0-0.21.0-0.51.01.0 $5.38 \pm 0.28$ 51.0-1.0-1.0-1.0-1.0-1.00.47 \pm 0.086-1.0-1.0-1.01.0-1.00.47 \pm 0.0370.30.3-1.00.50.50.00.83 \pm 0.0380.00.00.0-0.20.00.03.00 \pm 0.089-1.01.0-1.00.51.0-1.01.05 \pm 0.1310-1.01.0-1.01.0-1.01.01.05 \pm 0.13110.00.00.0-0.20.00.02.98 \pm 0.03121.01.0-1.01.01.01.05 \pm 0.15130.31.01.00.51.00.05.12 \pm 0.06141.0-1.01.01.0-1.01.06.73 \pm 0.1316-1.01.01.0-1.01.06.73 \pm 0.1316-1.01.01.00.5-0.81.06.86 \pm 0.16170.00.00.0-0.20.00.03.55 \pm 0.22181.0-1.0-1.0-1.0-1.00.06.13 \pm 0.1320-1.0-1.01.00.51.01.00.57 \pm 0.0622-1.01.01.00.5-0.81.04.62 \pm 0.1923-1.0-1.0-1.00.5-1.0-1.00.53 \pm 0.0224	3	1.0	1.0	0.5	-1.0	0.9	1.0	6.83±0.03
5         1.0 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.0$ $0.47 \pm 0.03$ 7         0.3         0.3 $-1.0$ 0.5         0.5         0.0 $0.83 \pm 0.03$ 8         0.0         0.0         0.0 $-0.2$ 0.0         0.0 $3.00 \pm 0.08$ 9 $-1.0$ 1.0 $-1.0$ $0.5$ $1.0$ $-1.0$ $1.05 \pm 0.13$ 10 $-1.0$ $1.0$ $-1.0$ $1.0$ $1.0$ $1.05 \pm 0.13$ 11 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $2.98 \pm 0.03$ 12 $1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $1.0$ $1.00 \pm 0.05$ 13 $0.3$ $1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $1.0$ $5.15 \pm 0.05$ 14 $1.0$ $-1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $6.73 \pm 0.13$ 16 $-1.0$ $1.0$ $1.0$ $-1.0$ <	4	-1.0	-0.2	1.0	-0.5	1.0	1.0	5.38±0.28
6         -1.0         -1.0         -1.0         1.0         -1.0         0.47 $\pm 0.03$ 7         0.3         0.3         -1.0         0.5         0.5         0.0         0.83 $\pm 0.03$ 8         0.0         0.0         0.0         -0.2         0.0         0.0         3.00 $\pm 0.08$ 9         -1.0         1.0         -1.0         0.5         1.0         -1.0         1.05 $\pm 0.13$ 10         -1.0         1.0         -1.0         1.0         1.0         1.05 $\pm 0.13$ 11         0.0         0.0         0.0         -0.2         0.0         0.0         2.98 $\pm 0.03$ 12         1.0         1.0         -1.0         1.0         -1.0         1.00         1.00 $\pm 0.05$ 13         0.3         1.0         1.0         -1.0         1.0         -1.0         1.0         5.15 $\pm 0.05$ 14         1.0         -1.0         1.0         -1.0         -1.0         1.0         6.73 $\pm 0.13$ 16         -1.0         1.0         1.0         0.5         -0.8         1.0         6.13 $\pm 0.13$ 10         -1.0         -1.0         -	5	1.0	-1.0	-1.0	-1.0	-1.0	-1.0	0.47±0.08
7 $0.3$ $0.3$ $-1.0$ $0.5$ $0.5$ $0.0$ $0.83 \pm 0.03$ 8 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $3.00 \pm 0.08$ 9 $-1.0$ $1.0$ $-1.0$ $0.5$ $1.0$ $-1.0$ $1.05 \pm 0.13$ $10$ $-1.0$ $1.0$ $-1.0$ $1.0$ $1.0$ $1.05 \pm 0.13$ $10$ $-1.0$ $1.0$ $-1.0$ $1.0$ $1.0$ $1.35 \pm 0.12$ $11$ $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $2.98 \pm 0.03$ $12$ $1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $1.00 \pm 0.05$ $13$ $0.3$ $1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $1.00 \pm 0.05$ $14$ $1.0$ $-1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $5.15 \pm 0.05$ $15$ $1.0$ $1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $6.73 \pm 0.13$ $16$ $-1.0$ $1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $6.73 \pm 0.13$ $16$ $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $6.86 \pm 0.16$ $17$ $0.0$ $0.0$ $0.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $0.0$ $19$ $1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $0.5$ $20$ $-1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.57 \pm 0.06$ $22$ $-1.0$ $1.0$ $1.0$ $0.5$ $1.0$ $1.0$ $0.57 \pm 0.06$ <td>6</td> <td>-1.0</td> <td>-1.0</td> <td>-1.0</td> <td>-1.0</td> <td>1.0</td> <td>-1.0</td> <td>0.47±0.03</td>	6	-1.0	-1.0	-1.0	-1.0	1.0	-1.0	0.47±0.03
8         0.0         0.0         -0.2         0.0         0.0         3.00±0.08           9         -1.0         1.0         -1.0         0.5         1.0         -1.0         1.05±0.13           10         -1.0         1.0         -1.0         -1.0         1.0         1.35±0.12           11         0.0         0.0         0.0         -0.2         0.0         0.0         2.98±0.03           12         1.0         1.0         -1.0         -1.0         1.0         -1.0         1.00±0.05           13         0.3         1.0         1.0         -1.0         1.0         -1.0         5.15±0.05           15         1.0         1.0         1.0         -1.0         1.0         6.73±0.13           16         -1.0         1.0         1.0         0.5         -0.8         1.0         6.86±0.16           17         0.0         0.0         0.0         -1.0         -1.0         1.0         6.73±0.33           16         -1.0         1.0         1.0         0.5         1.0         1.0         5.12±0.03           19         1.0         -1.0         0.5         1.0         1.0         6.13±0.13 <td>7</td> <td>0.3</td> <td>0.3</td> <td>-1.0</td> <td>0.5</td> <td>0.5</td> <td>0.0</td> <td>0.83±0.03</td>	7	0.3	0.3	-1.0	0.5	0.5	0.0	0.83±0.03
9 $-1.0$ $1.0$ $-1.0$ $0.5$ $1.0$ $-1.0$ $1.05 \pm 0.13$ 10 $-1.0$ $1.0$ $-1.0$ $1.0$ $1.0$ $1.35 \pm 0.12$ 11 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $2.98 \pm 0.03$ 12 $1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $1.00 \pm 0.05$ 13 $0.3$ $1.0$ $1.0$ $0.5$ $1.0$ $0.0$ $5.12 \pm 0.06$ 14 $1.0$ $-1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $5.15 \pm 0.05$ 15 $1.0$ $1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $6.73 \pm 0.13$ 16 $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $6.86 \pm 0.16$ 17 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $3.55 \pm 0.22$ 18 $1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.52 \pm 0.03$ 19 $1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77 \pm 0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77 \pm 0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $1.0$ $0.77 \pm 0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $1.0$ $0.77 \pm 0.06$ 22 $-1.0$ $1.0$ $1.0$ $0.5$ $-1.0$ $1.0$ $0.77 \pm 0.06$ 23 $-1.0$ $1.0$ $1.0$ $0.5$ $-1.0$ $1.0$ $0.77 \pm 0.06$ 24 $-1.0$	8	0.0	0.0	0.0	-0.2	0.0	0.0	3.00±0.08
10 $-1.0$ $1.0$ $1.0$ $1.0$ $1.35\pm0.12$ 110.00.00.0 $-0.2$ 0.00.0 $2.98\pm0.03$ 121.01.0 $-1.0$ $-1.0$ $1.0$ $-1.0$ $1.00\pm0.05$ 130.31.01.0 $0.5$ $1.0$ 0.0 $5.12\pm0.06$ 141.0 $-1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $5.15\pm0.05$ 15 $1.0$ $1.0$ $1.0$ $-1.0$ $1.0$ $6.73\pm0.13$ 16 $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $6.86\pm0.16$ 17 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $3.55\pm0.22$ 18 $1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.55$ 20 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.55$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77\pm0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77\pm0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $-1.0$ $0.57\pm0.06$ 22 $-1.0$ $1.0$ $1.0$ $0.5$ $-1.0$ $-1.0$ $0.57\pm0.06$ 23 $-1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.77\pm0.18$ 24 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.53\pm0.06$ 25 $1.0$ $1.0$ $-1.0$ $-1.0$ $-1.0$ $0.77\pm0.18$ 26 <t< td=""><td>9</td><td>-1.0</td><td>1.0</td><td>-1.0</td><td>0.5</td><td>1.0</td><td>-1.0</td><td>1.05±0.13</td></t<>	9	-1.0	1.0	-1.0	0.5	1.0	-1.0	1.05±0.13
110.00.0-0.0-0.20.00.0 $2.98\pm0.03$ 121.01.0-1.0-1.01.0-1.01.00\pm0.05130.31.01.00.51.00.05.12\pm0.06141.0-1.01.0-1.01.0-1.05.15\pm0.05151.01.01.0-1.0-1.01.0 $6.73\pm0.13$ 16-1.01.01.00.5-0.81.0 $6.86\pm0.16$ 170.00.00.0-0.20.00.0 $3.55\pm0.22$ 181.0-1.0-1.0-1.0-1.00.5520-1.0-1.00.90.40.81.0 $6.13\pm0.13$ 20-1.0-1.0-1.00.51.01.0 $0.77\pm0.18$ 211.0-1.0-1.00.51.0-1.0 $0.57\pm0.06$ 22-1.01.01.00.5-0.81.0 $4.62\pm0.19$ 23-1.0-1.0-1.00.5-1.00.97\pm0.08251.01.00.9-1.0-1.00.97\pm0.0826-1.01.0-1.0-1.0-1.00.92±0.13270.91.0-1.0-1.01.00.74±0.08301.01.0-1.00.5-1.01.00.58±0.06301.01.0-1.00.5-1.01.00.58±0.0633-1.01.0-1.00.5 <td>10</td> <td>-1.0</td> <td>1.0</td> <td>-1.0</td> <td>-1.0</td> <td>1.0</td> <td>1.0</td> <td>1.35±0.12</td>	10	-1.0	1.0	-1.0	-1.0	1.0	1.0	1.35±0.12
121.01.0 $-1.0$ 1.0 $-1.0$ 1.0 $-1.0$ 1.00 $-0.0$ 130.31.01.00.51.00.05.12±0.06141.0 $-1.0$ 1.01.0 $-1.0$ 1.0 $-1.0$ 5.15±0.05151.01.01.0 $-1.0$ $-1.0$ 1.0 $6.73\pm0.13$ 16 $-1.0$ 1.01.00.5 $-0.8$ 1.0 $6.86\pm0.16$ 170.00.00.0 $-0.2$ 0.00.0 $3.55\pm0.22$ 181.0 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.55$ 20 $-1.0$ $-1.0$ $0.9$ 0.40.81.0 $6.13\pm0.13$ 20 $-1.0$ $-1.0$ $0.5$ 1.01.0 $0.77\pm0.18$ 211.0 $-1.0$ $-1.0$ $0.5$ 1.0 $-1.0$ $0.57\pm0.06$ 22 $-1.0$ 1.01.0 $0.5$ $-1.0$ $-1.0$ $0.57\pm0.06$ 23 $-1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $-1.0$ $0.77\pm0.18$ 211.0 $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.77\pm0.08$ 22 $-1.0$ 1.0 $1.0$ $0.5$ $-1.0$ $-1.0$ $0.97\pm0.08$ 251.01.0 $-1.0$ $-1.0$ $-1.0$ $0.97\pm0.08$ 26 $-1.0$ 1.0 $-1.0$ $-1.0$ $-1.0$ $0.2\pm0.13$ 270.91.0 $-1.0$ $-1.0$ $1.0$ $0.5\pm0.06$ 30<	11	0.0	0.0	0.0	-0.2	0.0	0.0	2.98±0.03
13 $0.3$ $1.0$ $1.0$ $0.5$ $1.0$ $0.0$ $5.12\pm0.06$ 14 $1.0$ $-1.0$ $1.0$ $-1.0$ $1.0$ $-1.0$ $5.15\pm0.05$ 15 $1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $6.73\pm0.13$ 16 $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $6.86\pm0.16$ 17 $0.0$ $0.0$ $0.0$ $-0.2$ $0.0$ $0.0$ $3.55\pm0.22$ 18 $1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.55$ 20 $-1.0$ $-1.0$ $0.9$ $0.4$ $0.8$ $1.0$ $6.13\pm0.13$ 20 $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77\pm0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $-1.0$ $0.57\pm0.06$ 22 $-1.0$ $1.0$ $1.0$ $0.5$ $-1.0$ $-1.0$ $0.57\pm0.06$ 23 $-1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $-1.0$ $0.57\pm0.06$ 24 $-1.0$ $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.45\pm0.01$ 24 $-1.0$ $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.97\pm0.08$ 25 $1.0$ $1.0$ $-1.0$ $-1.0$ $-1.0$ $0.97\pm0.08$ 26 $-1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.79\pm0.08$ 27 $0.9$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.58\pm0.06$ 30 $1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.58\pm0.$	12	1.0	1.0	-1.0	-1.0	1.0	-1.0	1.00±0.05
141.0 $-1.0$ 1.0 $-1.0$ $1.0$ $-1.0$ $-1.0$ $5.15 \pm 0.05$ 151.01.01.0 $-1.0$ $-1.0$ $1.0$ $6.73 \pm 0.13$ 16 $-1.0$ 1.01.00.5 $-0.8$ 1.0 $6.86 \pm 0.16$ 170.00.00.0 $-0.2$ 0.00.0 $3.55 \pm 0.22$ 181.0 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.55$ $1.0$ $0.52 \pm 0.03$ 191.0 $-1.0$ $0.9$ 0.40.81.0 $6.13 \pm 0.13$ 20 $-1.0$ $-1.0$ $-1.0$ $0.55$ $1.0$ $1.0$ $0.77 \pm 0.18$ 211.0 $-1.0$ $-1.0$ $0.55$ $1.0$ $1.0$ $0.57 \pm 0.06$ 22 $-1.0$ 1.0 $1.0$ $0.55$ $-0.8$ $1.0$ $4.62 \pm 0.19$ 23 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.45 \pm 0.01$ 24 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.97 \pm 0.08$ 25 $1.0$ $1.0$ $0.9$ $-1.0$ $-1.0$ $0.97 \pm 0.08$ 26 $-1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.70 \pm 0.99$ 28 $0.2$ $1.0$ $0.2$ $0.5$ $1.0$ $1.0$ $0.58 \pm 0.06$ 30 $1.0$ $1.0$ $-1.0$ $0.5$ $-1.0$ $1.0$ $0.58 \pm 0.06$ 33 $-1.0$ $1.0$ $-1.0$ $-1.0$ $1.0$ $7.45 \pm 0.13$ 34 $1.0$ $0.4$ <t< td=""><td>13</td><td>0.3</td><td>1.0</td><td>1.0</td><td>0.5</td><td>1.0</td><td>0.0</td><td>5.12±0.06</td></t<>	13	0.3	1.0	1.0	0.5	1.0	0.0	5.12±0.06
151.01.01.0-1.0-1.01.0 $6.73 \pm 0.13$ 16-1.01.01.00.5-0.81.0 $6.86 \pm 0.16$ 170.00.00.0-0.20.00.0 $3.55 \pm 0.22$ 181.0-1.0-1.0-1.0-1.0-1.00.52 \pm 0.03191.0-1.00.90.40.81.0 $6.13 \pm 0.13$ 20-1.0-1.00.51.01.0 $0.77 \pm 0.18$ 211.0-1.0-1.00.51.0-1.0 $0.57 \pm 0.06$ 22-1.01.01.00.5-0.81.0 $4.62 \pm 0.19$ 23-1.0-1.0-1.0-1.0-1.0 $0.55 \pm 0.8$ 1.0 $4.62 \pm 0.19$ 24-1.0-1.0-1.00.5-1.0-1.0 $0.45 \pm 0.01$ 24-1.01.00.9-1.0-1.0-1.0 $0.97 \pm 0.08$ 251.01.00.9-1.0-1.0-1.0 $0.97 \pm 0.08$ 26-1.01.0-1.0-1.0-1.0 $0.70 \pm 0.09$ 280.21.00.20.5-1.01.0 $0.70 \pm 0.08$ 301.01.0-1.00.5-1.01.0 $0.58 \pm 0.06$ 301.01.0-1.00.5-1.01.0 $0.58 \pm 0.06$ 31-1.01.0-1.0-1.01.0 $0.58 \pm 0.06$ 33-1.0-1.0-1.01.0 <td< td=""><td>14</td><td>1.0</td><td>-1.0</td><td>1.0</td><td>-1.0</td><td>1.0</td><td>-1.0</td><td><math>5.15 \pm 0.05</math></td></td<>	14	1.0	-1.0	1.0	-1.0	1.0	-1.0	$5.15 \pm 0.05$
16-1.01.01.00.5-0.81.0 $6.86\pm0.16$ 170.00.00.0-0.20.00.0 $3.55\pm0.22$ 181.0-1.0-1.0-1.0-1.00.52\pm0.03191.0-1.00.90.40.81.0 $6.13\pm0.13$ 20-1.0-1.0-1.00.51.01.0 $0.77\pm0.18$ 211.0-1.0-1.00.51.0-1.0 $0.57\pm0.06$ 22-1.01.01.00.5-0.81.0 $4.62\pm0.19$ 23-1.0-1.0-1.0-1.01.0-1.0 $0.45\pm0.01$ 24-1.0-1.0-1.00.5-1.0-1.0 $0.45\pm0.01$ 24-1.01.00.9-1.0-1.0 $0.97\pm0.08$ 251.01.00.9-1.0-1.0 $0.97\pm0.08$ 26-1.01.0-1.0-1.0-1.0 $0.70\pm0.09$ 280.21.00.20.5-1.0-1.0 $0.70\pm0.09$ 280.21.00.20.51.01.0 $0.58\pm0.06$ 301.01.0-1.0-1.01.0 $0.58\pm0.06$ 31-1.01.0-1.0-1.01.0 $0.58\pm0.06$ 33-1.0-1.0-1.01.0 $0.58\pm0.06$ 33-1.0-1.0-1.00.5-0.11.0 $0.58\pm0.06$ 33-1.0-1.0-1.00.5	15	1.0	1.0	1.0	-1.0	-1.0	1.0	6.73±0.13
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	16	-1.0	1.0	1.0	0.5	-0.8	1.0	6.86±0.16
18         1.0 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.52 \pm 0.03$ 19         1.0 $-1.0$ $0.9$ $0.4$ $0.8$ $1.0$ $6.13 \pm 0.13$ 20 $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77 \pm 0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.57 \pm 0.06$ 22 $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $4.62 \pm 0.19$ 23 $-1.0$ $-1.0$ $-1.0$ $1.0$ $1.0$ $4.62 \pm 0.19$ 24 $-1.0$ $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.5$ $-1.0$ $-1.0$ $0.45 \pm 0.01$ 24 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $-1.0$ $0.4 \pm 4.02$ $0.8$ $2.0$ $0.4$ $-1.0$ $0.97 \pm 0.8$ 25 $1.0$ $1.0$ $-1.0$ $-1.0$ $-1.0$ $0.7 \pm 4.03$ $0.7 \pm 4.03$ $0.7 \pm 4.03$	17	0.0	0.0	0.0	-0.2	0.0	0.0	3.55±0.22
191.0 $-1.0$ $0.9$ $0.4$ $0.8$ $1.0$ $6.13\pm0.13$ 20 $-1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $1.0$ $0.77\pm0.18$ 21 $1.0$ $-1.0$ $-1.0$ $0.5$ $1.0$ $-1.0$ $0.57\pm0.06$ 22 $-1.0$ $1.0$ $1.0$ $0.5$ $-0.8$ $1.0$ $4.62\pm0.19$ 23 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $1.0$ $-1.0$ $0.45\pm0.11$ 24 $-1.0$ $-1.0$ $-1.0$ $0.5$ $-1.0$ $-1.0$ $0.9\pm0.13$ 25 $1.0$ $1.0$ $0.9$ $-1.0$ $-0.4$ $-1.0$ $0.97\pm0.08$ 26 $-1.0$ $1.0$ $-1.0$ $-1.0$ $-1.0$ $0.92\pm0.13$ 27 $0.9$ $1.0$ $-1.0$ $-1.0$ $1.0$ $0.72\pm0.09$ 28 $0.2$ $1.0$ $0.2$ $0.5$ $-1.0$ $-1.0$ $0.72\pm0.09$ 28 $0.2$ $1.0$ $0.2$ $0.5$ $-1.0$ $1.0$ $0.72\pm0.09$ 28 $0.2$ $1.0$ $0.2$ $0.5$ $-1.0$ $1.0$ $0.5\pm0.06$ 30 $1.0$ $1.0$ $-1.0$ $0.5$ $-1.0$ $1.0$ $0.5\pm0.06$ 33 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $1.0$ $0.5\pm0.06$ 33 $-1.0$ $-1.0$ $-1.0$ $-1.0$ $1.0$ $0.5\pm0.06$ 34 $1.0$ $0.4$ $1.0$ $0.5$ $-0.1$ $-1.0$ $2.8\pm0.03$ 35 $-1.0$ $-1.0$ $1.0$ $0.5$	18	1.0	-1.0	-1.0	-1.0	-1.0	-1.0	0.52±0.03
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	19	1.0	-1.0	0.9	0.4	0.8	1.0	6.13±0.13
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	20	-1.0	-1.0	-1.0	0.5	1.0	1.0	0.77±0.18
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	21	1.0	-1.0	-1.0	0.5	1.0	-1.0	0.57±0.06
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	22	-1.0	1.0	1.0	0.5	-0.8	1.0	4.62±0.19
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	23	-1.0	-1.0	-1.0	-1.0	1.0	-1.0	$0.45 \pm 0.01$
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	24	-1.0	-1.0	-1.0	0.5	-1.0	-1.0	5.03±0.08
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	25	1.0	1.0	0.9	-1.0	-0.4	-1.0	$0.97\pm0.08$
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	26	-1.0	1.0	-1.0	-1.0	-1.0	-1.0	0.92±0.13
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	27	0.9	1.0	-1.0	-1.0	-1.0	1.0	0.70±0.09
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	28	0.2	1.0	0.2	0.5	-1.0	-1.0	3.12±0.08
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	29	0.1	0.1	-1.0	0.5	-1.0	1.0	0.58±0.06
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	30	1.0	1.0	-0.2	0.5	1.0	1.0	6.23±0.08
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	31	-1.0	1.0	1.0	-1.0	1.0	-1.0	4.42±0.08
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	32	1.0	-1.0	-1.0	-0.2	-0.1	1.0	0.58±0.06
34         1.0         0.4         1.0         0.5         -0.1         -1.0         2.83±0.03           35         -1.0         -1.0         1.0         0.5         1.0         -1.0         5.43±0.06           36         -1.0         -0.2         1.0         -1.0         -1.0         0.0         6.60±0.05           37         1.0         1.0         -1.0         0.5         -1.0         0.0         0.67±0.06           38         1.0         -1.0         1.0         0.5         -1.0         1.0         5.77±0.23           39         -0.3         -1.0         1.0         -1.0         0.1         1.0         7.10±0.13	33	-1.0	-1.0	-0.6	-0.9	-1.0	1.0	7.45±0.13
35         -1.0         -1.0         1.0         0.5         1.0         -1.0         5.43 ±0.06           36         -1.0         -0.2         1.0         -1.0         -1.0         0.0         6.60 ±0.05           37         1.0         1.0         -1.0         0.5         -1.0         0.0         0.67 ±0.06           38         1.0         -1.0         1.0         0.5         -1.0         1.0         5.77 ±0.23           39         -0.3         -1.0         1.0         -1.0         0.1         1.0         7.10±0.13	34	1.0	0.4	1.0	0.5	-0.1	-1.0	2.83±0.03
36         -1.0         -0.2         1.0         -1.0         -1.0         0.0         6.60±0.05           37         1.0         1.0         -1.0         0.5         -1.0         0.0         0.67±0.06           38         1.0         -1.0         1.0         0.5         -1.0         1.0         5.77±0.23           39         -0.3         -1.0         1.0         -1.0         0.1         1.0         7.10±0.13	35	-1.0	-1.0	1.0	0.5	1.0	-1.0	5.43±0.06
37         1.0         1.0         -1.0         0.5         -1.0         0.0         0.67±0.06           38         1.0         -1.0         1.0         0.5         -1.0         1.0         5.77±0.23           39         -0.3         -1.0         1.0         -1.0         0.1         1.0         7.10±0.13	36	-1.0	-0.2	1.0	-1.0	-1.0	0.0	6.60±0.05
38         1.0         -1.0         1.0         0.5         -1.0         1.0         5.77±0.23           39         -0.3         -1.0         1.0         -1.0         0.1         1.0         7.10±0.13	37	1.0	1.0	-1.0	0.5	-1.0	0.0	$0.67 \pm 0.06$
39 -0.3 -1.0 1.0 -1.0 0.1 1.0 7.10±0.13	38	1.0	-1.0	1.0	0.5	-1.0	1.0	5.77±0.23
	39	-0.3	-1.0	1.0	-1.0	0.1	1.0	7.10±0.13

Concentration in coded format for the factors: A = potassium phosphate; B = masgnesium sulfate; C = ammonium sulfate; D = calcium sulfate;E = Iron(II) sulfate, and F = temperature. CDW = cell dry weight.

The difference between the predicted R-squared (0.7659) and the adjusted R-squared (0.8415) was < 0.2. In a confirmation run, a cell dry weight of 8.18±0.58 g L<sup>-1</sup> was achieved with the vitamin and salt adjusted medium at 30 °C, based on the following salt concentrations: KH<sub>2</sub>PO<sub>4</sub>, 9.95 g L<sup>-1</sup>; MgSO<sub>4</sub>·H<sub>2</sub>O, 1 g L<sup>-1</sup>; CaSO<sub>4</sub>·H<sub>2</sub>O, 1 g L<sup>-1</sup>; FeSO<sub>4</sub>, 0.19 g L<sup>-1</sup>; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 g L<sup>-1</sup>. In shake flasks, the cell dry weight increased by 186%. Similar increases in production yield (210%) have been achieved

[22] by reducing the concentration of salts and trace elements compared to the original FM22 medium [26].



Figure 2. Contour plot of the D-optimal model with cell dry weight as the response. Actual concentration of the remaining factors:  $KH_2PO_4$ , 9.95 g L<sup>-1</sup>; MgSO<sub>4</sub>·H<sub>2</sub>O, 1 g L<sup>-1</sup>; CaSO<sub>4</sub>·H<sub>2</sub>O, 1 g L<sup>-1</sup>; FeSO<sub>4</sub>, 0.19 g L<sup>-1</sup>.

#### C. Enzyme Production

Four duplicate fermentation runs were conducted with the vitamin and salt adapted FM22 medium at pH 5 and 6. The cell dry weights increased to  $17.48\pm0.20$  g L<sup>-1</sup> at pH 5 and to  $16.53\pm0.55$  g L<sup>-1</sup> at pH 6 (Fig. 3). The higher cell dry weights were achieved due to the adjusted carbon source concentrations at the larger reactor scale. The transfer activities of the cell-free fermentation broth in each case reflect the expressed fructosyltransferase. Compared to shake flask cultures, a new 80-kDa protein band was identified by SDS-PAGE (Fig. 4), corresponding to the glycosylated form of the expressed fructosyltransferase in *K. lactis*, whereas this band is not expressed by wild-type *K. lactis* GG799 [21].



Figure 3. Cell dry weight under four different fermentation conditions, each fermentation conducted in duplicate, and each cell dry weight measured in triplicate.

Fermentation broths without galactose showed no measurable transfer activity (data not shown). By measuring the expression level of the enzymes, the activities in adapted FM22 medium were found to be comparable to the results in YPD medium at both pH values (Fig. 5).



Figure 4. SDS-PAGE analysis of proteins in the fermentation broth. M = Pre-stained Protein Standards (250-25 kDa). Lane 1 = Cell-free fermentation broth of vitamin and salt adapted medium in shake flask cultures. Lane 2 = Cell-free fermentation broth of vitamin and salt adapted medium in reactor scale cultures (30 g L<sup>-1</sup> glucose, 7.5 g L<sup>-1</sup> galactose, pH 6).



Figure 5. Enzyme activity under four different fermentation conditions, each fermentation conducted in duplicate, and each activity assay conducted in triplicate.



Figure 6. HPLC profile of cell-free fermentation broth comprising vitamin and salt adjusted FM22 medium at pH 6, with 30 g  $L^{-1}$  glucose and 7.5 g  $L^{-1}$  glucose. The peaks correspond to (1) fructose, (2) glucose, (3) sucrose, (4) a putative isomeric trisaccharide of 1-kestose, (5) 1-kestose, (6) nystose, and (7) 1<sup>F</sup>-fructofranosyl-nystose.

In fermentations with the vitamin and salt adapted FM22 medium at pH 6, the maximum enzyme activity in the cell-free fermentation broth was  $133.8\pm16.5$  U mL<sup>-1</sup>.

The crude supernatant was analyzed by HPLC, revealing peaks corresponding to 1-kestose, nystose and  $1^{\text{F}}$ -fructofranosylnystose by reference to calibration standards (Fig. 6). These results indicate that the recombinant fructosyltransferase in this study does not exclusively produce  $\beta$ (2-1)-linked FOS. A putative isomeric trisaccharide of 1-kestose was also identified (Fig. 6).

#### IV. CONCLUSIONS

Batch fermentations in a stirred tank reactor with adapted FM22 medium achieved a maximum cell dry weight of 17.48 g L<sup>-1</sup>. In comparison, *P. pastoris* batch cultivations with adapted FM22 medium have achieved cell dry weights of 11.25 g L<sup>-1</sup> with a yield coefficient of 0.55 g<sub>biomass</sub> g<sub>glycerol</sub><sup>-1</sup> and a growth rate of 0.23 h<sup>-1</sup> [27]. Comparing the influence of vitamins, it is remarkable that the utilization of selected B-vitamins leads to a significant increase in biomass compared to the provision of a complete set of vitamins, suggesting the unnecessary vitamins are not only superfluous but may also be responsible for substrate inhibition.

In previous studies, an enzyme activity of 71.3 U ml<sup>-1</sup> was reported for the production of FOS by solid-state fermentation using Aspergillus japonicus ßfructofuranosidase [28]. Furthermore, an A. aculeatus βfructofuranosidase was purified from the technical enzyme solution Viscozyme L (Novozymes, Denmark) with a maximum yield ratio (grams of FOS per gram of initial sucrose) of 55%, after 100 min [29]. We achieved a maximum vield of 17.0±2.4 grams of FOS per gram of initial sucrose by fermentation in adjusted FM22 medium at pH 6. The lower yield may reflect the shorter reaction time of only 20 min in our study. We also used a crude supernatant, so the enzyme was not concentrated or purified prior to the reactions. Given these considerations, the enzyme activity we observed was remarkably high.

We have therefore developed a robust production method using a fructosyltransferase in chemically defined medium, yielding comparable cell dry weights and enzyme activities to the nutrient-rich YPD fermentation medium.

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- [1] A. P. Rauter, P. Vogel, and Y. Queneau, *Carbohydrates In* Sustainable Development I, Berlin: Springer Berlin Heidelberg, 2010.
- [2] F. V. A. Risso, M. A. Mazutti, H. Treichel, F. Costa, F. Maugeri, and M. I. RoRodrigues, "Comparison between systems for synthesis of fructooligosaccharides from sucrose using free inulinase from Kluyveromyces Marxianus NRRL Y-7571," *Food* and Bioprocess Technology, vol. 5, no. 1, pp. 331-337, 2012.

- [3] Z. KovÁCs, E. Benjamins, K. Grau, A. Ur Rehman, M. Ebrahimi, and P. Czermak, "Recent developments in manufacturing oligosaccharides with prebiotic functions," *Advances in Biochemical Engineering/Biotechnology*, vol. 143, pp. 257-295, 2014.
- [4] S. P. Singh, J. S. Jadaun, L. K. Narnoliya, and A. Pandey, "Prebiotic oligosaccharides: Special focus on fructooligosaccharides, its biosynthesis and bioactivity," *Applied Biochemistry and Biotechnology*, vol. 183, no. 2, pp. 613-635, 2017.
- [5] J. J. Virgen-Ort Ź, V. Ibarra-Junquera, P. Escalante-Minakata, S. Centeno-Leija, H. Serrano-Posada, J. de Jesús Ornelas-Paz, et al., "Identification and functional characterization of a fructooligosaccharides-forming enzyme from *aspergillus aculeatus*," *Applied Biochemistry and Biotechnology*, vol. 179, no. 3, pp. 497-513, 2016.
- [6] A. Ur Rehman, Z. Kovacs, H. Quitmann, M. Ebrahimi, and P. Czermak, "Enzymatic production of fructo-oligosaccharides from inexpensive and abundant substrates using a membrane reactor system," *Separation Science and Technology*, vol. 58, no. 4, P. 548, 2016.
- [7] B. Erdős, M. Grachten, P. Czermak, and Z. Kovás, "Artificial neural network-assisted spectrophotometric method for monitoring fructo-oligosaccharides production," *Food and Bioprocess Technology*, vol. 8398, no. 2014, P. 37, 2017.
- [8] N. Duchateau, K. Bortlik, U. Simmen, A. Wiemken, and P. Bancal, "Sucrose: Fructan 6-fructosyltransferase, a key enzyme for diverting carbon from sucrose to fructan in barley leaves," *Plant Physiology*, vol. 107, no. 4, pp. 1249-1255, 1995.
- [9] S. Kilian, S. Kritzinger, C. Rycroft, G. Gibson, and J. du Preez, "The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonic microbiota," *World Journal of Microbiology* and Biotechnology, vol. 18, no. 7, pp. 637-644, 2002.
- [10] A. J. J. Van Ooyen, P. Dekker, M. Huang, M. M. A. Olsthoorn, D. I. Jacobs, and P. A. Colussi, *et al.*, "Heterologous protein production in the yeast *kluyveromyces lactis*," *FEMS Yeast Research*, vol. 6, no. 3, pp. 381-392, 2006.
- [11] F. J. Bonekamp and J. Oosterom, "On the safety of kluyveromyces LACTIS: A review," Applied Microbiology and Biotechnology, vol. 41, no. 1, pp. 1-3, 1994.
- [12] S. Das And C. P. Hollenberg, "A high-frequency transformation system for the yeast *kluyveromyces lactis*," *Current Genetics*, vol. 6, no. 2, pp. 123-128, 1982.
- [13] J. A. Van Den Berg, K. J. Van Der Laken, A. J. J. Van Ooyen, T. C. H. M. Renniers, K. Rietveld, A. Schaap, *et al.*, "Kluyveromyces as a host for heterologous gene expression: Expression and secretion of prochymosin," *Nature Biotechnology*, vol. 8, no. 2, pp. 135-139, 1990.
- [14] R. Fleer, X. J. Chen, N. Amellal, P. Yeh, A. Fournier, F. Guinet, et al., "High-level secretion of correctly processed recombinant human interleukin-1 beta in *kluyveromyces lactis*," *Gene*, vol. 107, no. 2, pp. 285-295, 1991.
- [15] X. Chen, B. Gao, W. Shi, and Y. Li, "Expression and secretion of human interferon Alpha a in yeast kluyveromyces lactis," Yi Chuan Xue Bao=Acta Genetica Sinica, vol. 19, no. 3, pp. 284-288, 1992.
- [16] T. Iwata, R. Tanaka, M. Suetsugu, M. Ishibashi, H. Tokunaga, and M. Kikuchi, "Efficient secretion of human lysozyme from the yeast, *kluyveromyces lactis*," *Biotechnology Letters*, vol. 26, no. 23, pp. 1803-1808, 2004.
- [17] M. Saliola, C. Mazzoni, N. Solimando, A. Crisà, C. Falcone, G. Jung, *et al.*, "Use of the kladh4 promoter for ethanol-dependent production of recombinant human serum albumin in *kluyveromyces lactis*," *Applied and Environmental Microbiology*, vol. 65, no. 1, pp. 53-60, 1999.
- [18] Y. M. Feng, B. Y. Zhang, Y. S. Zhang, and H. Fukuhara, "Secretory expression of porcine insulin precursor in *kluyveromyces lactis* and its conversion into human insulin," *Acta Biochimica et Biophysica Sinica*, vol. 29, no. 2, pp. 129-134, 1997.
- [19] B. Hahn-Hägerdal, K. Karhumaa, C. U. Larsson, M. Gorwa-Grauslund, J. Görgens, and W. H. van Zyl, "Role of cultivation media in the development of yeast strains for large scale industrial use," *Microbial. Cell Factories*, vol. 4, no. 31, 2005.

- [20] G. G. Moulton, Fed-Batch Fermentation: A Practical Guide to Scalable Recombinant Protein Production in Escherichia Coli, 2014.
- [21] S. C. Spohner And P. Czermak, "Heterologous expression of Aspergillus Terreus fructosyltransferase in Kluyveromyces Lactis," New Biotechnology, vol. 33, no. 4, pp. 473-479, 2016.
- [22] B. A. Plantz, J. Sinha, L. Villarete, K. W. Nickerson, and V. L.Schlegel, "*Pichia pastoris* fermentation optimization: Energy state and testing a growth-associated model," *Applied* Microbiology and Biotechnology, vol. 72, no. 2, pp. 297-305, 2006.
- [23] P. P. Jacobs, M. Inan, N. Festjens, J. Haustraete, A. Van Hecke, R. Contreras, *et al.*, "Fed-batch fermentation of GM-CSF-producing glycoengineered *pichia pastoris* under controlled specific growth rate," *Microbial Cell Factories*, vol. 9, no. 93, 2010.
- [24] S. Van Hijum, M. Van Der Maarel, and L. Dijkhuizen, "Kinetic properties of an inulosucrase from *lactobacillus reuteri* 121," *FEBS Letters*, vol. 534, no. 1-3, pp. 207-210, 2003.
- [25] M. G. Borines, R. L. De Leon, and J. L. Cuello, "Bioethanol production from the macroalgae sargassum spp," *Bioresource Technology*, vol. 138, pp. 22-29, 2013.
- [26] J. Stratton, V. Chiruvolu, and M. Meagher, "High cell-density fermentation," *Methods in Molecular Biology (Clifton, N. J.)*, vol. 103, pp.107-120, 1998.
- [27] A. Ghosalkar, V. Sahai, and A. Srivastava, "Optimization of chemically defined medium for recombinant *pichia pastoris* for biomass production," *Bioresource Technology*, vol. 99, no. 16, pp. 7906-7910, 2008.
- [28] S. I. Mussatto, C. N. Aguilar, L. R. Rodrigues, and J. A. Teixeira, "Fructooligosaccharides and β-fructofuranosidase production by *aspergillus japonicus* immobilized on lignocellulosic materials," *Journal Of Molecular Catalysis B: Enzymatic*, vol. 59, no. 1-3, pp. 76-81, 2009.
- [29] A. S. G. Lorenzoni, L. F. Aydos, M. P. Klein, R. C. Rodrigues, and P. F. Hertz, "Fructooligosaccharides synthesis by highly stable immobilized β-fructofuranosidase from aspergillus aculeatus," Carbohydrate Polymers, vol. 103, pp. 193-197, 2014.



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