CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Microorganism

Gluconobacter oxydans NCIMB 4943 was obtained from the National Collection of Industrial Food and Marine Bacteria (Aberdeen, UK).

1.2 Chemicals

Unless otherwise stated, all chemicals and reagents were supplied by Sigma-Aldrich Co. Ltd., UK and bacteriological growth media supplements were supplied by Oxoid Ltd., UK.

1.2.1 Reagents for enzyme activity assay

A. For α-amylase assay, sodium phosphate buffer (20 mM) with sodium chloride (6.7 mM) was prepared using anhydrous sodium phosphate (NaH_2PO_4) and sodium chloride in reverse osmosis (RO) water. Solution was adjusted to pH 4, 5, 6, 7 and 8 with 1 M NaOH or 1 M HCl.

B. For β-amylase assay, sodium acetate buffer (16 mM) was prepared using sodium acetate (trihydrate) in RO water. Solution was adjusted to pH 4, 5, 6, 7 and 8 with 1 M NaOH or 1 M HCl.

C. 1.0% (w/v) Oligodextran solution was prepared in either sodium phosphate (reagent A) or sodium acetate buffer (reagent B).

D. Sodium potassium tartrate solution was prepared by dissolving 12.0 g of sodium potassium tartrate (tetrahydrate) in 8.0 ml of 2 M NaOH. Solution was heated directly on a heating/stir plate using constant stirring to dissolve but did not boil.

E. 96 mM 3,5-Dinitrosalicylic acid (DNS) solution was prepared in 20 ml in RO water. Solution was heated directly on a heating/stir plate to dissolve but did not boil.

F. Color reagent solution for reducing sugar analysis was prepared by addition of reagent D to reagent E slowly while stirring. Solution was diluted to 40 ml with RO water. The solution should be stored in the amber bottle at room temperature. The color reagent solution was stable for 6 months.

G. 0.2% (w/v) Maltose standard solution was prepared in RO water using maltose (monohydrate).

H. Enzyme solution was immediately prepared before use. Enzyme solution contained 1 and 2 unit ml^{-1} of α-amylase or β-amylase in cold RO water.

1.2.2 Reagents for acid stability assay

1.2.2.1 HCl

HCl was prepared from 5 M HCl to give pH 1 (1.5×10^{-1} M), 2 (1.5×10^{-2} M), 3 (1.5×10^{-3} M), 4 (1.5×10^{-4} M) and 5 (1.5×10^{-5} M) by addition of 1N NaOH.

1.2.2.2 HCl buffer

HCl buffer contained (g l^{-1}): NaCl, 8; KCl, 0.2; $Na_2HPO_4.2H_2O$, 8.25; $NaHPO_4$, 14.35; $CaCl_2.2H_2O$, 0.1; $MgCl_2.6H_2O$, 0.18. Buffer pH was adjusted to 1, 2, 3, 4 and 5 using 5 M HCl (Korakli *et al*., 2002).

1.2.3 Reagents for FISH technique

1.2.3.1 Phosphate buffered saline (PBS)

Phosphate buffered saline (PBS) with concentration of 0.1 mol l^{-1} was prepared using premade tablets (10 tablets l^{-1}) or it contained (g l^{-1}): NaCl, 8; KCl, 0.2; $Na_2HPO_4.2H_2O$, 1.44; KH_2PO_4, 0.24. Solution was adjusted to pH 7 then filter-sterilized through 0.2 μm filter.

1.2.3.2 4% Paraformaldehyde

2 g of paraformaldehyde and 100 μl of 1 N NaOH were added to 30 ml of HPLC-water and gently warmed until fully dissolved. 1 N HCl (100 μl) and 16.6 ml of 0.3 mol l^{-1} PBS (30 tablets l^{-1}) were added. The solution volume was adjusted to 50 ml

with HPLC-grade water, adjusted to pH 7.2 then filter-sterilized through a 0.2 μm filter. The solution was stored at 4°C before use.

1.2.3.3 Wash buffer

Wash buffer contained 20 mM Tris-HCL, 20mM NaCl, pH 7.2. The buffer was filter-sterilized through 0.2 μm filter.

1.2.3.4 Hybridization buffer

Hybridization buffer contained 30 mM Tris-HCl, 30 mM NaCl and 1.5% sodium dodesyl sulphate (SDS), pH 7.2.

1.3 Media

1.3.1 Glucose yeast extract-chalk (GYC) medium

The glucose yeast extract-chalk (GYC) medium contained (g l^{-1}): glucose, 50; yeast extract, 10; $CaCO_3$, 30 and agar, 25 without pH adjustment (6.8±0.2) (Mountzouris *et al*., 1999).

1.3.2 Glucose complex (GY) medium

The glucose complex (GY) medium contained (g l^{-1}): glucose, 50 and yeast extract, 10 without pH adjustment (6.8±0.2) (Mountzouris *et al*., 1999).

1.3.3 Maltodextrin complex medium

The maltodextrin complex medium contained (g l^{-1}): maltodextrin, 50 and yeast extract, 10 without pH adjustment (6.8±0.2) (Mountzouris *et al*., 1999).

1.3.4 Basal medium for stirred pH-controlled batch culture

The basal medium for stirred pH-controlled batch culture contained (g l^{-1}): peptone water, 2; yeast extract, 2; NaCl, 0.1 (Fisher, Loughborough, UK); $NaHCO_3$, 2; cysteine-HCl, 0.5; KH_2PO_4, 0.04 (BDH, Poole, UK); K_2HPO_4, 0.04 (BDH); bile salts, 0.5; $CaCl_2.6H_2O$, 0.01 (BDH); haemin, 0.005; $MgSO_4.7H_2O$, 0.01; Tween 80, 2 ml

(BDH); vitamin K_1, 10 μl; resazurin, 4 ml. 135 ml of basal medium was aliquoted into Duran bottles and sterilized by autoclaving (Rycroft *et al*., 2001b).

1.3.5 Basal medium for three-stage continuous system (gut model)

The basal medium for the three-stage continuous system contained (g l^{-1}): soluble potato starch, 5; peptone water, 5; tryptone, 5; yeast extract, 4.5; NaCl, 4.5; KCl, 4.5; pig porcine mucin, 4; milk casein, 3; pectin, 2; larch wood xylan, 2; arabinogalactan, 2; $NaHCO_3$, 1.5; $MgSO_4$, 1.25; guar gum, 1; cysteine-HCl, 0.8; KH_2PO_4, 0.5; K_2HPO_4, 0.5; bile salts, 0.4; $CaCl_2.6H_2O$, 0.15; $FeSO_4.7H_2O$, 0.005; haemin, 0.05; Tween 80, 1 ml; vitamin K_1, 10 μl; resazurin, 4 ml. 135 ml of basal media was aliquoted into 3 serum bottles and sterilized. 5 litres of basal medium was sterilized in a 10-l glass bottle and used as a medium reservoir (Sghir *et al*., 1998).

Oligodextran (10 g l^{-1}) produced from maltodextrin Glucidex 19 (Section 3.5) was added into basal medium aseptically before feeding started from day 10 to day 20.

1.4 Carbohydrate substrates

Maltodextrins were supplied by two companies; Goldex 15, 20, 30 and 37 were supplied by ABR Foods Ltd., UK and Glucidex 12, 19 and 29 were supplied by Roquette Ltd, France.

Commercially available prebiotics, used as carbohydrate references on fermentation studies, included inulin (Raftiline ST, Orafti, Belgium), high performance inulin (Raftiline HP, Orafti, Belgium) and levan (Magnolia, USA).

1.5 Sources of enzyme

Five sources of α-amylase (EC 3.2.1.1) derived from *Aspergillus oryzae, Bacillus sp.*, *B. licheniformis*, human pancreas and human saliva and one of β-amylase (EC 3.2.1.2) derived from barley were used in this study. These enzymes were supplied by Sigma-Aldrich Co. Ltd., UK.

2. General Methods

2.1 Ultrafiltration system

A labscale™ TFF system (Millipore Corporation, UK) was used to separate high molecular weight product (oligodextrans) from low molecular weight substrate (maltodextrins) in the sample. A tangential flow Pellicon XL filtre unit containing an Ultracel regenerated cellulose membrane, 5 kDa nominal molecular weight cut-off with a filtration area of 50 cm^2 was used. This modular system included a 500 ml acrylic reservoir with pressure gauges, device docking manifold and retentate valve. The system included a magnetic stirrer and diaphragm pump.

The pilot-scale ultrafiltration system consisted of a sample reservoir (50-l), a pump and a membrane module (1.2 m long). The membrane had a nominal molecular weight cut-off of 10 or 25 kDa and consisted of 18 tubular membranes with a total surface area of 1.08 m^2 attached to the module.

2.2 Fermentation system for oligodextran production

Pilot-scale production of oligodextran was performed on a 100-l scale in a 150-l fermenter (CH-8620 Wetzikon, MBR bioreactor AG, Switzerland). The fermenter was equipped with a controller for pH, stirring speed, and temperature and was equipped with steam sterilization (Appendix Figure 10.3). A 20-l feed reservoir (Millipore Corporation, USA) was used. Growth medium was filter sterilized using a 0.1 μm hydrophilic Durapore filter system (Millipore, UK).

2.3 Determination of cell concentration

Cell concentrations were determined by absorbance measurement (A_{620}), with conversion to dry weight by means of a calibration curve (Appendix Figure 1.1) and photo of *G. oxydans* NCIMB 4943 grown on GYC is shown in Appendix Figure 10.1. Cells were harvested by centrifugation (17,000×*g*) for 15 min at room temperature (25°C) and the cells were dried at 105°C for 24 h in a hot air oven (Adapted from Mountzouris *et al*., 1999).

2.4 Reducing sugar determination

Reducing sugar concentration was also determined using the dinitro-salicylic acid (DNS) assay and expressed as maltose equivalents (1.2.1 and 2.10). The assay was

calibrated with maltose standards from 0 to 2 mg ml^{-1} (Appendix Figure 3.1). This assay was used for the enzyme activity assay and acid stability study.

2.5 Determination of total carbohydrates

The total sugar concentration, expressed as glucose equivalents, was determined using the phenol-sulphuric acid assay (Dubois *et al*., 1956). The assay was calibrated with D-glucose standards from 0 to 100 μg ml^{-1} (Appendix Figure 2.1). Sample or glucose standard solution (200 μl) was added to 5% (w/v) phenol solution (200 μl). Concentrated sulphuric acid (1 ml) was then added and left for 10 min prior to vigorous mixing. The samples and standards were left for 30 min at room temperature before reading the absorbance at 490 nm. The concentration of carbohydrate in the sample was calculated by comparison with the standard curve of glucose.

2.6 Molecular weight analysis by high performance size exclusion chromatography (HPSEC)

After the cells were removed by centrifugation (17,000x*g* for 15 min at 4°C), the supernatant fluids were analyzed by HPSEC with refractive index detection. The analytical system consisted of two TSK G4000 PW columns and one TSK G6000 PW_{XL}, connected in series with decreasing pore size. The flow rate was 0.6 ml min^{-1}, the eluent was 0.02% (w/v) sodium azide in HPLC water and the operating temperature was 30°C. Samples and standards (dextran with different molecular weight) were filtered through a 0.45 μm syringe filter and the injection volume was 100 μl. The system was calibrated with at least six standards consisting of dextrans with average molecular weights of 38000 kDa, 2000 kDa, 464 kDa, 68.4 kDa, 42 kDa, 19.5 kDa, 9.5 kDa, maltoheptaose (1.15 kDa) and glucose (0.18 kDa). External standards of dextran (9.5 kDa) at a concentration of 1-10 mg ml^{-1} were used to establish the area response for quantification of the products. Oligodextran yield was calculated by the area of oligodextran divided by the respective curve area of its maltodextrin substrate and multiplying by 100 (Appendix Figure 5.1). The molecular

weight (MW) of oligodextran was determined by comparing sample peak K_{av} values with those of the standards in Appendix Figure 4.2 (Mountzouris *et al*., 1999).

2.7 Fractionation and purification of oligodextran

Oligodextran was recovered by gel filtration chromatography using a Sephadex G-50 column (2.5 cm x 50 cm) with a molecular weight filtration range of 1,000 to 10,000 Da and a gel bead pore size of 50-150 μm. Eluent (water) flow rate was 0.40 ml min^{-1}. Freeze dried samples were re-dissolved at 0.5% (w/v) and 12.3 ml were loaded onto the column. Fractions (1.5 ml) were collected in a fraction collector. The high molecular weight fraction from the Sephadex G-50 column was further fractionated on a Sephadex G-200 column (0.5 cm x 25 cm) with bead pore size of 40-120 μm. Eluent (water) flow rate was 0.2 ml min^{-1} (Kennedy and Paliuga, 1994). Carbohydrate samples were prepared at 0.5% (w/v) and 2 ml were loaded. Each fraction was lyophilized prior to further structural analysis.

A labscale ultrafiltration (Labscale™ TFF system, Section 2.1 and Appendix Figure 10.2) was used for larger scale separation and purification of oligodextran. The system was initially washed with RO water for over 1 h to elute preservative materials from the membrane. Cell-free culture medium (500 ml) was added to the sample reservoir and the magnetic stirrer and pump were turned on to give inlet and retentate pressures of 2-3 bars. The high molecular weight (>5 kDa) fraction was retained and recycled into the feed reservoir. The low molecular weight fraction (containing residual maltodextrin) was collected as the permeate. When the retentate volume in the feed reservoir was reduced to 50 ml RO water was added to the feed reservoir to make the volume up to 500 ml and filtration was resumed. This procedure was repeated a further two times. The oligodexran fraction was then freeze dried. Cleaning of membrane was required after daily use to prevent blocking and microbial contamination. The system was washed with 0.1 N NaOH for at least 1 h and the membrane stored in 0.05 N NaOH.

Pilot-scale ultrafiltration was used to purify G19 oligodextran obtained from pilot-scale fermentation. 20-l of cell-free culture medium was added to the feed tank, the pump was turned on and inlet and outlet pressures adjusted to 6 and 2 bar, respectively. The retentate fraction was recycled into the feed tank whereas the

permeate fraction was discarded. Filtration was continued until the retentate volume was reduced to 5-l then water was added to make the volume up to 20-l. This diafiltration step was repeated a further two times. The retentate fraction was freeze dried for fermentation studies.

2.8 Methylation analysis

The purified products were lyophilized and the linkage positions of the sugar residues were determined by methylation analysis (Mountzouris, 1999). Samples (5 mg) were dissolved in 0.4 ml of dry dimethylsulfoxide (DMSO), then 20 mg of powdered sodium hydroxide and 0.1 ml of methyl iodide were added. The mixture was then vortex-mixed in a closed vial for 5 min and left at room temperature (25°C) for 1 h (Ciucanu and Kerek, 1984; MacCormick *et al*., 1993). Water (1 ml) and chloroform (1 ml) were added and this mixture was vortex-mixed again. After phase separation, the water phase was removed with a micropipette and discarded. The chloroform layer was washed five times with water. After the final wash, any remaining water was removed by micropipette followed by evaporation to dryness under a stream of nitrogen. The partially methylated sample was hydrolyzed in 5 M trifluoroacetic acid (0.3 ml) by heating to 100°C for 15 h in a sealed vial (Blakeney *et al*., 1983). The hydrolyzed sample was cooled before being evaporated to dryness under stream of nitrogen. The dried hydrolyzed sample was reduced by adding sodium borohydride (1 ml of 0.5 M sodium borohydride in 2 M ammonia), and heating at 60° C for 1 h (Albersheim *et al*., 1967). Acetone (0.5 ml) was added to stop the reaction and the sample was evaporated to dryness under stream of nitrogen. The methylated and reduced products were acetylated by dissolving the dried sample in 0.2 ml of 18 M acetic acid, followed by addition of 1 ml of ethyl acetate, 3 ml of acetic anhydride, and 100 µl of 70% perchloric acid. The reaction mixture was vortex-mixed and allowed to stand for 5 min. After this, the mixture was cooled on ice before 5 ml of water were added, which was followed by addition of 200 µl of 1-methylimidazole (Albersheim *et al*., 1967). The reaction mixture was again allowed to stand for 5 min. Finally, 1 ml of dichloromethane was added and mixed on a vortex-mixer. After phase separation, the

organic phase was removed with a micropipette and stored in a sealed vial prior to analysis by gas chromatography and mass spectrometry (Mountzouris *et al*., 1999).

The partially methylated alditol acetates (PMAAs) were analyzed by GC-MS on a cross-bonded 50% cyanopropyl methyl- 50% phenyl methyl polysiloxane column (Thames Chromatography, Maidenhead, UK) using a flame ionization detector and a temperature program of 55°C for 2 min, increasing at 45°C min^{-1} for 1.9 min, 140°C for 2 min, increasing at 2°C min^{-1} for 35 min and 210°C for 40 min. The PMAAs were identified by measuring their retention times relative to *myo*-inositol hexaacetate, and comparing the relative retention times with those of external standards (maltose indicated 1,4 linkage and isomaltose indicated 1,6 linkage). Peak areas were represented as relative molar quantities compared to the terminal residues (Appendix Figure 6.1). Identities of PMAAs were confirmed by their electron-ionization mass spectra using a Fisons Analytical Trio 1S mass spectrometer, using a source temperature of 200°C and ionization potential of 70eV (Mountzouris *et al*., 1999).

2.9 NMR analysis

Spectra were obtained on a Bruker ARX 400 spectrometer operating at 400 MHz for ^{1}H. Chemical shifts (δ) were expressed in parts per million (ppm) from TMS (tetramethylsilane) as internal reference for ^{1}H. Samples (1 to 4 mg) were dissolved in 0.6 ml deuterated water (D_2O), placed in 5 mm o.d. NMR tubes, and the temperature was regulated at room temperature (25°C). The ^{1}H chemical shifts for the anomeric protons were compared to previous published data (terminal residue of α-form, δ=5.22; terminal residual of β-form, δ=4.64; α-1-6-D-glucose, δ=4.96; α-1-4-D-glucose, δ=5.35) (Jodelet *et al*., 1998). Example of calculation of degree of polymerization (DP) is shown in Appendix Figure 7.1.

2.10 Enzyme activity assay

The enzyme activity was determined by the Sigma quality control test procedures for α-amylase (EC. 3.2.1.1) and β-amylase (EC. 3.2.1.2).

Enzyme was prepared in solution containing 1 and 2 unit ml^{-1} using sodium phosphate buffer (20 mM) in sodium chloride (6.7 mM), pH 4, 5, 6, 7 or 8 for α-

amylase assay or dissolved in sodium acetate buffer (16 mM) for β-amylase assay, pH 4, 5, 6, 7 or 8. Oligodextran was prepared to 0.5% (w/v) solution in sodium phosphate buffer for α-amylase or sodium acetate buffer for β-amylase assay. 1 ml of enzyme solution was added to 1 ml of oligodextran solution. The reaction mixture was incubated at 37°C for 15 min, 1, 2, 4 or 6 h then 1 ml of color reagent (Reagent F) solution (Section 1.2.1) was added. Reaction was stopped by placing in a boiling water bath for exactly 15 min. The mixture was cooled to ambient temperature (25°C) then RO water (9 ml) was added. The mixture was mixed by vortexing and the A_{540} was measured and compared to that of a blank where the reaction was stopped immediately after the enzyme solution was added by boiling. The amount of maltose released was calculated from a standard curve. Percentage hydrolysis (adopted from Doyle *et al*., 1999) was calculated based on reducing sugar liberated (Section 2.4) and the total sugar content of the sample (Section 2.5).

$$\text{Hydrolysis (\%)} = [\text{Reducing sugar released}] / [\text{Total sugar content}] \times 100$$

Numeric values of the responses used in the persistence of the G19 oligodextran to human salivary amylase and human pancreatic amylase are shown in Table 8.1 and Table 8.2 of Appendix, respectively using response surface methodology.

2.11 Acid stability assay

Sample of oligodextran product (0.25%, w/v) was dissolved in RO water. Either HCl or HCl buffer at pH 1, 2, 3, 4 or 5 (1 ml) was added to the sample solution (1 ml) and the reaction mixture was incubated at 37°C for 30 min, 1, 2, 4 or 6 h. Color reagent (Reagent F, 1 ml, Section 1.2.1) was added to the mixture and boiling for 15 min. After cooling to ambient temperature (25°C), RO water (9 ml) was added before measuring absorbance at 540 nm. Percentage hydrolysis (Korakli *et al*., 2002) was calculated based on reducing sugar liberated (Section 2.4) and total sugar content of sample (Section 2.5).

$$\text{Hydrolysis (\%)} = [\text{Reducing sugar released}] / [\text{Total sugar content}] \times 100$$

2.12 Short-chain fatty acid (SCFA) analysis

Sample (1.5 ml) was centrifuged (17,000x*g*) for 15 min to remove particulate materials and cells then the supernatant (20 µl) was injected onto an HPLC system attached to a UV detector set at 210 and 214 nm. The column was an ion-exclusion Aminex HPX-87H 150×7.8 mm ID (BioRad, Watford, Herts) maintained at 50°C with a column heater. The eluent, 0.005 mol l^{-1} sulphuric acid in HPLC-grade water, was pumped through the column at a flow rate of 0.6 ml min^{-1}. Data from the UV detector were integrated using the 'ValueChrom™, software package (Bio-Rad) (Rycroft *et al*., 2001). Using external calibration curves, lactate, formate, acetate, propionate and butyrate were quantified in the sample (Appendix Figure 9.1).

2.13 Fluorescent *in situ* hybridization (FISH) technique

Genus-specific 16S rRNA-targeted oligonucleotide probes labeled with fluorescent dye Cy 3 (supplied by Eurogentec Ltd., UK) were used for enumeration of *Bifidobacterium* (Bif 164), *Bacteroides* (Bac 303), *Lactobacillus/Enterococcus* spp. (Lab 158), *Clostridium perfringens/histolyticum* subgroup (His 150) and *Clostridium coccoides/Eubaterium rectale* group (Erec 482). The DNA sequences of Bif 164, Bac 303, Lab 158, His 150 and Erec 482 probes were 5'-CAT CCG GCA TTA CCA CCC-3', 5'-CCA ATG TGG GGG ACC TT-3', 5'-GGT ATT AGC A(T/C)G TGT TTC CA-3', 5'-TTA TGC GGT ATT AAT CT(C/T) CCTTT-3' and 5'-GCT TCT TAG TCA GGT ACC G-3', respectively (Rinne *et al*., 2005). To obtain total bacterial counts, the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) was added to each sample. Cells were fixed on a slide and those stained with DAPI or hybridized with probe were enumerated under a microscope as described below.

Samples (375 µl) were removed from the culture and added to 1.125 ml filtered 4% (w/v) paraformaldehyde solution (pH 7.2), mixed and stored at 4°C overnight to fix the cells. The fixed cells were washed twice in filtered PBS (pH 7) and resuspended in 150 µl filtered PBS. Ethanol (150 µl) was added and the sample mixed and stored at -20°C for at least 1 h or until needed, but no longer than 3 months. The fixed cells (16 µl) were added to 264 µl prewarmed hybridization buffer then filtered

(0.2 μm membrane). The suitable volume of this mixture and probe (50 ng μl^{-1}) were mixed and placed in the hybridization oven at appropriate temperature overnight (Bif 164, Erec 482 and His 150 at 50°C; Bac 303 and Lab 158 at 45°C; DAPI at any temperature.

The hybridization samples (5-120 μl) were washed in 7 ml prewarmed, filtered, wash buffer and filtered through 0.2 μm filter) containing 20 μl DAPI solution (500 ng μl^{-1}) for 30 min at appropriate hybridization temperatures. Samples were vacuum filtered onto a 0.2 μm isopore membrane filter (Millipore Corporation, UK) and the filters mounted in 'SlowFade' (Molecular Probes, The Netherlands) on a clean slides. Microscope (Nikon Eclipse, E400, Japan) fitted with appropriate filters for the DAPI stain (excited at 550 nm and emits at 461 nm) and the Cy3 dye (excited at 550 nm and emits at 564 nm). A minimum of 15 fields, each containing 10-100 cells, was counted for each slide.

2.14 Calculation of prebiotic index (PI)

Equation for calculation of prebiotic index is as following:

$$\text{Prebiotic index (PI)} = (\text{Bif/Total})-(\text{Bac/Total})+(\text{Lac/Total})-(\text{Clos/Total})$$

Where Bif is bifidobacterial numbers at sample time/number at inoculation
Bac is bacteroides numbers at sample time/numbers at inoculation
Lac is lactobacilli numbers at sample time/number at inoculation
Clos is clostridia numbers at sample time/number at inoculation
Total is total bacteria numbers at sample time/numbers at inoculation
The equation assumed that an increase in the populations of bifidobacteria and/or lactobacilli has a positive effect in contrast to an increase in bacteroides and clostridia has negative (Palframan *et al*., 2003).

2.15 Statistic analysis

Differences between bacterial counts at 0, 6, 24 and 48 h of batch culture fermentations and at day 0, 9, 11 and 20 for three-stage continuous system, were tested for significance using paired *t*-tests, assuming equal variance and considering both

sides of the distribution (two-tailed distribution). Differences were considered at 99% and 95% significance if $P< 0.01$ and $P< 0.05$, respectively using SPSS for Windows software.

3. Experimental Procedures

3.1 Optimization for production of oligodextran from *G. oxydans* NCIMB 4943

3.1.1 Preparation of cell suspension

The cell suspensions were prepared by inoculating one loopful of *G. oxydans* NCIMB 4943 (prepared by growing on GYC agar for 72 h) into 100 ml of the GY medium in 250 ml Erlenmeyer flasks. Cultivation was performed on a rotary shaker (175 rev min^{-1}) for 24 h at 30°C. Cells were harvested by centrifugation at 17,000x*g* for 15 min at 4°C, then washed three times with sterilized 0.025 mol l^{-1} sodium acetate buffer (pH 4.5) and filter-sterilized cell pastes on 0.2 μm nylon membrane filter. The wet cell pastes were mixed with the above buffer to yield cell suspensions for production of oligodextran by the cell suspension method.

3.1.2 Cell growth characteristics

3.1.2.1 Shake-flask culture

Growth of *G. oxydans* was tested in GY medium. Cultivation was performed in shake-flasks on a rotary shaker (175 rev min^{-1}) at 30°C for 48 h (Mountzouris *et al*., 1999). Samples were taken at 0, 4, 8, 16, 24, 32 and 48 h of cultivation. Cell growth (absorbance was measured at 620 nm and converted to cell dry weight using standard curve, Appendix Figure 1.1) and pH were measured in triplicate samples.

3.1.2.2 Batch fermenter culture

Growth of *G.oxydans* was also tested in GY medium. Cultivation was performed in pH-controlled (6.8) batch fermenter (10-l) and non-pH controlled batch fermenter (10-l), with agitation speed of 150 rpm, 30°C for 48 h. Samples were taken at 0, 4, 8, 16, 24, 32 and 48 h of cultivation. Cell growth and pH were measured in triplicate samples using the same methods as of shake-flask culture.

3.1.3 Comparison of oligodextran production by the culture medium

method and the cell suspension method

3.1.3.1 Culture medium method

In the culture medium method, the microorganism was grown on GYC agar for 72 h at 30°C then one loopful of the microorganism was transferred into a 250 ml Erlenmeyer flask containing 100 ml maltodextrin complex medium. Cultivation was carried out for 72 h at 30°C and 175 rev min^{-1} on a rotary shaker. Samples were taken at 0, 4, 8, 16, 24, 32, 48 and 72 h. Bacterial cells were removed from the viscous culture by centrifugation at 17,000x*g* for 15 min at 4°C. The yield of oligodextrans in the cell-free supernatant was determination by HPSEC (Mountzouris *et al*., 1999).

3.1.3.2 Cell suspension method

For the cell suspension method, cell suspensions were prepared as described in Section 3.1.1. The substrate was dissolved in 0.025 mol l^{-1} sodium acetate buffer (pH 4.5) and filter-sterilized on a 0.2 μm nylon membrane filter. Substrate solution (90 ml) was mixed with stock cell suspension (10 ml) to yield 10 and 5 g l^{-1} of the initial substrate and cell concentrations, respectively. Reactions were performed in 250 ml Erlenmeyer flasks at 30°C and 200 rev min^{-1} on a rotary shaker for 72 h. Samples were taken at the same time intervals as before and oligodextran production was determined by HPSEC (Mountzouris *et al*., 1999). The experiments were performed in triplicate.

3.1.4 Effect of cell concentration, buffer pH and temperature

Experiments were performed in 100 ml reaction volume in 250 ml Erlenmeyer flasks using cell suspension conditions as described in section 3.1.3.2. Effects of cell concentrations (5, 10, 15, 30 g l^{-1}), sodium acetate buffer (0.025 mol l^{-1}), pH (3.5, 4.5, 5.5) and temperature (25, 30, 35°C) were tested. Samples were taken at 0, 4, 8, 16, 24, 32, 48 and 72 h of reaction time. The oligodextran production and substrate utilization were evaluated by HPSEC.

3.1.5 Effect of substrate type and its concentration

Experiments were performed as described above using cell suspension method. The maltodextrins tested were Goldex 15, 20, 30 and 37 and Glucidex 12, 19 and 29 (the numbers refer to the dextrose equivalent (DE) value) at concentrations of 10, 25,

50, 100 g l^{-1}. Samples (100 ml) were taken at 0, 4, 8, 16, 24, 32, 48 and 72 h of reaction time. Oligodextran production and substrate utilization were determined by HPSEC.

3.2 Studies on the influence of reaction parameters on the molecular weight distribution and chemical structure of oligodextran

Molecular weight distribution of oligodextrans using various conditions (i.e. production method, buffer pH, cell concentration, temperature) and type and concentration of substrate, were determined by high performance size exclusion chromatography (HPSEC). Chemical structures were analyzed by methylation analysis followed by gas chromatography-mass spectrometry (GC-MS) and proton nuclear magnetic resonance (^{1}H-NMR).

The samples (10 ml) were taken at 0, 16, 24, 48, 72 and 120 h cultivation. The cell-free supernatants of culture fluids were kept at –20°C until use. Molecular weight of sample was determined by HPSEC and oligodextran production and substrate utilization were determined as previously described in analytical methods. Samples were methylated and the type of linkages was analyzed by GC-MS. In addition, their chemical structure was further confirmed by NMR.

3.3 Studies on the susceptibility of oligodextrans to enzymatic digestion

The sensitivity of oligodextrans to enzymatic digestion was determined. The experiment was performed by addition of 1 ml of each enzyme solution (1 and 2 unit ml^{-1}) to 1% (w/v) oligodextran solution (1 ml) in suitable buffer (pH 4, 5, 6, 7 and 8) and the reaction mixture was incubated at 37°C for 0, 1, 2, 3, 4, 5 and 6 h (Doyle *et al*., 1999). The reaction was stopped by boiling for 15 min. After cooling to ambient temperature (25°C), the sample was withdrawn and added to 9 ml RO water prior to determination of the absorbance at 540 nm, using maltose as standard as previously described (Section 2.4). The molecular weight distribution of sample was determined by HPSEC (Section 2.6).

3.4 Studies on the stability of oligodextran to acid

The acid stability of oligodextran was determined. Two types of acidic solution were used; HCl solution at pH 1 (1.5×10^{-1} M), 2 (1.5×10^{-2} M), 3 (1.5×10^{-3} M), 4 (1.5×10^{-4} M) and 5 (1.5×10^{-5} M) (Section 1.2.2.1) and HCl buffer solution at the same pH ranges were prepared. HCl buffer solution had similar components to human gastric juice in the small intestine (Section 1.2.2.2).

Samples were taken at 0, 1, 2, 3, 4, 5 and 6 h for measurement of reducing sugar released and the molecular weight distribution was determined by HPSEC.

3.5 Pilot-scale production of oligodextran

Production of oligodextran was performed in batch system using a 150-l fementer (Appendix Figure 10.3) in two steps as follows. For the first step, 100 liters of culture medium was sterilized by steam and, after cooling, inoculated with *G. oxydans* (5%, v/v). Culture pH, temperature and stirring speed were controlled at 6.8, 30°C and 150 rpm, respectively without aeration. Cultivation was performed for 24 h to reach maximum cell yield and the cells was removed by centrifugation (17,000x*g*), for 15 min at 4°C. Cells were resuspended in sodium acetate buffer solution (pH 4.5) to make cell slurries. For the second step, the fermenter was sterilized and emptied then substrate solution was filter-sterilized through a 0.1 μm filter and filled into the sterile fermenter. After the substrate was sterilized and filled into the fermenter, cell slurries were aseptically introduced using a pump. Conditions of reaction were the same as in first step except for the cultivation time was 48 h. The viscous culture (100 liters) was harvested and cells were removed by centrifugation (17,000x*g*), for 15 min at 4°C. Separation and purification of oligodextrans were performed using a pilot-scale ultrafiltration system (Section 2.7).

3.6 The prebiotic properties of oligodextrans in batch and three-stage continuous systems

3.6.1 Preparation of faecal slurries

Human faecal slurries at concentrations of 10% and 20% (w/w) were prepared by diluting in PBS for use in batch culture and the three-stage continuous system, respectively. Fresh stool samples were weighed in pre-weighed container then added PBS to get desired concentration of faecal slurries then blended in a stomacher

(Seward, UK) at normal speed for 120 seconds. Faecal slurries for three-stage continuous system were filtered through stomacher sieve bags to remove solid particles to prevent blocking in the tube between vessels during operation (Rycroft *et al*., 2001b).

3.6.2 Stirred pH-controlled batch culture fermentation

Sterile batch culture fermenter (300 ml capacity) was filled with sterile basal medium and pre-reduced overnight by purging with oxygen-free nitrogen gas (Appendix Figure 10.4). Carbohydrate samples (1.5 g) were dissolved in pre-reduced, sterilized basal medium to give a final concentration of 10 g l^{-1} and returned to the sterile fermenter aseptically. Faecal slurries (15 ml) were added to each fermenter and the closed fermenter maintained under a headspace of oxygen-free nitrogen gas. Fermentation was carried out at 37°C, magnetically stirred and culture pH was controlled at 6.8±0.1 by addition of 0.5 N NaOH or HCl. Each carbohydrate sample was tested in duplicate fermenters. Samples (6 ml) were removed at 0, 6, 24 and 48 h for the enumeration of bacteria using FISH technique and short-chain fatty acid (SCFA) was analyzed by HPLC. The prebiotic index (PI) of each carbohydrate tested at each time point was calculated followed an equation given in analytical methods (section 2.14). Their values were compared to those of commercially available prebiotics.

3.6.3 The three–stage continuous system (gut model)

The system comprised of a cascade of three fermenters connected in series (Appendix Figure 10.5). This system had been validated and proved to be suitable for study on prebiotic effect *in vitro* which mimic human colon (Macfarlane *et al*., 1998). The system was operated in batch culture mode in basal medium for 24 h to allow the system to reach equilibrium. Basal medium for gut model was pumped into vessel (V1), which sequentially fed to V2, V3 and ultimately a waste unit. V1 and V2 had operating volumes of 280 ml, whist the volume in V3 was 300 ml. V1, V2 and V3 simulated proximal, transverse and distal colon conditions respectively. The pH set points (±0.1) of V1, V2 and V3 were 5.5, 6.2 and 6.8, respectively. Each vessel was magnetically stirred and maintained under a headspace of oxygen-free nitrogen gas.

Temperature (37°C) was maintained by a water-cooling system and culture pH was controlled automatically by addition of 1 N NaOH or HCl. Flow rate of basal medium was controlled by a pump set to 28±2 ml h^{-1} to give system-wide dilution rate of 0.10 h^{-1} to give system transit time of 27.1 h. Dilution rates of V1, V2 and V3 were 0.10, 0.10 and 0.093 h^{-1}, respectively. After 10 days of fermentation, basal medium was supplemented with G19 oligodextran (10 g l^{-1} final concentration) and fed from day 10 to day 20 of fermentation. Samples (6 ml) were taken at day 0, 9, 11 and 20 fermentation then samples were analyzed for bacterial enumeration by FISH and for SCFA by HPLC. Prebiotic index (PI) of oligodextran produced from maltodextrin (Glucidex 19) at each time point in vessel 1, 2 and 3 was calculated using equation given Section 2.14.