Table 4. Percentage distribution of the dominant bacterial species isolated from the mucous layer and digesta from the ceca of five rabbits

Species	Mucous scraping %	Lumen contents %
Bacteroides vulgatus	34	27
Bacteroides distasonis	9	3
Streptococcus intermedius	8	13
Bacteroides ruminicola	4	8
Bacteroides uniformis	7	3
Peptostreptococcus productus	5	6
Fusobacterium mortiferum	5	4
Coprococcus eutactus	4	nil
Bacteroides ovatus	nil	3
Clostridium innocuum	3	2
Clostridium ramosum	3	nil
Eubacterium rectale	3	4
Clostridium sticklandii	2	nil
Fusobacterium necrophorum	2	2
Unidentified Clostridium sp.	nil	6
Ruminococcus albus	2	3
Bacteroides praeacutus	1	nil
Bacteroides capillosus	1	1
Bacteroides oris	nil	5
Leptotrichia buccalis	nil	2
Unclassified	4	11

202 isolates were grouped into genera on Grams'reaction, morphology, metabolic-end products and motility. 59 isolates representing each of the groups were presumptively identified to species level.

Table 5. Distribution of the dominant bacterial species isolated from the mucous layer and lumen contents of the ceca of each of five rabbits.

Species	No. of isolates from mucus (M) and lumen (L) of rabbit No: 1		2		3		4		5	
	M	L	M	L	M	L	M	L	M	L
Bacteroides vulgatus	8	5	9	6	6	6	5	6	6	4
Bacteroides distasonis	-	-	2	-	2	1	-	-	5	2
Streptococcus intermedius	3	3	2	3	1	3	2	1	-	3
Bacteroides ruminicola	-	3	1	2	1	-	1	3	1	-
Bacteroides unifo[illegible]s	1	-	3	3	2	-	1	-	-	-
Peptostreptoco[illegible]roductus	1	1	-	-	1	3	3	2	-	-
Fusobacteriu[illegible]iferum	2	-	-	-	-	-	2	1	2	3
Coprococcus eutactus	1	-	-	-	1	-	2	-	-	-
Bacteroides ovatus	-	-	-	2	-	-	-	-	-	1
Clostridium innocuum	2	2	-	-	1	-	-	-	-	-
Clostridium ramosum	1	-	-	-	1	-	1	-	-	-
Eubacterium rectale	2	-	1	-	1	2	-	-	-	1
Clostridium sticklandii	-	-	-	-	-	-	-	-	2	-
Fusobacterium necrophorum	-	-	-	-	-	-	1	-	1	2
Unidentified Clostridium sp	-	2	-	1	-	-	-	3	-	-
Ruminococcus albus	-	-	1	1	1	-	-	1	-	1
Bacteroides praeacutus	-	-	-	-	-	-	-	-	1	-
Bacteroides capillosus	-	-	-	-	-	1	-	-	-	1
Bacteroides oris	-	-	-	2	-	-	-	3	-	-
Leptotrichia buccalis	-	-	-	-	-	1	-	-	-	1
Unclassified	1	3	-	3	2	2	1	2	-	1
Total	22	19	19	23	20	19	19	22	18	20

Approximately 20 isolates were cultured from the mucus layer and lumen contents of each of five rabbits. The isolates are listed as the numbers of each species recovered from the different habitats in the five animals.

Table 4 shows the overall percentage distribution of the bacterial species isolated and identified from the ceca of 5 rabbits. It is appreciated that although the techniques used probably recovered most of the viable bacteria present in the samples, there are several species in complex systems such as the gut, that do not grow on general-purpose media or have never been cultivated. With this proviso, *Bacteroides sp.* constituted over half the species isolated from the rabbit cecum, with *Bacteroides vulgatus* being most frequently isolated from all rabbits. There were marked variations in the dominant species distribution among the individual animals and between the two habitats sampled. Some bacterial species were found in all rabbits and in both lumen contents and mucous scrapings (Table 5). However only nine species, mainly *Bacteroides*, were found in rabbit number two, whereas the flora of rabbit mumber three was made up of smaller populations of over thirteen species. Several species were also confined to either of the two habitats. *Coprococcus* for example, was only recovered from mucus and *Bacteroides oris* was found only in lumen contents.

3.5 Chemical composition of mucin from rabbit small intestine

The results of chemical analyses of pronase-digested rabbit mucin and subunit pig gastric mucin are compared in Table 6. Small intestine mucus was pooled from 5 or 6 animals and three batches were prepared at different times. The data are presented as the average values for the three samples. Sulphate esters were not detected in rabbit mucin preparations, and the protein components of all three samples analysed were very much higher than the concentrations found in pig gastric mucin. Rabbit intestinal mucus retained its gel-forming properties even after two sequential treatments with β-mercaptoethanol, although full solubilization was obtained after 48 h proteolytic digestion with pronase. Table 7 shows the ABO blood group status, and therefore terminal structures, on the two mucin molecules. Only group A structure was detected on pig mucin,

Table 6. Chemical analysis of subunit mucins from pig stomach and rabbit intestines

Source	Chemical composition						
	GlcNAc	GalNAc	Gal	Fuc	NANA	Sulph	Prot
	-------Molar ratios-------				--------%--------		
Pig:							
stomach	1.22	1.0	1.2	0.7	0.2	5.6	21
Rabbit:							
small intestine	0.87	1.0	1.2	0.3	0.1	nil	59

Commercial pig gastric mucin was dialysed before analysis. Pooled mucus from rabbit small intestines was digested with pronase. Samples were derivatized for gas-liquid chromatography for analysis of carbohydrates. The concentrations of proteins, sulphates and sialic acids were estimated by chemical methods as described in section 2.8.

Table 7. Agglutination of homologous red cells by ABO antisera after absorption with mucin preparations.

	Antibody dilutions					Substance
	1:2	1:4	1:8	1:16	1:32	on mucin
Anti-A	+	+	+	+	-	-
Anti-B	+	+	+	+	-	-
Anti-A abs.with pig mucin	+	-	-	-	-	A
Anti-B abs.with pig mucin	+	+	+	+	-	none
Anti-A abs.with rabbit mucin	+	-	-	-	-	A
Anti-B abs.with rabbit mucin	+	-	-	-	-	B

Antisera to blood groups A and B were absorbed with pig and rabbit mucins. Residual titres were tested by agglutination against homologous red cells and compared with the titres of unabsorbed antisera.

but both A and B were found on rabbit mucin by absorption of antibody titre from both antisera. In reporting the molar ratios of the sugar components of mucin, the N-acetylgalactosamine value is set to unity. Rabbit mucin has approximately equimolar amounts of N-acetylgalactosamine and galactose, but less N-acetylglucosamine and fucose, with essentially A-type terminal structure. These findings suggest shorter carbohydrate side-chains than on the pig mucin, and with fewer fucose residues, assuming that the N-acetylgalactosamine is only to be found at both the non-reducing and reducing terminals.

3.6 Bacterial degradation of mucins *in vitro*.

All isolates were tested for their ability to degrade mucins by cleavage of glycoside bonds as indicated by the Periodic acid-Schiffs reagent (PAS-reactive mucin). Thirty four of 202 strains tested (17%) reduced initial concentrations of PAS-reactive pig mucin in culture media by over 25% after 4 d incubation in pure culture. W22, W6, L36, L25, L34, L40, L63, W12 and L52 were strains of *Bacteroides vulgatus*. Three isolates of *Fusobacterium mortiferum* (W5, W85 and W99), and all 5 isolates of *Peptostreptococcus productus*; W3, W68, W69, L50 and L53, were weakly, but consistently, positive. The three strains of *Bacteroides vulgatus*, W12, W13 and L34, were most strongly mucinolytic, consistently reducing the levels of PAS-reactive rabbit mucin by over 40%.

In an experiment to determine the relative digestibilities of the rabbit mucin preparation and the pig gastric mucin, four strains of *Bacteroides vulgatus*-like strains were cultivated for four days in media in which the two mucins provided the only carbohydrate energy sources. Table 8 shows that isolates L34, W12 and W13 digested pig mucin more readily than rabbit mucin, and reached higher cell density, although growth was severely restricted with both substrates. The changes in ABO serological specificity of the two mucins at the end of the culture period are shown

Table 8. Comparative degradation of pig and rabbit mucins by four mucinolytic strains of *Bacteroides vulgatus*.

	Isolate			
	L52	L34	W11	W13
Culture on pig mucin:				
Culture density	0.69	0.54	0.27	0.52
% reduction of mucin	20	45	36	54
Culture on rabbit mucin:				
Culture density	0.36	0.22	0.16	0.27
% reduction of mucin	4	31	9	44

Isolates were grown on M10 medium containing 500 mg dl^{-1} of the respective mucins. After 4 d, culture densities were measured at 420 nm and the mucin concentrations compared with those of uninoculated media by the Periodic acid-Schiff's method.

Table 9. Changes in ABO status of mucins after digestion by *Bacteroides vulgatus* strains.

Absorbent	Antibody	Antibody titres after absorption with mucins from cultures of:				
		M10 medium	L52	L34	W12	W13
Pig mucin	Anti-A	[illegible]	1:4	1:16	1:16	1:32
Rabbit mucin	Anti-A	1:2	1:2	1:8	1:8	1:16
Rabbit mucin	Anti-B	1:2	1:2	1:2	1:2	1:2

Isolates were cultured in M 10 medium containing 500 mg dl^{-1} pig or rabbit mucins. After 4 d, the mucins were precipitated with alcohol. Antisera were added to the deposits and allowed to absorb for 30 min. Human red cells of A or B blood groups were added and agglutination scored microscopically.

in Table 9. Although the A terminal structure of both pig and rabbit mucin was modified by digestion, as shown by restoration of antibody titre after absorption, the B-absorbing structure in rabbit mucin was unaffected, even when the mucinolytic activity of the strain was high.

Mucins remaining at the end of the experiment discussed above, were precipitated with alcohol and derivatized for gas-liquid chromatography. Figure 13 compares th chromatograms of rabbit intestinal mucin from culture media before and after digestion. In Table 10, the carbohydrate molar ratios, derived from similar chromatograms, are presented for comparison with the data for undegraded mucin from the same batch of medium. It was not possible to estimate quantitative losses of individual sugars because of the derivatization process for GLC analysis, but considering the changes in molar ratios and the relative digestibilities given in Table 8 it can be concluded that pig mucin lost more galactose and N-acetylglucosamine, but less fucose than the rabbit mucin, after digestion by mucinolytic bacteria.

In a similar experiment, strain W13 was grown on pig mucin and the levels of PAS-reactive mucin monitored throughout the growth period, together with the optical density of the culture. The graph of mucin concentration plotted against culture density given in Figure 14, shows that much of the mucin degradation took place towards the end of the growth cycle in batch culture.

3.7 Fermentation of mucin hexoses

Monosaccharides released by enzyme digestion of rabbit mucin may be available as fermentable energy scurces for microorganisms. The extent to which the component hexoses are utilized by cecal bacteria was investigated by using mucin sugars as single substrates in fermentation tests. The reactions of mucinolytic and non-mucinolytic strains from

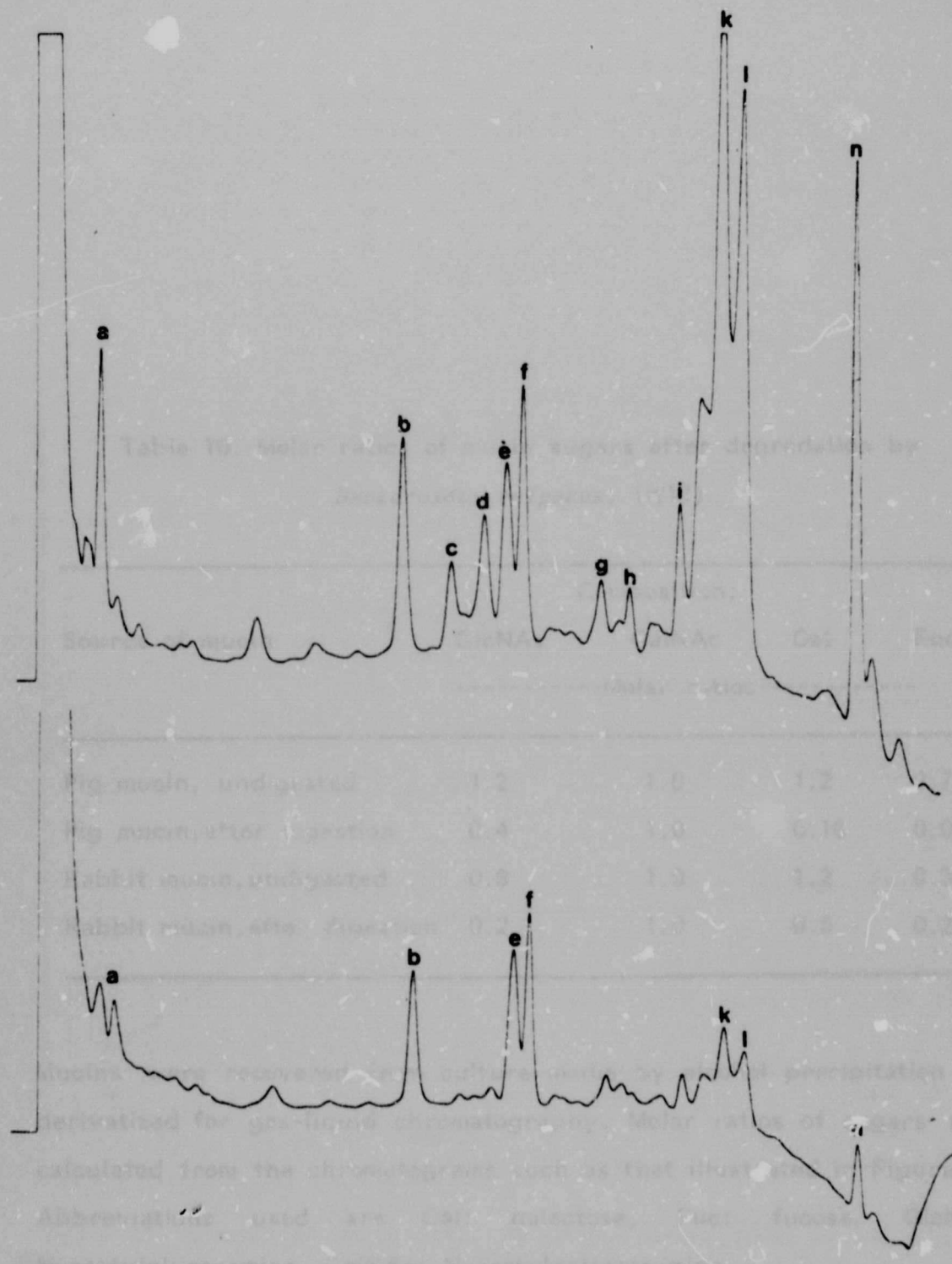

Figure 13. GLC chromatograms of silyl-derivatized rabbit mucin before (top) and after (bottom) digestion by mucinolytic isolate (W13) *Bacteroides vulgatus*. The peaks are identified as follows: a: fucose, b,c,d: galactose, e,f: glucose, h.l: N-acetylgalactosamine, g,i,k: N-acetylglucosamine, n: silalic acids

Table 10. Molar ratios of mucin sugars after degradation by *Bacteroides vulgatus*, (W13)

Source of mucin	Composition: GlcNAc	GalNAc	Gal	Fuc
	Molar ratios			
Pig mucin, undigested	1.2	1.0	1.2	0.7
Pig mucin, after digestion	0.4	1.0	0.16	0.02
Rabbit mucin, undigested	0.8	1.0	1.2	0.3
Rabbit mucin, after digestion	0.2	1.0	0.5	0.2

Mucins were recovered from culture media by alcohol precipitation and derivatized for gas-liquid chromatography. Molar ratios of sugars were calculated from the chromatograms such as that illustrated in Figure 13. Abbreviations used are Gal: galactose, Fuc: fucose, GlcNAc: N-acetylglucosamine, GalNAc: N-acetylgalactosamine.

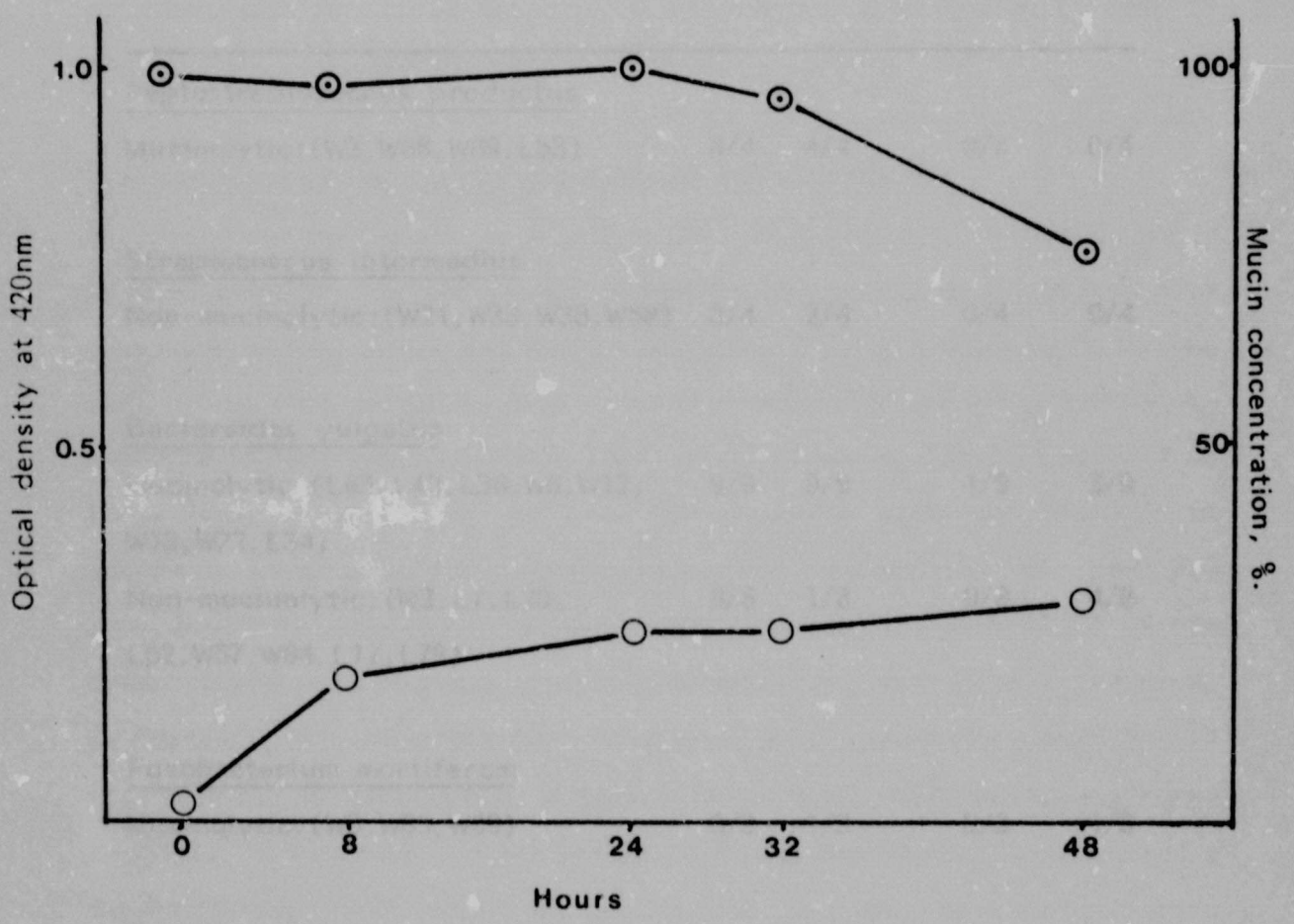

Figure 14. Growth of *Bacteroides vulgatus* (strain W13) on pig gastric mucin. Optical density of culture at 420 nm: ○——○, concentration of mucin reactive to the Periodic acid-Schiff's regent: ⊙——⊙.

Table 11. Fermentation reactions of strains grown on single mucin hexoses.

	Substrates:*			
	Gal	Fuc	GalNAC.	GlcNAc.
Peptostreptococcus productus				
Mucinolytic: (W3, W68, W69, L53)	4/4	4/4	0/4	0/4
Streptococcus intermedius				
Non-mucinolytic: (W21, W33, W38, W58)	3/4	2/4	0/4	0/4
Bacteroides vulgatus				
Mucinolytic: (L63, L40, L36, W6, W12, W13, W22, L34)	9/9	9/9	1/9	3/9
Non-mucinolytic: (W2, L7, L25, L52, W57, W64, L17, L79)	8/8	1/8	0/8	4/8
Fusobacterium mortiferum				
Mucinolytic: (W5, W85, W99)	3/3	1/3	0/3	1/3
Fusobacterium necrophorum				
Non-mucinolytic: (L18, L80)	2/2	2/2	0/2	1/2

*Abbreviations used are, Gal: galactose, Fuc: fucose, GalNAc: N-acetylgalactosamine, GlcNAc: N-acetylglucosamine. Strains were incubated with substrates in M10 medium for 2 d. The pH was measured and compared with that of uninoculated media. A drop of one pH unit was considered to be a positive reaction. Results are expressed as Number of strains positive / Number of strains tested.

similar genera are given in Table 11. *Streptococcus* strains were used as controls for the *Peptostreptococcus* strains which were all weakly mucinolytic. The substrates used were galactose, fucose, N-acetylgalactosamine and N-acetylglucosamine. The results show that N-acetylglucosamine was utilized by one third of the strains tested whereas only a single strain grew on N-acetylgalactosamine. In contrast, galactose was fermented by 97% of all strains and fucose by 87% of mucinolytics but only 36% of non-mucinolytic strains.

3.8 Detection of cell-bound glycosidases by APIZYM

A comparison of the cell-bound glycolytic hydrolases of mucinolytic and non-mucinolytic strains was made using the APIZYM system with washed cells of isolates grown in pre-reduced trypticase soy broth in the absence of added carbohydrate. The results given in Table 12 show that there was no correlation between the glycoside hydrolase reactions in *Bacteroides vulgatus*-like strains and the ability to degrade the polysaccharide component of the mucin molecule. The APIZYM system detects cell-bound enzymes in washed preparations and there is no growth of the bacteria during the test. Table 12 also shows therefore that all the strains of *Bacteroides vulgatus* tested contained the same enzyme profiles. In contrast, strains similar to *Fusobacterium mortiferum* had less α-galactosidase and none of the strains of this species contained β-glucuronidase.

3.9 Production of glycosidases by bacterial cultures

Four strains similar to *Bacteroides vulgatus* were selected for further study of the glycosidases produced in GIT bacteria when grown on different carbohydrates. Strains W12 and W13 were strongly mucinolytic but W2 and L7 had no effect on either pig or rabbit mucins. The strains were grown in bulk volumes of media containing glucose, lactose,

galactose and rabbit mucin as described in section 2.3. Growth curves were drawn from biomass measurements and the activities of different glycosidases in culture supernatants and cells estimated by release of chromogen from p-nitrophenol glycoside substrates.

Figure 15 shows the general growth curves of the strains grown on galactose (W12,W13 and L7), the same strains grown on fucose and W13 grown on N-acetylglucosamine. Stationary phase was reached by all cultures at between 6 and 7 h. The mean specific activities on p-nitrophenol glycosides of β-D-galactosidase, α-fucosidase and N-acetylglucosaminidase, detected in cell-free culture supernatants during the growth cycle of isolates W12 and W13 were plotted against their respective growth curves (Figure 16). Although different levels of the three enzymes were produced, they all reached a plateau as the cultures approached stationary phase.

Cultures were harvested in late logarithmic phase of the growth cycle for determination of cell-bound and extracellular glycosidase activity. Very little activity was detected in cell-free culture supernatants at this stage of growth, but the influence of substrates on the relative enzyme activities of the two non-mucinolytic and two mucinolytic strains could be compared. Figures 17 and 18 illustrate these differences in histogram form. Lactose exerted a marked stimulatory effect on the production of enzymes by the mucinolytic strains, particularly, the hexosaminidase and fucosidase. Glucose and galactose were mildly stimulatory, but the effect of rabbit mucin was difficult to gauge, as the amounts of enzymes in both mucinolytic and non-mucinolytic isolates grown on this substrate were very similar except for elevated fucosidase in the non-mucinolytics

3.10 Inhibition of glycosidases *in vitro*

Enzyme sources prepared as described in section 3.9, were also tested in the standard p-nitrophenol assay in which the buffer component was replaced by solutio s of different carbohydrates in test buffer at pH 6.5. The monosaccharides galactose and fucose, the disaccharide lactose and the polysaccharide component of rabbit intestinal mucin were chosen to compare their effects on the activities of four bacterial glycosidases, and to validate the use of synthetic substrates in these enzyme studies. Carbohydrates were added to the test system in 0.5 ml volumes of buffer to give final concentrations of 10 and 20 mM. Rabbit mucin additions of 4 and 8 mg were calculated to give approximately the same sugar cconcentrations in the reaction volumes, allowing for 50% protein component. The minimal inhibition of enzymes by different sugars is shown in Figure 13. Fucose (20 mM) inhibited α-L-fucosidase acting on its p-nitrophenol glycoside by 75% and galactose (10 mM) inhibited β-galactosidase by 28%. Cross inhibition was shown by fucose (20 mM) on galactosidase by 17%, galactose (10 mM) on the fucosidase by 15% and lactose (10 mM) affecting both enzymes by over 20% reduction in activity. The effect of mucin at 8 mg in the reaction mixture was confined to 27% inhibition of fucosidase and the activity of the two hexosaminidases was not affected by any of the carbohydrates used in the test.

Table 12. Cell-bound glycosidases of *Bacteroides vulgatus* strains detected by the APIZYM system

Enzyme	Strain No.									
	W6	W11	W13	W22	L36	L52	L63	W15	W31	W45
	Strength of reaction graded 0-5									
α-galactosidase	0	2	3	5	3	2	2	2	4	2
β-galactosidase	5	5	5	5	5	5	5	5	5	5
β-glucuronidase	0	1	0	1	0	1	0	0	1	0
α-glucosidase	5	5	5	4	5	5	4	5	5	5
β-glucosidase	0	0	0	0	0	0	0	0	0	0
β-hexosaminidase	5	4	5	5	4	4	2	3	2	3
α-mannosidase	0	0	0	0	0	0	0	0	0	0
α-fucosidase	3	4	3	3	4	5	3	2	3	4

Columns show the colour intensity graded from 0 to 5 with 0 = no activity and 5 = activity of over 40 nM. Strains were grown in Trypticase soy broth, without any added carbohydrate, and cells were washed by centrifugation in ADF before addition to the APIZYM trays. Columns 1 to 6 are mucin-degrading strains and columns 7 to 10 are non-mucinolytic.