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PHOSPHORILASES INDUCTION BY DIFFERENT CARBON SOURCES IN FOUR PREDOMINANT RUMEN BACTERIA

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ABSTRACT

The activities of some phosphorylase enzymes e.g. cellobiose phosphorylase, cellotriose phosphorylase, maltose phosphorylase and lactose phospphorylase, have been examined in some rumen bacteria e.g. *R.flavefaciens* 17, *B.fibrisolvenes* OR77, *P.bryantii* B_14 and *S.bovis* A30 in vitro . The cellotriose phosphorylase activity was observed in *R.flavefaciens* 17 and *B.fibrisolvenes* OR77. The activity of cellobiose phosphorylase was detected in *R.flavefaciens* 17, *B.fibrisolvenes* OR77 and *P.bryantii* B_14. Lactose phosphorylase activity was found in *B.fibrisolvenes* OR77, *P.bryantii* B_14 and *S.bovis* A30. While maltose phosphorylase activity was detected only in *S bovis* A30.

The induction of phosphorylases by some carbon sources was investigated. Cellobiose phosphorylase in R flavefaciens 17 and P.bryantii B_14 is a constitutive enzyme but in B.fibrisolvenes OR77, it is inducible. Maltose has induced the maltose phosphorylase in S.bovis A30. The time-course experiment revealed that the cell growth and the pattern of enzyme production in R.flavefaciens 17 occurred within 24 hr incubation time. The relationship between endoglucanase and cellotriose phosphorylase was observed in R.flavefaciens 17 and it was concluded that the production of cellotriose phosphorylase is parallel with the production of endoglucanase. All phosphorylases differ in their affinity and specificity for substrate and physiological roles.The maximum growth was observed in the period between 18-21 hr incubation in all carbon sources.

The high level of cellotriose phoshorylase activity was present in cellulose medium after 24 hr of incubation at 37^{0}C. Cellobiose phosphorylase activity was found in cellobiose,cellodextrins,xylan or cellulose-grow cells. The high level of activity was with cellobiose, celiodextrins and xylan.

Keywords: Rumen bacteria, phosphorylase, fiber, cellulose, cellobiose,cellodextrins, xylan.

INTRODUCTION

The rumen bacteria are members of a microbial consortium that perform several functions vital to the well-being of the host animals such as the degradation of fiber and other polymeric plant material which are not degraded by the host animal, synthesis of microbial protein, synthesis of certain vitamins and degradation of some toxic compounds of the diet.

Since bacterial energy sources in the rumen are often limited and the yield of ATP is generally low in anaerobic rumen bacteria, efficient utilization of the substrate is important for growth of rumen bacteria.(Martin and Wani ,2000).

Considerable research efforts have been devoted to the manipulation of rumen metabolism to improve the transformation of poor quality feeds into

milk and meat. The developments in recombinant DNA techniques and molecular biology have led rumen microbiologists to apply these techniques to the rumen microflora (Smith and Hespell,1983).The main aims of the manipulation of the rumen microbial ecosystem are :

1- To improve fiber digestion.
2- To reduce protein degradation or to produce amino acids.
3- To modify the ratio of the fermentation products.
4- To inhibit the growth and metabolic activities of undesirable organisms.
5- To obtain bacterial production of substances that are of benefit to the host metabolism

Plant cell wall is the most abundant energy source in the world. Improvements of the plant materials utilization may be play an important role for narrowing the energy gap. Ruminants can change the plant cell walls to protein food (meat, milk, cheese etc.) by the help of microorganisms, which inhabits their rumen. Contribution of the cellulolytic bacteria to this function is especially important. Cellulose degradation is realized by a mixture of cellulolytic enzymes, such as endo-β-1,4- glucanase (EC. 3.2.1.4), cellobiohydrolase (3.2.1.91) and β–glucosidase. It is important to clarify the enzyme system employed in the control of cellulose degradation in the rumen. Unlike soil clostridium or soil fungi that are used for industrial cellulase production, little is known about the enzyme system of the rumen cellulolytic bacteria.

Utilization of plant materials by ruminants depends upon the microbial consortium of the rumen. This process is slow and incomplete, and enhancement of the fiber-hydrolyzing activity of the rumen bacteria to increase the efficiency of ruminal metabolism and animal reproduction. The microbial degradation of plant cell wall material in the rumen is the key to the nutrition of ruminant animal, the energy source in forages exists primarily as structural polysaccharides. The cellulose microfibrils are linked to xylans by hydrogen bonds and therefore the accessibility of the cellulosic materials to hydrolytic enzymes requires the removal of the matrix embedding the cellulose microfibrils. Thus, ruminal bacteria are challenged with a complex heterogeneous substrate requiring the elaboration of an array of enzymes, notably cellulases and xylanases. The predominant cellulose-degrading bacteria in the rumen are *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*, whereas the major hemicellulose utilizers include the *Ruminococcus* sp., *Prevotella ruminicola* and *Butyrivibrio fibrisolvens*. The physiology, enzymology, and genetics of cellulose degradation by rumen bacteria has been studied, but many questions remained unanswered. The cellulolytic enzyme system has been examined in some depth and has been shown to be complex, involving numerous enzymes. Therefore, the objectives of this study are investigating a) the distribution of some phosphorylases enzymes in some rumen bacterial species b) phosphorylases induction by some carbon source and c) selecting of the highest activity for cellobiose phosphorylase in selected predominant rumen bacteria .

MATERIALS AND METHODS

Ruminococcus flavefaciens as a cellulolytic rumen bacteria ,*Butyvibrio fibrisolvenes* as a hemicellulolytic rumen bacteria, *Prevetolla bryantii* as a hemicellulolytic rumen bacteria and *Sterptococcus bovis* as an amylolytic rumen bacteria are four of the most predominant bacteria isolated from the rumen were used in this present work in order to investigate a) the distribution of some phosphorylases enzymes in some rumen bacterial species b) phosphorylases induction by some carbon source and c) selecting of the highest activity for cellobiose phosphorylase in selected predominant rumen bacteria all bacteria are tested *in vitro* .

1. Bacterials strains and culture media

1.1. *Ruminicoccus Flavefaceins* 17

Ruminicoccus flavefaceins 17 kindly provided by H.J.Flint, Rowett Research Institute, Greenburn, Bucksburn, Aberdeen AB2 9SB, UK, as a source of genomic DNA. It was grown anaerobically at 37°C in M_2 medium (Bryant, 1972) as described previously (Flint *et al.*, 1989) whose composition /l is as follows mineral slution (a)150 ml, mineral solution (b) 150 ml, rumen fluid 150 ml, deionized water 550 ml. bacto casitone 10g,yeast extract 10g, cellobiose 0.2% and resazurin 100µl . The pH is adjusted to 6.8 in addition to 0.5 cystein-HCl and 2.5 g $NaHCO_3$ were added to the medium.

1.2. *Prevetolla bryantii* B_14

Prevetolla bryantii B_14 is provided kindly from H.J.Flint, Rowett Research Institute, Greenburn, Bucksburn, Aberdeen AB2 9SB, UK.

1.3. *Butyrivibrio fibrisolvens* OR77 and *Streptococcus bovis* A30

These strains were provided from Dr.H.Minato, Ibaraki University, Japan. *Prevetolla bryantii* B_14, *Butyrivibrio fibrisolvens* OR77, and *Streptococcus bovis* A30 were grown anaerobically at 37°C on Ruminal Glucose Medium (RGM) which is composed of 170 ml Hungate solution A (6.0 g NaCl, 3.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.6 g $MgSO_4$, 0.6 g $CaCl_2$ per liter), 170 ml Hungate solution B (3.0 g K_2HPO_4 / l), 300 ml rumen fluid, 560 ml distilled H_2O, 1.0 g trypticase, 0.5 g yeast extract, 0.5 g L-cysteine, 5.0 g $NaHCO_3$, 100 ul resazurine, 2.0 g glucose. The carbon sources were added as separate sterile solutions, and cultures were grown at 37°C.

2. Cell fractionation

The cells were harvested by centrifugation (15000 rpm for 10 min at 4°C). The pellet was washed two times with either 50mM Tris-HCl buffer plus 2 mM DTT (dithiothreitol) (pH 6.8, 4°C) for endoglucanase assay. The pellet was suspended in 1 ml of 50mM Tris-HCl buffer plus 2 mM DTT (pH 6.8, 4°C) and 25 µl of toluene was added for the cells disruption. Then the endo-glucanase activity was measured. For cellobiose-phosphorylase (Cellobiose, orthophosphateα-D glyucosyltransferase, EC2.4.1.20), cellotriosephosphorylase (1,4-β-D-triglucan:orthophosphateα-D-glyucosyl-transferase, EC2.4.1.49), β-

glucosidase, the pellets suspended in the buffer (5ml). The suspension ultrasonicated (20 times, 30 sec.), and centrifuged for10 min, 15000rpm at 4°C. The precipitate discarded and the supernatant (cell extract fraction) was kept at 4°C for enzyme assays (Helaszek and White, 1991; Lou *et al.*, 1997)

3. Enzyme assays.

Cellobiose and cellotriose phosphorylase activities assays were performed by measuring Pi (inorganic phosphorus) formation (Alexander, 1968).

Maltose-phosphorylase and **Lactose** phosphorylase activities were assayed according to the procedure of Lou *et al.*, 1996.

Endoglucanase (CMCase) was assayed according to procedure of Matsushita *et al.*, 1990; Pittepher and Latham, 1979) (-amylase activity was measured by adding rice starch instead of carboxmethycellulose in CMCase reaction mixture (Petterson and Porath, 1966). The protein was determined by the procedure of Lowery *et.al.*, (1951). This study is carried out at the Kyoto Prefecural University, Laboratory of Animal Science, Kyoto, Japan.

Cellodextrins preparation

Cellodextrins were prepared by a modification of the method of Freer and Detroy (1982). Sigmacell 20 microcrystalline cellulose (10g) was first mixed with 100 ml of Ice-cold concentrated HCl in 500 ml flask and stirred to give a uniform suspension; Ice-cold fuming HCl (100ml) was then poured into the flask to completely dissolve cellulose. A slightly yellow, viscous solution formed after 2 hr of incubation at the room temperature. The HCl was partially removed by application of vaccum for 30 minutes. Cellodextrins were precipitated by the addition of 10 volumes of acetone.The precipitate was washed four times with 5 volumes of acetone and collected by centrifugation (10.000 rpm ,0^0 C for 15 munutes).The pellet was suspended in 600 ml of deionized distilled water to extract water-soluble cellodextrins, and the solution was stirred overnight in a chemichal hood to remove residual acetone. The supernatant was collected after centrifugation, neutralized by passage through an aninon exchange colunn (1.7by 7.5 cm; Dowex AG1-X8; Bio-Rad) and concentrated to 10-15 ml on a rotary evaporator (45^0 C).

Statistical analysis:

The statistical analysis was carried out according to Snedecor and Cochran (1967).

RESULTS

1. Bacterial growth

Using several carbon sources the bacterial cells growth and phosphorylases induction system were tested. The used carbon sources were glucose, cellobiose, lactose, or maltose. R.flavefaciens 17 grew on cellobiose as a growth substrate but no growth was found in glucose, lactose or maltose medium. P.brynatii B14 is one of the most numerous ruminal

bacteria and it is a noncellulolytic bacteria rather, hemicellulolytic. It utilized glucose, cellobiose, lactose or maltose as a sole energy source. *B.fibrisolvens* OR77 is among the predominant species present in the rumen and extensively solubilised the hemicellulose more than cellulose. It was able to grow on glucose, cellobiose, lactose or maltose medium. *S.bovis* A30 is among the most amylolytic bacteria found in the rumen. It was able to grow in glucose, cellobiose, lactose or maltose medium.

2. Phosphorylases induction by substrates.

The activity of cellobiose phosphorylase and cellotriose phosphorylase were detected in *R.flavefaciens* 17 in the cellobiose-grown cells. On the other hand, maltose phosphorylase and lactose phosphorylase were not produced in this organism (Table1). The release of cellobiose phosphorylase in glucose, lactose or maltose-grown cells in *P.brynatii* B_14 was low, the activity was decreased 75% for the cell grown on glucose and lactose, 45 % for the cells grown on maltose compared to cellobiose-grown cells. No activity was detected for cellotriose phosphorylase or maltose phosphorylase in this organism. Lactose phosphorylase activity was observed in lactose-grown cells or maltose-grown cells but no activity in the glucose or cellobiose-grown cells. The lactose phosphorylase activity was twice higher in the lactose grown-cells than in maltose grown-cells (Table 2).

Table 1: Effect of carbon source on CBP, CTP, MalP or LacP activities (nmole/min/mg protein) in *R.flavefaciens* 17.

Carbon source	CBP	CTP	MalP	LacP
Glucose	NG	NG	NG	NG
Cellobiose	212.80±1.24	22.66± 0.12	NG	NG
Lactose	NG	NG	NG	NG
Maltose	NG	NG	NG	NG

NG, no growth. Means± SE
CBP, Cellobiose phosphorylase CTP, Cellotriose phosphorylase
MalP, Maltose phosphorylase LacP, Lactose phosphorylase

Table 2: Effect of carbon source on CBP, CTP, MalP or LacP activities (nmole/min/mg protein) in *P.brynatii* B_14.

Carbon source	CBP	CTP	MalP	LacP
Glucose	4.10 ±0.20	ND	ND	ND
Cellobiose	16.30 ±0.08	ND	ND	ND
Lactose	4.41 ±0.38	ND	ND	33.23±0.07
Maltose	9.21 ±0.38	ND	ND	17.74±0.05

NG, no growth. Means± SE
CBP, Cellobiose phosphorylase CTP, Cellotriose phosphorylase
MalP, Maltose phosphorylase LacP, Lactose phosphorylase

B.fibrisolvens OR77 showed the cellobiose phosphorylase activity in cellobiose, lactose or maltose grown-cells but the activity was too low in glucose-grown cells. The highest activity for cellobiose phosphorylase was found in case of lactose grown-cells followed by cellobiose grown-cells. The

activity was about half that of *R.flavefaciens* 17. Cellotriose phosphorylase activity was observed in glucose, cellobiose, lactose or maltose medium and the highest activity was observed in lactose or cellobiose-grown cells of *B.fibrisolvens* OR77. This organism has shown high activity for cellotriose phosphorylase than expected. Its activity was greater more than twofold the activity in *R.flavefaciens* 17 which grew on cellobiose. Lactose phosphorylase activity was detected in *B.fibrisolvens* OR77, which grew on glucose, cellobiose, lactose or maltose as a sole energy source. The highest specific activity for lactose phosphorylase was found in lactose or maltose-grown cells but the lowest activity was in cellobiose medium. This bacterium did not produce maltose phosphorylase (Table 3).

Table 3: Effect of carbon source on CBP, CTP, MalP or LacP activities (nmole/min/mg protein) in *B.fibrisolvens* OR77.

Carbon source	CBP	CTP	MalP	LacP
Glucose	9.20 ±0.05	54.00 ±3.38	ND	62.26 ±1.74
Cellobiose	91.37 ±0.60	75.88 ±0.11	ND	15.06 ±1.76
Lactose	123.43±0.62	134.13±1.48	ND	137.79 ±0.28
Maltose	31.39 ±0.33	71.71 ±0.16	ND	136.14 ±0.53

NG, no growth. Means± SE
CBP, Cellobiose phosphorylase CTP, Cellotriose phosphorylase
MalP, Maltose phosphorylase LacP, Lactose phosphorylase

The cellobiose phosphorylase and cellotriose phosphorylase activities were not detected in *S.bovis A30*. This organism produced the lactose phosphorylase and maltose phosphorylase. The activity of lactose phosphorylase was found only in lactose-grown cells. Maltose phosphorylase activity was much higher on maltose-grown cells than glucose, cellobiose or lactose grown-cells (Table 4).

Table 4: Effect of growth substrates on CBP, CTP, MalP or LacP activities (nmole/min/mg protein) in *S.bovis A30*.

Carbon source	CBP	CTP	MalP	LacP
Glucose	ND	ND	43.13 ±0.57	ND
Cellobiose	ND	ND	48.32 ±0.78	ND
Lactose	ND	ND	28.52 ±0.11	75.48 ±0.28
Maltose	ND	ND	102.04 ±1.39	ND

NG, no growth. Means± SE
CBP, Cellobiose phosphorylase CTP, Cellotriose phosphorylase
MalP, Maltose phosphorylase LacP, Lactose phosphorylase

R.flavefaciens 17 showed the highest activity for cellobiose phosphorylase compared to *B.fibrisolvens* OR77 and *P.brynatii* B_14. The activity in *R.flavefaciens* 17 was two fold or 13 fold greater than *B.fibrisolvens* OR77 and *P.brynatii* B_14, respectively when the cellobiose was used as a growth substrate. Cellotriose phosphorylase activity was detected in *R.flavefaciens* 17 and *B.fibrisolvens* OR77. The activity of cellotriose

phosphorylase in *B.fibrisolvens* OR77 was 6 fold greater than in *R.flavefaciens* 17.

3. Production of endo-glucanase

Endoglucanase (1,4-β-D-glucan glucano-hydrolase, EC 3.2.1.4) activity was examined in *R.flavefaciens* 17, *B.fibrisolvens* OR77, and *P.bryantii* B_14 under various growth conditions. The results reported that all these bacterial strains have the endo-glucanase activity but the activity in *R.flavefaciens* 17 was higher than those in *B.fibrisolvens* OR77, and *P.bryantii* B_14 (Table 5).

Table 5: Bacterial growth with different carbon sources.

Carbon source	Bactrial strains			
	R.flavefaciens	*P.brynatii*	*B.fibrisolvens*	*S.bovis*
Glucose	-	+	+	+
Cellulose	+	+	+	+
Lactose	-	+	+	+
Maltose	-	+	+	+

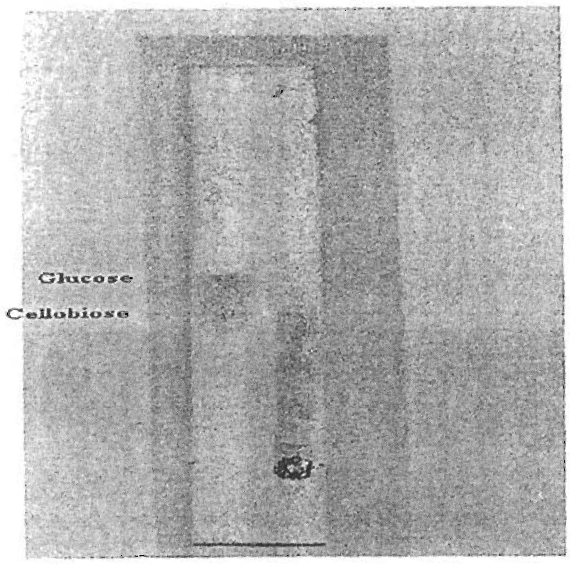
+ growth - no growth

4.Effect of carbon sources and time course on both phosphorylase induction and endo-glucanase activities in *Ruminicoccus Flavefaceins* 17.

From previous results, *Ruminicoccus Flavefaceins* 17 was selected to study some physiological aspects, which are related to substrate regulation (specifity), phosphorylases induction system and the relation ship between phosphorylases and endo-glucanase. Cellulose, cellodextrines, cellobiose or xylan was used as a sole energy source for the growth of *R. Flavefaceins* 17. The composition of prepared cellodextrins was shown in (Fig1). The maximum growth was observed in the period between 18-21 hr incubation with al carbon sources (Fig 2). The highest growth was observed in cellobiose medium. *Ruminicoccus Flavefaceins* 17 preferentially utilized cellobiose and xylan then cellodextrins and cellulose as a sole energy source.

The activity of cellotriose phosphorylase were found in cell extract in *R. Flavefaceins* 17 and influenced by the fiber source. The high level of activity was present in cellulose medium after 24 hr of incubation (Fig 3). Cellobiose phosphorylase activity was found in cellobiose, cellodexrtins, xylan or cellulose-grown cells. The high level of activity was found with cellobiose, cellodextrins and xylan (Fig 4,5 and 6).

The ß-glucosidase activity was detected in cellulose ,cellodextrins, cellobiose or xylan –grown cells. The maximum activity of the ß- glucosidase was found at 24 hr in cellulose or cellodextrins grown cells at 18-21 hr in cellobiose or xylan grown –cells. The activity in cellobiose medium was twice in cellobiose medium as much as cellulose medium. The production of ß-glucosidase in cellodextrins or xyla medium was less than in cellobiose medium by 20%



a b

Fig. 1 : Thin layer chromatography (TLC) for the composition of prepared cellodextrins, a ; glucose and celobise standard, b; prepared cellodextrins.

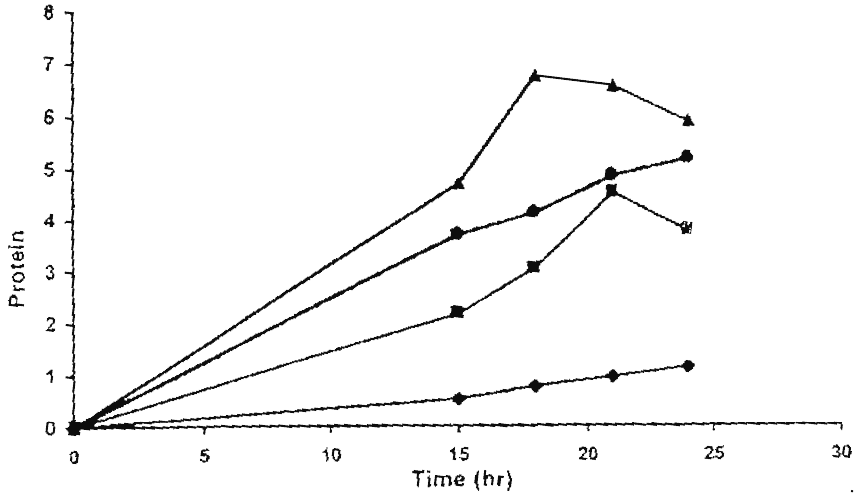


Fig 2: Effect of carbon source on cell growth in R.flavefaciens 17.
♦ cellulose ■ cellodextrins ▲ cellobiose • xylan

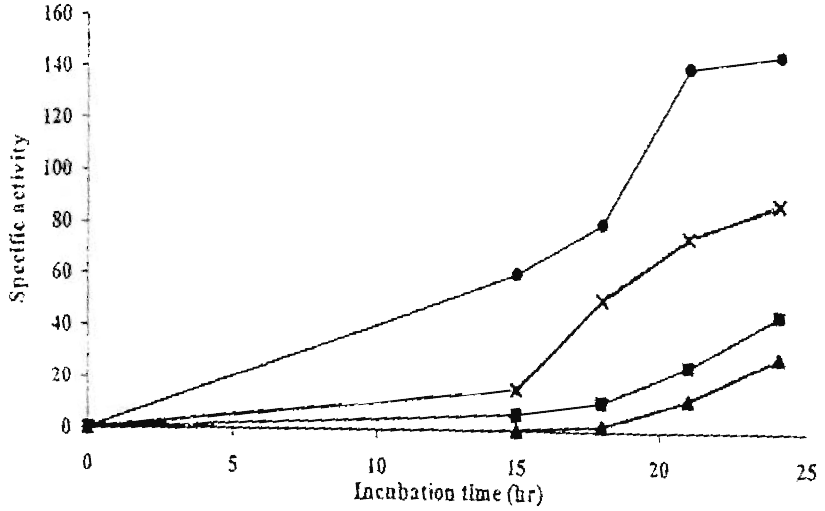


Fig. 3: Specific activities (n mol/mg protein)of CTP,CBP, ß –glucosidase and CMCase in R.flavefaciens 17 grown on cellulose.

X CTP ■ CBP ▲ ß –glucosidase • CMCase

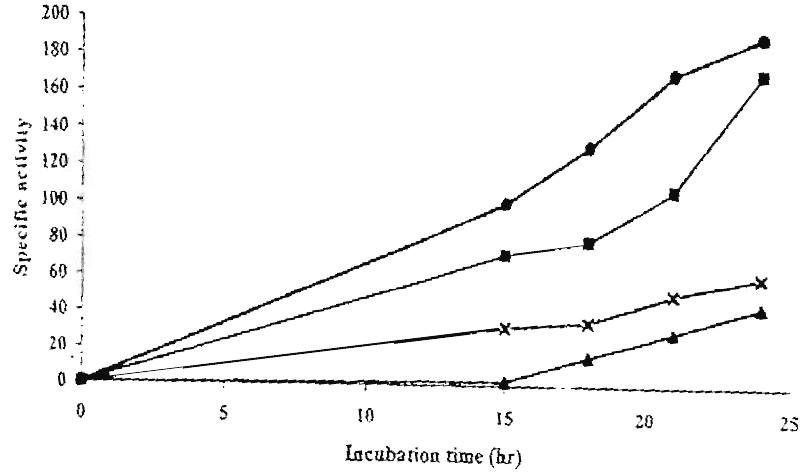


Fig 4: Specific activities (n mol/mg protein)of CTP,CBP, ß –glucosidase and CMCase in R.flavefaciens 17 grown on cellodxtrins.

X CTP ■ CBP ▲ ß –glucosidase • CMCase

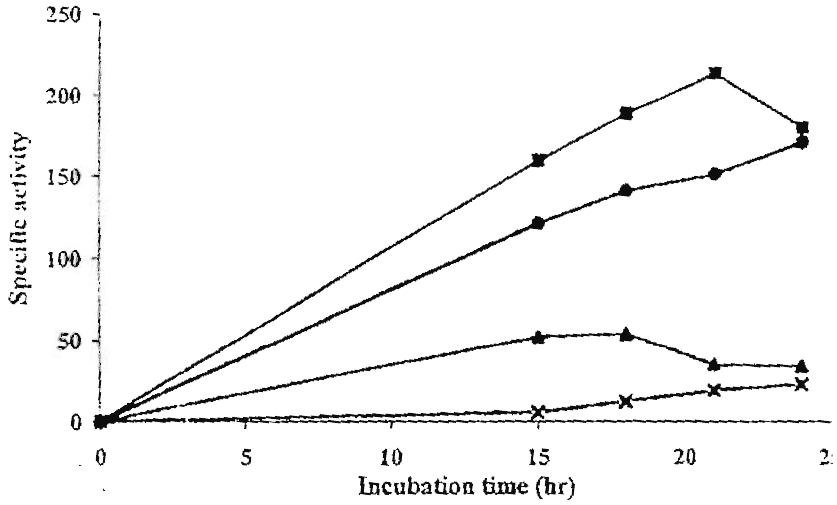


Fig. 5: Specific activities (n mol/mg protein)of CTP,CBP, ß –glucosidase and CMCase in R.flavefaciens 17 grown on celloblose.

X CTP ■ CBP ▲ ß –glucosidase • CMCase

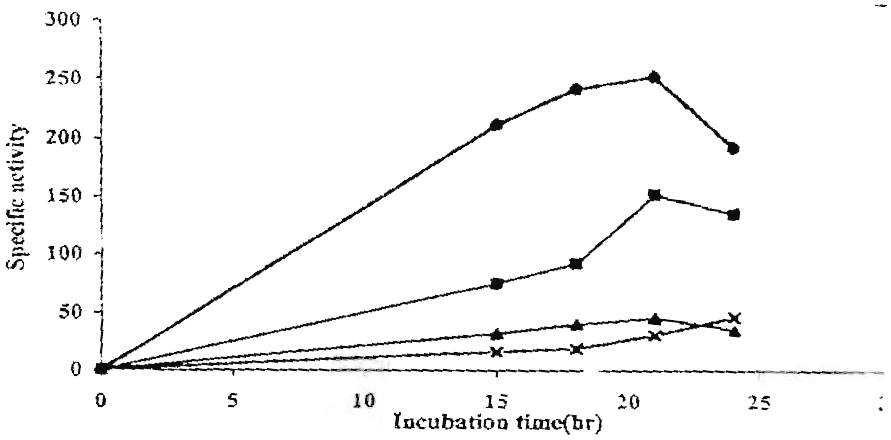


Fig. 6: Specific activities (n mol/mg protein)of CTP,CBP, ß –glucosidase and CMCase in R.flavefaciens 17 grown on xylan

X CTP ■ CBP ▲ ß –glucosidase • CMCase

DISCUSSION

Phosphorylases are omnipresent enzymes that are found in many organisms, including bacteria, yeast, slime mold, plants and vertebrates. There are many phosphorylases identified e.g. maltodextrine phosphorylase, maltose phosphorylase, cellobiose phosphorylase, cellodextrins phosphorylase, sucrose phosphorylase and glycogen phosphorylase. These enzymes catalyse the reversible cleavage of polysaccharides, oligosaccharides, or disaccharides into α-D-glucose-1-phosphate and hence play a central role in carbohydrate metabolism. Phosphorylase enzymes from plant, mold, yeast and human origins have been studied in some details but a few studies have been focused on phosphorylases in rumen bacteria. Therefore, information on phosphorolysis will help elucidate its potential role in cellulolysis and is also necessary for a comprehensive understanding of carbohydrate utilization by rumen bacteria. Cellulolytic bacteria usually degrade cellulose by the synergistic action of endo- and exo-glucanase (Ohmyia *et al.*, 1982/87), but this process is potentially inhibited by accumulation of soluble end products e.g. cellobiose and cellotriose (Johnson *et al.*, 1982).

Therefore, the activity of cellobiose, cellotriose, maltose and lactose phophorylases were investigated in this study in some rumen bacteria e.g. *R.flavefaciens* 17, *P.bryantii* B_14, *B.fibrisolvens* OR77, or *S.bovis* A30. These bacterial species were selected in this study to represent cellulolytic, hemicellulolytic, noncellulolytic and amylolytic bacteria in the rumen. The induction system of cellulases still remains unclear. Recently, it was found that most of induction system are carried out by two components regulatory system. When sensor and regulatory protein perceives an inducer (input signal), histidine residue of the protein is phosphorylated. Then, the phosphoryl group is transferred to the regulatory protein. Finally, the phosphorylated regulatory protein is converted to active form and induces the target gene expression. This experiment also, investigated the phosphorylases induction in various rumen bacteria by some carbon sources. We have hypothesized that cellooligosaccharides induced cellulase production in *R.flavefaciens* and phosphorylases are related to polymer degradation and bacterial growth. *R.flavefaciens* 17 cannot utilize glucose, lactose or maltose as growth substrates but it was grown on cellulose, cellodextrins, cellobiose or xylan as a sole energy source. Helaszek and White (1991) reported that *R.flavefaciens* FD-1 is unable to transport and utilize extracellular glucose. The highest growth rate was observed in cellobiose medium indicating that this bacterium had affinity and preference for cellobiose. Ayers, (1958) reported that cellobiose is taken up by the cell of *R.flavefaciens* and undergoes a phosphorolytic cleavage yielding glucose-1-phosphate and glucose, both of which are metabolised. Also, Hungate, (1963) predicted that high growth yield on cellobiose could be attributed to the action of cellobiose phosphorylase and this is in agreement with the current results which showed that this organism has cellobiose phopshorylase activity. *B.fibrisolvens* OR77,*P.bryantii* B_14 and *S.bovis* A30

were grown and utilized glucose, cellobiose, lactose or maltose as a sole energy source.

In this study, the activity of cellobiose, cellotriose phosphorylases was found in *R.flavefaciens* 17, *P.bryantii* B_14 and *B.fibrisolvens* OR77. In addition, the activity of lactose phosphorylase was detected in *P.bryantii* B_14, *B.fibrisolvens* OR77 and *S.bovis* A30. *S.bovis* A30 is the bacterium that produced the maltose phosphorylase. The cellotriose phosphorylase activity in *R.flavefaciens* 17 was detected as measured by release of Pi. Further more, cellulose or cellodextrins were found to be required for the maximum production of cellotriose phosphorylase in *R.flavefaciens* 17 but xylan or cellobiose cannot induce the production of cellotriose phosphorylase indicating that cellodextrins induced the production of cellotriose phosphorylase. The explanation of induction of cellotriose phosphorylase in the cellulose-grown cells, the cellulose was initially metabolised by endo-glucanases producing cello-oligosaccharides, which induced the production of cellotriose phosphorylase in *R.flavefaciens* 17. The highest activity of cellotriose phosphorylase was found after 24 hr incubation time which supported this explanation. The activity of cellotriose phosphorylase was low in cellobiose or xylan-grown cells due to substrate preference and specificity. This is in agreement with Schenzil and Nidetzky, (1999) who stated that bacterial phosphorylases differed in their substrate specificity. Surprisingly, cellotriose phosphorylase activity in *B.fibrisolvens* OR77 was twofold greater than the activity in *R.flavefaciens* 17 which grew on cellobiose. Lactose or cellobiose induced the production of cellotriose phosphorylase in *B.fibrisolvens* OR77 may be due to phosphorylase enzyme playing an important role in the growth of this bacterium by preventing the accumulation of soluble end products. This enzyme was semi-purified. This is first trial for the prediction and semi-purification of cellotriose phosphorylase in *R.flavefaciens* 17

When cellobiose, glucose, lactose or maltose were used as a sole energy source, the cellobiose phosphorylase activity was induced by cellobiose in *R.flavefaciens* 17. *B.fibrisolvens* OR77 has the cellobiose phosphorylase activity in cellobiose, lactose, or maltose-grown cells but the activity was low in glucose-grown cells indicating that the enzyme was regulated by the carbon source and induced by either lactose or cellobiose. Then, cellulose, cellodextrins, cellobiose or xylan were used as a sole energy source in *R.flavefaciens* 17 and showed that the activity of cellobiose phosphorylase in cellulose-grown cells of *R.flavefaciens* 17 was low. Highest activity was found in cellobiose-grown cells indicating that *R.flavefaciens* 17 had high affinity and preference for cellobiose, while *B.fibrisolvens* OR77 had higher affinities for cellobiose or lactose than for glucose or maltose and the phosphorylase can improve the growth of these bacteria. Another explanation is due to substrate specificity and cellobiose is required for the maximum production of cellobiose phosphorylase. The highest bacterial growth rate was found in cellobiose-grown cells after 18 hr incubation and at the same time from 18-21 hr incubation, the highest activity for cellobiose phosphorylase was observed indicating that production of such enzyme encourages the bacterial growth rate. Hungate, (1963) predicted that high

growth yield on cellobiose could be attributed to the action of cellobiose phosphorylase, and the results indicated that such activity was presented. In cellodextrins medium, the activity of cellobiose phosphorylase was high because the prepared cellodextrins contains large amounts of cellobiose result in an increase of the production of celiobiose phopshorylase in *R.flavefaciens* 17.

The results concluded that *R.flavefaciens* 17 has the highest activity of cellobiose phopshorylase comparing to *B.fibrisolvens* OR77 and *P.bryantii* B_14. Surprisingly, the cellotriose phosphorylase production in *B.fibrisolvens* OR77 was higher than in *R.flavefaciens* 17 and it was induced by lactose notably.

Finding of cellotriose phosphorylase and cellobiose phosphorylase is logically acceptable and essential because the cellobiose and cellotriose are the main end products of cellulolysis by the cellulase complex of *R.flavefaciens* (Rasmussen *et al.*, 1988 and Russell, 1985). On the other hand, the activity of cellobiose phosphorylase in *P.bryantii* B_14 was low indicating that the simple hydrolysis of disaccharides by production of β-glucosidase is much greater than phosphorolytic activity by cellobiose phosphorylase.

Enzymes capable of degrading cellobiose and cellotriose were found as essential components of microbial celluloytic enzyme systems. They converted the cellobiose and cellotriose formed during the enzymatic degradation of cellulose by the synergistic action of endglucanase and exoglucanases to fermentable sugars e.g. glucose and glucose-1-phosphtae and initiates the Embden-Myerhoff-Parnas pathway. As cellulolytic enzymes are generally subject to end product inhibition and the cellotriose and cellobiose are the main end products for cellulose degradation by *R.flavefaciens* (Rasmussen *et al.*, 1988 and Russell, 1985) so; an adequate level of cellobiose and cellotriose phosphorylases are required for efficient breakdown of cellulose.

The metabolism of soluble cellulose degradation products involves phosphorylitic and hydrolytic cleavage (Coughlan and Mayer ,1992).Hydrolysis is catalyzed by ß-glucosidases releasing glucose from the disaccharides and the non reducing ends of the oligosaccharides. Phosphorolysis is energetically advantageous and constitute the primary route of disaccharides and cellodextrins utilization in particular anaerobic environments. The cellobiose degradation was investigated by either hydrolytic cleavage through ß-glucosidase or phosphororylatic cleavage in *R.flavefaciens* 17. The phosphorolytic cleavage was about threefold greater than hydrolytic cleavage acitivity in *R.flavefaciens* 17 indicating that phosphorolyses were key enzymes in the initial metabolism of the soluble products of cellulose degradation. The metabolic pathways, which use phosphorolytic cleavage , conserve more energy, through investment of ATP, than those utilizing simple hydrolysis. Since bacterial energy sources in the rumen are often limited and the yield of ATP is generally low in anaerobic microorganisms, efficient utilization of the substrate is important for the growth of ruminal bacteria. On the other hand Lou *et al* , (1997) reported that the hydrolytic cleavage of cellobiose was three-folds greater than the

phosphorolytic activity in *P. bryantii* B_{14} as measured by an enzymatic assay suggesting that this organism degraded cellobiose mainly through hydrolytic rather than phosphorolytic cleavage. The results concluded that there are two factors affecting cellobiose cleavage , either hydrolytic or phosohorylitic in *R.flavefaciens* 17, including the carbon source and time course.

REFERENCES

Alexander, J.K. (1968). Synthesis of 4-O-B-D-Glucopyranosyl-D-Xylose,4-O-B-D-Glucopyranosyl-D-arabinose,4-O-B- D-Glucopyranosyl-D-Deoxy-D-Glucose, 4-O-B-D-Glucopyranosyl-D-Glucosamine by cellbiose phosphorylase from the *Clostridium thermocellium*. Arch. Biochem. Biophys.,123:240-246.

Ayers, W.A. (1958) Phosphorylation of cellobiose and glucose by *Ruminicoccus flavefaciens*. J.Bacteriol.76:515-517.

Bryant, M.P. (1972). Commentary on the hingate techniques for cultivation of anaerobic bacteria. Am/J.Clin. Nutr., 25:1324-1328.

Coughlan ,M.P. and M. Mayer(1992).The cellobiose-decomposing bacteria and their enzyme systems .In: The Prokaryotes, Balows ,A.; H.G. Truper; M. Dworkin ; W.Harder and K.H. Schleifer. (Eds) 2nd Edition Springer-Verlag, New York.

Flint, H.J.;C.A. McPherson and j. Bisset (1989). Molecular cloning of genes from *Ruminicoccus flavefaciens* encoding xylanase and β(1,3-1,4)-glucanase activities. Appl.Environ. Microbiol., 55:230-233.

Freer, S. N.and R.W.Detroy (1982). Direct fermentation of cellodextrins to ethanol by *Candida wickerhamii* and *Candida lusitaniae*. Biotechnol.Lett., 4:453-458.

Helaszek, C.T. and S.A. White (1991) Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. Appl.Environ Micrbiol, 57:64-68.

Hungate, R.E. (1963). Polysaccharide storage and growth efficiency in *Ruminococcus albus*. J. Bacteriol., 86:848-854.

Johnson,E.A.; G. Sakajoh;G. Halliwell;A. Madia and A.L. Demain (1982). Saccharification of complex cellulosic substrates by the cellulase system from Clostridium thermocellum. Appl.Environ.Microbiol., 43:1125-1132.

Lou, J.;K.A. Dawson and H.J. Strobel (1996). Role of phosphorolytic cleavage in cellobiose and cellodextrin metabolism by the rumina bacterium *Prevetolla ruminicola*. Appl.Environ.Microbiol, 62:1770-1773.

Lou, J.;K.A. Dawson and H.J. Strobel (1997). Cellobiose and cellodextrin metabolism by the ruminal bacterium *Ruminococcus albus*. Cur.Micrbiol., 35: 221-227.

Lowry, O.H.;N.J. Rosebrough;A.L. Farr and J. Randall (1951). Protein measurment with folin phenol reagent. J.Biol.Chem.,19-265-275.

Martin , S. A. and E.L. Wani (2000). Factors affecting glucose and maltose phoshorylation by ruminal bacterium *Megashaera elsdenii.*, Curr. Microbiol., 40: 387-391

Matsushita, O.;J.B. Russel and D.B. Wilson (1991). A *Bacteriodes succinogenes* 1,4-β-endoglucanase is encoded in two reading frames. J.Bacteriol., 173:6919-6926.

Ohmiya, K.;M. Shimizu,M. Taya and S.Shimizu (1982). Purification and properties of cellobiosidase from *Ruminoncoccus albus*. J Bacteriol., 150. 407-409.

Ohmiya, K. K.Maeda,. and S.Shimizu (1987) Purification and properties of endo-β-1,4-g;ucanase from *Ruminincoccus albus*. Carbohydr.Res., 166: 145-155.

Petterson, G. and J. Porath (1966). A cellulolytic enzyme from *Penicillium notatum*. Methods Enzymol., 8:603-607.

Pittipher, G.L. and M.J. Latham (1979). Production of enzymes degrading plant cell walls and fermentation of cellobiose by *Ruminococcus flavefaciens* in batch and continuous culture. J.Gen.Microbiol., 110:29-38.

Rasmussen, M.A.;R.B. Hospell;B.A. White, B.A. and R.J. Bothast (1988). Inhibitory effects of methylcellulose on cellulose degradation by *Ruminococcus flavefaciens*. Appl.EnvironMicrobiol., 54: 890-897

Russel,J.B. (1985). Fermentation of cellodextrins by cellulolytic and non-cellulolytic rumen bacteria. Appl.EnvironMicrobiol., 49:572-576.

Schenzil, R. and B. Nidetzky. (1999). Bacterial α–glucan phosphorylases. FEMS Microbiol. Lett., 171:73-79.

Smith,C.J. and R.B. Hespell (1983). Prospects for the development and use of recombinant deoxyribonucleic acid techniques with ruminal bacteria. J.Dairy Sci., 66: 1536-1546.

Snedecor , G.W and W.H. Cochran (1967). Statistical Method. 5th Ed. The Iowa State Univ. Press ,Ames. Iowa. USA.

تحفيز إنزيمات الفسفرة بواسطة مصادر كربونية في أربع أنواع من بكتريا الكرش

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اجريت هذه الدراسة معمليا بغرض دراسة تأثير بعض المصادر الكربونية الجلوكوز ، السليوبيوز ، اللاكتوز والمالتوز على نشاط بعض انزيمات الفسفرة مثل Cellobiose phosphorylase, cellotriose phosphorylase, maltose phosphorylase and lactose phospphorylase على بعض بكتريا الكرش مثل *R.flavefaciens* 17, *B.fibrisolvenes* OR77, *P.bryantii* B_14 and *S.bovis* A30ولقد لوحظ نشاط إنزيم cellotriose phosphorylase في بكتريا *R.flavefaciens* 17 and *B.fibrisolvenes* OR77. Cellobiose phosphorylase بينما نشاط إنزيم وجد في بكتريا :*R.flavefaciens* 17, *B.fibrisolvenes* OR77 and *P.bryantii* B_14 أما نشاط أنزيم Lactose phosphorylase فلقد لوحظ في بكتريا *B.fibrisolvenes* OR77, *P.bryantii* B_14 and *S.bovis* A30. فقط. *S.bovis* A30 وجد في بكتريا Maltose phosphorylase و أخيرا نشاط إنزيم

كذلك تم دراسة تأثير بعض المصادر الكربونية على Induction of phosphorylases باستخدام بعض المصادر الكربوهيدراتية مثل الجلوكوز والسيلوبيوز واللاكتوز والمالتوز ولقد وجد أن إنزيم Cellobiose phosphorylase كان constitutive enzyme في بكتريا R flavefaciens 17 and P.bryantii B_14 بينما في بكتريا B.fibrisolvenes OR77 وجد أنه inducible enzyme في بكتريا S.bovis A30 لوحظ أن المالتوز حفز نشاط إنزيم maltose phosphorylase.

ولقد أظهرت تجربة تأثير وقت التحضين أن أقصي نمو للخلايا و نظام إنتاج الإنزيمات يكون بعد ١٨-٢١ ساعة وأن أقصي نمو لوحظ عند إستخدام السيلوبيوز كمادة تفاعل في بكتريا *R.flavefaciens* 17 وكانت pH المثلى لنشاط انزيمي cellobiose and cellotriose phosphorylase في بكتريا *R.flavefaciens* 17 ما بين ٦,٤-٧,٠ بينما الحرارة المثلى ٥٠م°

وقد اوضح هذا الجزء من الدراسة اهمية انزيم phoshorylase cellobiose في بكتريا *R.flavefaciens* 17.

و التي تتلخص في ثلاث نقاط هي:

١- يتم هدم السيليلوز بواسطة التآثر بين انزيمي endo and exo glucanase و نواتج الهدم هي السكريات الثنائية والثلاثية وتراكمها يؤدي إلى تثبيط هدم السيليلوز ولكن انتاج انزيمي cellobiose and cellotriose phosphorylase يمنع تراكم هذة المواد.

٢- من مميزات عملية الفسفرة هي توفير الطاقة والتي تمثل المسار المبدئي في الاستفادة من السكريات الثنائية والثلاثية خصوصا في بيئة الكرش اللاهوائية و هذة الطاقة هامة جدا لمحدودية مصادر الطاقة البكتيرية وانخفاض انتاج ATP.

٣- على الرغم من عدم وجود نظام PEP-PTS في بكتريا *R.flavefaciens* 17 و لكن هذة البكتريا تملك انزيمات الفسفرة cellobiose and cellotriose phosphorylase و حيث أن هناك تشابة بين نظام PEP-PTS وعملية الفسفرة والتي تستطيع تعويض غياب النظام الذي يمكنة توفير الطاقة للبكتريا. فقد تم اختيار هذة البكتريا وتم اجراء القياسات عليها من حيث تأثير مصادر الكربون على نموها وكذلك اثر المصدر الكربوهيدراتي على نشاط بعض انزيمات الفسفرة.

ويتضح مما سبق اهمية اجراء مزيد من الدراسات على هذا النوع من البكتريا ومحاولة عزل الجين المسئول عن انتاج cellobiose phosphorylase في بكتريا *R.flavefaciens* 17.