# ROLE OF IONIZED CALCIUM AND MAGNESIUM IN CELLULOSE DEGRADATION BY RUMINAL BACTERIA

DISSERTATION

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By

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# ABSTRACT

To establish the ionized calcium ($Ca^{+2}$) requirement for growth (G) and cellulose degradation (CD) by rumen bacteria, cultured strains representing the principal cellulolytic rumen bacteria were used: *F. succinogenes* (FS) A3c and S85, *R. albus* (RA) 7 and 8, and *R. flavefaciens* (RF) B34b and C94. They were incubated in complete liquid media with cellobiose for G experiments and complete media with cellulose for CD experiments; both varied in $Ca^{+2}$ concentrations. Growth was measured by OD (600 nm); and CD, by substrate disappearance. Results obtained with both substrates were analyzed by a logistic function, using nonlinear regression (PROC NLIN-SAS); with cellobiose, max growth (MG), rate of growth (RG) and lag time (LT) were calculated; and with cellulose, extent of degradation (ED), rate of degradation (RD) and lag time (LT) were determined. These coefficients were analyzed by ANOVA and LSM were compared by linear and quadratic contrasts (PROC MIXED-SAS) and the maximum of the first derivative was used to establish the cation requirement. Different strains responded differently to the $Ca^{+2}$ treatments with cellobiose substrate. $Ca^{+2}$ concentrations had a significant effect ($P<0.05$) in all the parameters evaluated, either for G (MG, RG, LT) or CD (ED and LT), with the exception of RD ($P>0.05$); the interaction strain within species*$Ca^{+2}$ concentrations always was significant ($P<0.05$) for G and CD for all parameters. For MG, $Ca^{+2}$ requirements were estimated as follow: FS-S85: 0.47mM, and RA-7: >0.64 mM (tendency); whereas no estimation for A3c, B34b and C94. FS A3c and S85 showed an absolute requirement for CD. $Ca^{+2}$ requirements for ED

were: FS-Ac3: >0.36; RA-7: 0.28; RF B34b and C94: >0.64 mM; whereas no estimation for FS-S85. FS showed considerable variation in ED, perhaps due to interactions of G and CD; therefore, $NH_3$-free complete cellulose media was used to separate the effect of G on CD, confirming the absolute $Ca^{+2}$ requirement of FS for CD; requirements calculated as: 0.42 and >0.64 mM for A3c and S85, respectively. A logistic function could not be fitted for RA 8, due to the slow G and limited CD; therefore, RA 8 was analyzed separately; by linear regression was determined RG and RD; and a positive effect on RG ($P<0.05$), and no effect on RD ($P>0.05$) were found related to $Ca^{+2}$ concentration. The averages from MG and ED were analyzed by ANOVA and no $Ca^{+2}$ concentration effect was found ($P>0.05$). Due to the response to $Ca^{+2}$ from RF strains, their G was tested for other specific cation requirements. These showed that RF has an absolute Mg requirement for G. Then all the strains used in the study were evaluated for Mg requirements for G; requirements were calculated as: S85: 0.56; RA-7: 0.52; RA-8: 0.51; RF-B34b: 0.54 and RF-C94: >0.82 mM; for FS-A3c no estimation. For RF, CD in $NH_3$-free media was used to evaluated the effect of $Ca^{+2}$ and Mg; thus no CD was observed with ($Ca^{+2}$–free and Mg-free) and (Mg-free, plus $Ca^{+2}$); and no differences in ED were found with (plus $Ca^{+2}$, plus Mg) and ($Ca^{+2}$-free, plus Mg) ($P>0.05$), reflecting a Mg requirement for CD. Thus, $Ca^{+2}$ and Mg requirements differ among the strains within species, as well as by the substrate available.

## DEDICATION

*With love and pride I dedicate this work:*
*In memorium of my Father*
*To my Family,*
*Don,*
*and friends.*

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## FIELD OF STUDY

Major field: Animal Sciences
Ruminal microbiology/Ruminant nutrition

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# CHAPTER 1

## INTRODUCTION

The high energy requirements of high producing dairy cows has moved nutritionists to search for alternative energy sources and feeding systems. Because forage alone cannot meet the higher energy requirements, concentrate feeds are added as supplements. However, with very high producers, feeding high levels of concentrate rich in soluble carbohydrates can lead to health and production problems (ruminal acidosis, laminitis, low-fat milk). Thus, fats and oils were explored as alternatives to cereals as a dense energy source. Successful research permitted advances in this front, and today fats and oils are widely used in diets of high producing cows. Although feeding of fats is successful, not all the questions related with rumen function have been answered.

Unsaturated fatty acids (UFA) are toxic for the rumen bacteria and protozoa, and ruminal biohydrogenation is the mechanism used by the bacteria to detoxify those compounds. As unsaturation of fatty acids increases, the toxicity to the rumen bacteria also increases (Galbraith et al, 1971); however, when fatty acids (FA) form salts with cations available in the rumen, toxicity is decreased. Jenkins and Palmquist, (1982) have reported that fiber digestion was

negatively affected when fats/oils were included in the diets (both *in vivo* and *in vitro*). When FA are toxic to the bacteria, they can not degrade the structural carbohydrates (Devendra and Lewis, 1974). Fat was postulated to physically coat the forage fiber, thus preventing bacterial attachment to the fiber (Devendra and Lewis, 1974; Harfoot et al, 1974). Fatty acids also have the capacity to chelate cations, such as $Ca^{+2}$, decreasing the availability of this cation to the cellulolytic bacteria that are believed to require it for growth (Devendra and Lewis, 1974). Numerous studies have investigated the depressed fiber degradation in presence of fat in the diet (Palmquist and Conrad, 1981; Jenkins and Palmquist, 1982; Ferlay and Doreau, 1995). The toxic effect of fats on bacteria was studied by Galbraith et al (1971) and Maczulack et al (1981), and the physical barrier effect was investigated by (Harfoot *et al*, 1974). Palmquist *et al* (1986) explored the aspects related with $Ca^{+2}$ availability in the rumen and observed that the depression of fiber degradation was overcome when a soluble source of calcium ($CaCl_2$) was added to the diet, increasing $Ca^{+2}$ concentration in rumen contents. Other authors (Wagner et al, 1993), using wheat middlings in place of corn silage, did not find increased a coefficient digestibility of the forage and milk production when they fed diets that included $CaCl_2$, although they were able to find differences in $Ca^{+2}$ concentration in rumen content.

Knowledge about rumen cellulolytic bacteria and the process of fiber degradation has advanced greatly in recent years. Mechanisms by which rumen cellulolytic bacteria attach to cellulose or structural carbohydrates are described from genetic and molecular points of view; thus, cellulosomes, pili and their

structures and encoding genes are described for many rumen bacteria (Miron et al, 2001, Bayer et al, 2004). The characterization of the structure and different components for attaching and degrading cellulose by bacteria have shown that cellulose binding proteins and dockerin domains have peptide sequences that resemble the EF-hand motif protein (Michiels et al, 2002), which has the capacity to bind cations, especially $Ca^{+2}$. This provides structural stability, heat resistance, and in some cases increased enzymatic activity (Chavaux et al, 1990, Chavaux et al, 1995, Bayer et al, 1998, Little et al, 2001).

Little information is available on the individual species of rumen cellulolytic bacteria or from *in vivo* rumen ecosystems related to the response to $Ca^{+2}$ and what could be the requirement for this cation for fiber digestion. Many authors (Gong and Forsberg, 1989, Roger et al, 1990, Van Soest, 1994) have suggested that different divalent cations play unspecific roles in bacterial attachment to the fiber, where the (+) charge of the cations interacts with the (-) charges of the plant cell walls.

Thus, one can relate the effect of increased $Ca^{+2}$ to the molecular biology, which suggests the potential role of $Ca^{+2}$ in fiber degrading systems. It is then apparent that little quantitative information is published that can be used to develop the idea. Hubbert et al (1958a) observed an increased cellulose degradation when washed rumen bacteria were exposed to increased levels of calcium; Bryant et al (1959) worked specifically with one of the predominant rumen cellulolytic bacteria (*Fibrobacter succinogenes* S85) and determined the calcium requirement for growth with a soluble carbohydrate substrate. No studies

have been reported on the effect of ionized calcium ($Ca^{+2}$) on growth or cellulolytic activity, or both, which is the form of Ca required by the microorganisms. Presumably $Ca^{+2}$ is incorporated into the cell for bacterial structures such as the cell wall or for structures used to attach and degrade plant fibers.

The lack of quantitative information moved me to investigate the role of $Ca^{+2}$ in cellulose digestion by the predominant rumen cellulolytic bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1. Maximizing cellulose degradation:

Palmquist and Conrad (1982), Lopez-Guisa (1985), Palmquist et al (1986), Wagner et al (1993), and Ferlay and Doreau (1995) have evaluated the role of divalent cations for several different parameters related with fiber digestion.

Palmquist and Conrad (1982) found that Ca supplementation improved the apparent digestibility of the fiber and all the nutrients of the diet, independently of the energy source. Lopez-Guisa (1985) evaluated the effect of supplementing a diet based on corn crop residues and alfalfa with Cu and Co. They observed an increased rate of both dry matter (DM) and cellulose digestion of supplemented corn crop residues when they were incubated *in situ*; however total DM degradation was not affected by the supplement.

Due to the negative effect on fiber degradation when ruminants are fed fats or oils because their toxic effect of fatty acid on rumen microorganism (Galbriath et al 1971), or their action as chelating agents decreasing cation availability to the rumen microorganisms (Devendra and Lewis, 1974), or because of the physical coating of the vegetable fiber (Harfoot et al, 1974), many studies were made to identify the factors that were reducing cell wall

degradation. Thus supplementing a 10% tallow diet with 0.96 % (3% in the concentrate) calcium chloride to cannulated cows resulted in a lower rumen pH at 12 hours post-feeding, a value that was highly correlated with the total VFA concentration. Ionized calcium ($Ca^{+2}$) concentration in the rumen was inversely related to rumen pH (r = - 0.73), whereas the proportion of long chain fatty acids (LCFA) in calcium-soap was positively related to pH (r = 0.29). $CaCl_2$ was dissociated and provided higher levels of $Ca^{+2}$ in rumen contents as compared with dicalcium phosphate or limestone, and also was associated with an increased rate of neutral detergent fiber (NDF) degradation (Palmquist et al, 1986). As mentioned, Devendra and Lewis (1974) suggested that LCFA inhibit fiber degradation because $Ca^{+2}$ availability is limited for growth of cellulolytic bacteria. Although LCFA bound rumen $Ca^{+2}$ to form insoluble soaps (Jenkins and Palmquist, 1982), the concentration of $Ca^{+2}$ in the rumen (Palmquist et al, 1986) exceeded the ruminal bacteria requirements proposed by Bryant et al (1959).

Wagner et al (1993) tested $CaCl_2$ (0.46% of DM) as a source of ionic calcium in wheat middling diets (replacing corn silage) fed to dairy cows. They found that, as $Ca^{+2}$ concentration increased, ruminal pH, C2:C3 ratio, and milk fat % all decreased; the acidic effect of $CaCl_2$ appeared to negate any beneficial responses of increased $Ca^{+2}$ concentration. These effects were not overcome by bicarbonate supplementation. Lack of response in this experiment was possibly due to a minimal amount of effective fiber in the diet.

When fats or oils are used as an energy source in livestock feeds, modifications occur in the rumen metabolism, among them being a decrease in cellulose digestion. One of the aspects studied by Galbraith et al (1971) was the beneficial effect of $Ca^{+2}$, $Mg^{+2}$, ergocalciferol and cholesterol concentrations on bacteria, which appeared to reverse the negative effect of LCFA on fermentation. Different fatty acids and different concentrations of $Ca^{+2}$ and $Mg^{+2}$ were evaluated. Toxicity of the fatty acids increased with chain length and level of unsaturation, apparently due the physicochemical properties. Both $Ca^{+2}$ and $Mg^{+2}$ affected lauric acid concentration, by formation of salts, but did not have the same effect as linoleic acid. The sensitivity of bacteria to fatty acids was higher in Gram (+) than Gram (-) cells, as well as by morphological differences, i.e., bacilli were more sensitive than cocci. The results suggested that supplementing fat could influence the requirement for $Ca^{+2}$ and $Mg^{+2}$; thus El-Hag and Miller (1972) had suggested increasing the supplementation of calcium when 3-5% of fat was added to the dairy cows diet. $CaCl_2$ has an increased capacity to form calcium soaps and reduce the toxic effect on rumen bacteria and improve the cell wall digestion, when it has compared with dicalcium phosphate (Jenkins and Palmquist, 1982).

**2.2. Cellulose degradation:**

The steps required for cellulose degradation by rumen bacteria were described by Miron et al (2001), and these include: transport of bacteria to the substrate, initial nonspecific adhesion, specific adhesion, proliferation, and

colonization of plant tisues. Different mechanisms of bacterial adhesion to cellulose/fiber have been described: cellulosome, fimbria or pili, and glycocalyx (Miron et al 2001; Bayer et al, 2004)

In some of the cellulolytic bacteria, adhesion/attachment, and hydrolysis of CHO involves a cellulosome structure, where an attachment system links the bacteria and a group of enzymes with the plant cell wall (Bayer et al, 1998; Shoham et al, 1999; Miron et al, 2001).

**Cellulosome**. The existence of a "large, discrete, multisubunit complex(es) which exhibits both antigenic and cellulolytic activities" was first described in *Clostridium thermocellum* twenty years ago by Lamed et al (1983). The authors proposed the concept of a "cellulosome", a structure by which the bacterium is attached to and degrades the insoluble substrate (Lamed et al, 1983, cited by Lamed et al, 1985). Later the cellulosome was further described, based on biochemistry and/or genetic evidence, in many other cellulolytic bacteria. Among these are *Clostridium cellulolyticum*, *C. cellulovorans*, *C. acetobutylicum*, *C. josui*, *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* (Mitsumori and Minato, 1997; Ohara et al, 2000; Ding et al, 2001; Bayer et al, 2004). Also the concept was broadened and today it includes, in addition to cellulases, enzymes that can degrade many other cell wall polysaccharides (Bayer et al, 2004).

*R. flavefaciens* and *R. albus* also possess a glycocalyx (Miron et al 2001), and *R. albus,* in addition, contains fimbria or pili (Miron et al, 2001; Rakotoarivonina et al, 2002) as attachment mechanisms.Whereas *Fibrobacter*

*succinogenes*, the principal cellulolytic bacterium, does not have a cellulosome, it has a glycocalyx (Miron et al, 2001) and presents enzymes (endoglucanases EG2, EGF and Cl-stimulated cellobiosidase) containing a cellulose binding protein that permits attachment to the substrate (Miron et al 2001). The presence of a cellulosome in this bacterium is questionable; however, by transmission electron microscopy it is possible to see protuberances around the surface of *F. succinogenes;* apparently these are only blebs arising from the outer membrane. The true mechanism for attachment of *F. succinogenes* remains unclear (Chesson and Forsberg, 1997).

The cellulosome is formed by different subunits: a scaffoldin that contains the cohesin domains, dockerins, anchoring proteins, cellulose-binding module, and catalytic domains. Different species have a different arrangement of the subunits, e.g., *C. cellulovorans* has multiple scaffoldins and *C. thermocellum* has a single scaffoldin, and *R. flavefaciens* has a scaffoldin without identifiable CBM and S-layer homology (Bayer et al, 2004). There are also different cohesins and dockerins, classified according to molecular characteristics and phylogenetic classification (Rincon et al, 2003; Bayer et al 2004).

Cohesin-dockerin interactions are species specific, first demonstrated for *C. thermocellum* and *C. cellulolyticum* by Pages et al (1997); $Ca^{+2}$ is involved in this interaction (Choi and Ljungdahl, 1996b).

The dockerin protein contains 70 amino acid residues with 2 duplicated segments, each with 22 amino acids. The 12 first amino acids in the duplicated sequence show a similar feature to the $Ca^{+2}$ binding loop present in EF-hand

motifs (Chavaux et al, 1990). EF-hand calcium-binding proteins are proteins with two perpendicular α-helices (E and F) separated by a 12-residue loop. Residues at 1, 3, 5, 7, 9 and 12 amino acid sequence of the loop provide the ligands for binding $Ca^{+2}$. The residues are represented by acid amino acids. EF-hand proteins can contain between two to eight helix-loop-helix structures organized in paired domains to facilitate their contact (Michiels et al, 2002). Prokaryotic proteins with EF-hand motifs are represented by extracellular polysaccharide degrading enzymes from *Clostridium*, *Ruminococcus* and *Bacteriodes* (Michiels et al, 2002).

The enzymes from *Clostridium thermocellum* have been described from the molecular point of view and many of them were shown to have Ca-binding motifs (like EF-hand motifs) related with their structural stability, permitting formation of the cellulosome structure. Ca-binding points are present at the dockerin and/or catalytic domains of the cellulosome (Bayer et al 1998; Shoham et al, 1999).

For *Clostridium thermocellum* the following have been described:

- cellulosome catalytic subunits: CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelJ, CelS, LicB, XynY, XynZ (Bayer et al, 1994).
- Endoglucanase CelD that contains 3 Ca-binding sites and one $Zn^{+2}$ binding site, when $Ca^{+2}$ is bound to CelD it increases the substrate binding affinity, protects against thermal denaturation (Chavaux et al, 1990), the enzyme has stable active conformation and has greater catalytic affinity (lower Km) (Chavaux et al, 1995).

- Choi and Ljungdahl (1996 a, b) demonstrated that “the intact cellulosome hydrolyzes crystalline cellulose in the presence of $Ca^{+2}$ and thiols”. The cellulosome tightly binds $Ca^{+2}$ from the growth medium; CipA is involved as the protein structure (IREs) with high affinity for $Ca^{+2}$ at the scaffolding of the cellulosome, binding several catalytic subunits at the carboxyl end of a conserved duplicated region.

- CelS (cellobiohydrolase subunit) of the cellulosome was shown to have a dockerin with a secondary structure that is folded into a stable tertiary structure, mediated by binding $Ca^{+2}$. This protein contains only one α-helix, comparable to the F hand of EF-hand motif and, therefore, is not the typical EF structure (Lytle et al, 2000). Later, Lytle et al (2001) proposed that it is possible that the dockerin undergoes a conformational change upon binding the $Ca^{+2}$ which could further expose hydrophobic and basic residues (side-chains). This is a new idea about cellulosome assembly.

- CBH-S8 and S11, the major cellobiohydrolase and endoglucanase, respectively, from the cellulosome of *C. thermocellum* were isolated and characterized by Bhat et al (2001).

*Ruminococcus albus* and *R. flavefaciens* also contain enzymes that have Ca-binding sites, similar to the EF-hand group of proteins:

- *Ruminococcus albus*: DocVII at the dockerin domain of EGVII shows Ca-binding sites (Ohara et al, 2000).

- *Ruminococcus flavefaciens*: EndA, XynD and XynB have dockerin-like sequences, with 23 amino acid sequences similar to those found in cellulolytic

*Clostridium sp*. that have Ca-binding residues (Kirby et al, 1997). CesA, XynE and XynB are esterases with Ca-binding motifs (Aurilia et al, 2000). *R. flavefaciens* has a cellulosome with similar characteristics to that of *C. thermocellum*, showing a Ca-binding motif at the dockerin domain (Ding et al, 2001); EndB, presenting two copies of $Ca^{+2}$ binding motif similar to other *Ruminococcus* domains and *Clotridium sp*. (Rincon et al, 2001).

*Fibrobacter succinogenes*, produces a number of cellulolytic enzymes and some cellulose-binding proteins that also could have enzymatic activity against cellulose (Chesson and Forsberg, 1997). McGavinMcGavin and Forsberg (1988) isolated and characterized two endoglucanases in *F. succinogenes* S85. Endoglucanase 1 (EG-1) was the most active enzyme, representing 32% of the total endoglucanase activity. The addition of $CaCl_2$ (1-10 mM) or $MgCl_2$ (1-10mM) increased its activity. Endoglucanase 2 (EG-2) represented 11% of total endoglucanase activity, and no variation in its activity was observed when $Ca^{+2}$ or $Mg^{+2}$ was increased (McGavin and Forsberg, 1988). EG-2 exhibited specific binding to acid-swollen cellulose, whereas neither EG-1 nor EG-2 bound crystalline cellulose (McGavin and Forsberg, 1988; McGavin et al, 1990). EG-2 was later renamed as endoglucanase F (EG-F), because the Cel F gene encodes this protein (Malburg et al, 1997). EG-F belongs to glycosyl hydrolase family 51 and has unique endoglucanase activity (Malburg et al, 1997; Mitsumori and Minato, 2000). The N-terminal cellulose-binding domain of EG-F has two repeated regions that have similar amino acid sequences arranged in tandem; the first region binds cellulose (Mitsumori and Minato, 2000). Mitsumori et al

(2002) demonstrated, using recombinant proteins of EG-F, that these repeated regions are involved in the binding capacity of the enzyme, and $Ca^{+2}$ improved its binding to carboxymethylcellulose. However, no calcium-binding motif was found in the amino acid sequence of the recombinant cellulose-binding protein (designated as AD2).

**2.3. Calcium and Magnesium requirements for rumen bacteria:**

Hungate (1966) considered that the mineral allowances for ruminants estimated from Blaxter (1952) should supply largely the Ca and Mg *in vitro* growth requirements of *B. succinogenes* in pure cultures. However, *in vitro* requirements may be lower than *in vivo* requirements, where much more complex substrates need to be metabolized. Also, he considered that rumen bacteria may not tolerate large unbalances of single minerals; although the typical mineral concentrations in the rumen content may be higher than the bacteria requirements, these requirements may be higher than those reported for single strains due the higher amount of substrate needing to be metabolized.

Bryant et al (1959), using *Bacteroides succinogenes* strain S85 (now *Fibrobacter succinogenes*), evaluated the mineral requirements for growth, and demonstrated the need for $PO_4^{-3}$, $NH_4^{+}$, Mg, Ca, K and Na in the medium. When $PO_4^{-3}$, K, Mg, $NH_4^{+}$ or Ca were deleted singly, little or no growth occurred. For Ca and Mg, they reported a requirement for maximum growth of 0.05 mg and 0.01 mg per 5ml of medium, respectively. This converts to a requirement of 0.25 mM of total Ca and 0.082 mM of total Mg.

Caldwell et al (1973) evaluated the inorganic growth requirements for *Bacteroides amylophilus* and found that this microorganism required for growth: Na, $PO_4$, K and small amounts of Mg, while no requirement for Ca, Co, Mn, Cl, or $SO_4$ could be demonstrated. In further studies, Caldwell and Hudson (1974) evaluated the sodium growth requirement for predominant rumen bacteria using pure cultures and found an obligate requirement for growth (both yield and growth rate) for all the species and strains tested. The deletion of Na from the media could not be replaced by Rb, Li or Cs.

Later, Caldwell and Arcand (1974) evaluated the mineral requirements for many species from the genus *Bacteroides*. When Ca was deleted from the media, growth of *B. fragilis, B. oralis* 7CM and *B. fundiliformis* 12290 was reduced. When they added only Ca (28 µM) or Mg (10 µM) to cation-depleted media, normal growth was not observed, but when Ca and Mg were added together, normal yield was achieved by *B. ruminicola* subsp. *ruminicola* B18, *B. fragilis* subsp. *vulgatus* 8483 and *B. succinogenes* S85. The addition of Co or Mn to a medium with Ca and Mg reduced the growth of *B. succinogenes* S85, suggesting a complex interaction among the different cations in this strain.

Mineral requirements for cellulose degradation were first investigated *in vitro* by Hubbert et al (1958a, 1958b). In the first study (1958a), the authors evaluated the addition of Ca, Mg, S, Mn, Fe, Cu, Co, Zn and B to *in vitro* systems, and in the second (1958b) they evaluated the effects of K, Na, Rb, Li, and Cs. In both studies the authors used washed suspensions of rumen microorganisms. Under these conditions they found that K, but not Na, was

necessary for cellulose digestion (Hubbert et al, 1958b). Among the minerals evaluated in the first study, Ca, Mg, and S were recognized as nutrients that must be added in artificial media. They suggested as optimal concentrations: 50-300 µg/ml of $CaCl_2$, 20-160 µg/ml of $MgSO_4$ and 10-500 µg/ml of $Na_2SO_4$. Higher concentrations 450, 320 and 1000 µg/ml, respectively, were considered to be toxic because cellulose degradation was decreased (Hubbert et al, 1958a). By calculation, the optimal range for Ca was 0.34 to 2.04 mM; and the toxic level,11.23 mM.

Later, Barth and Hansard (1962), using washed ruminal microorganisms as inoculum, confirmed that Ca is required for cellulose degradation *in vitro*; when phytin was the source of P, Ca had a detrimental effect on cellulose degradation, but not when P was from an inorganic source. The authors concluded that the ratio of Ca:P must be considered for supplementation. Whether *in vitro* or *in vivo*, they found the ratio must not be higher than 4:1.

Martin et al (1964) reported a depression in cellulose degradation when Mg was not supplemented to the ration (*in vivo*) or to the medium (*in vitro*).

Uesaka et al (1967), cited by Durand and Kawashima (1980) reported a positive effect on cellulose degradation when the concentration of $CaCl_2$ was increased from 10 to 100 µg /ml of medium.

Bales et al (1978) evaluated the degradation of DM from sorghum stalks incubated *in vitro* with different levels of Ca, P, Mg and urea, using washed rumen bacteria. As Ca or Mg concentrations increased (10 to 190 ppm, and 50 to 150 ppm, respectively) DM digestion was decreased ($P<0.05$), suggesting a

toxic effect from high Ca or Mg concentrations on DM digestion; considering that the principal components of the stalks are the structural carbohydrates, this decreased DM digestion can be interpreted as a decreased fiber digestion.

Gong and Forsberg (1989), studying the behavior of the principal cellulolytic bacteria, determined that *Fibrobacter succinogenes* requires either Ca or Mg for adhesion to cellulose. Conversely, Roger et al (1990) found in their experiments that *F. succinogenes* does not require $Ca^{+2}$ for adhesion, but *Ruminococcus flavefaciens* requires Ca and Mg for the cellulose adhesion process.

In 1973, Bryant remarked on the little research done about mineral requirements for cellulolytic bacteria, that this may be because of an assumption that they have similar requirements to other bacteria. However, he pointed out that, "contrary to most non-marine bacteria so far studied, *B. succinogenes* has a requirement for Na and relatively high need for Ca". He suggested that mixed cultures must need K, Na, Ca and phosphate for optimal cellulose digestion, and maybe Mo, Fe and Zn are also required. Subsequently, in their review, Durand and Kawashima (1980) commented: "It is not certain whether all bacteria have a Ca requirement for growth. Ca deficiency can cause growth defects and the formation of pleomorphic forms, and might alter metabolic processes requiring $Ca^{+2}$ activated exoenzymes as α-amylase. It can be considered that microorganisms require a smaller amount of $Ca^{+2}$ than $Mg^{+2}$."

Very little additional research on mineral requirements of bacteria occurred after this time, which is based on the idea that all microorganisms have the same requirements, as reflected in the review of Durand and Kawashima (1980). The research about mineral requirements by rumen bacteria and their different functions clearly has had a lack of direction; although certainly many individual efforts have been made, only isolated information has been obtained.

**2.4. Ca and Mg concentrations in ruminal fluid/content:**

Poutiainen (1970) measured Ca and Mg concentrations and their changes in the rumen fluid of cattle. Ca and Mg were determined by atomic absorption spectrometry. The author found significantly different concentrations among sampling times and also sampling location in rumen ($P<0.05$). The lower concentrations for Ca and Mg were obtained at 0 and 12 hours after feeding, and the peak was obtained at 3-4 hours after feeding. The Ca concentrations varied from 2 to 3.5 mM and for Mg from 2 to 4 mM (4 to 8 meq/l). Lower concentrations were found at the bottom of the rumen, probably due to dilution.

Nel and Moir (1974) evaluated the effect of supplying Ca and/or P by ruminal or duodenal infusion in sheep, with the idea to identify the site of absorption of these in the gastrointestinal tract. As part of the methodology they determined the total Ca concentration of centrifuged rumen liquor by atomic absorption spectrophotometry. Samples from control and non-ruminally infused Ca animals ranged from 0.46 to 0.68 mM of Ca (mean 0.60 mM). Concentration of Ca in the rumen was 1.04 mM when $CaCO_3$ was infused ruminally.

Playne et al (1978) evaluated the disappearance of different minerals from hays from *Medicago sativa, Stylosanthes humilis, Chloris barbata* and *Heteropogon contortus* using the nylon bag technique at 5 incubation times (12, 24, 48, 72 and 168 h). The minerals that remained in higher proportions after 168 h of incubation were P, Ca and N, (40% of DM) whereas Mg showed an intermediary resistance (20% of DM), and K had the least (~5% of DM). The minerals from *Medicago sativa* were the most soluble, whereas *Heteropogon* were the most insoluble. The most rapid release from the forage occurred during the first 24 h of incubation in rumen. Later relative concentrations increased because their release was slower than the loss of dry matter.

Bennink et al (1978) followed the postprandial changes in mineral composition of the rumen fluid of cattle and sheep fed with different diets. Ca and Mg concentrations were affected by their intake. As can be expected, Ca concentrations were increased 3 to 9 times when alfalfa was fed. The Ca and Mg concentrations at 24 h were related to intake. Negative correlations were observed for Ca and Mg concentrations and pH (increased solubility with lower pH). The ranges of Ca and Mg concentrations preprandially were: 0.4–1.7 and 1.0–4.4 mM, respectively. The range of means from all the samples from 24 hours from the 6 diets was: 0.39–10.0 and 1.79–10.10 mM, for Ca and Mg, respectively. Concentration peaks varied among diets, ranging from 1 to 3 hours postprandial. These values represent the total soluble minerals, because Ca and Mg were determined in the rumen fluid filtered through cheesecloth and determined by atomic absorption spectrometry.

Mackie and Therion (1984) reported a summary of studies that measured Ca and Mg concentrations in rumen; no information about methods used to measure the cations nor about conditions of sampling was provided. The ranges for Ca and Mg were 0.75 – 12 mM and 0.40 – 6.5 mM, respectively.

Rumen content of soluble Ca, determined by atomic absorption spectrometry, from steers fed 88% concentrate and varying levels and sources of calcium, no Ca, $CaCO_3$ (0.4%), $CaCl_2$ (0.4%), $CaCO_3$ (2.5%) were: 55; 165; 64; and 239 mg Ca/l, respectively (Goetsch and Owens, 1985).

Because the composition of plant species influences mineral availability in the rumen, Emanuele and Staples (1990) evaluated the minerals released from 6 different forage species using *in situ* technique. Immediate and maximal releases in rumen were 29 and 70 % for Ca, and 82 and 95% for Mg, respectively. The highest Ca concentration (1.46 % DM) was in alfalfa and 46.7% of this was released immediately and 12.6% was released slowly, requiring 12 to 18 hrs to reach the maximal release. Ca concentrations in other species were lower, and Ca was released over longer time. Dwarf elephant grass bound ruminal Ca, increasing the Ca concentration by ~20 %. The measurements for Ca and Mg forage content were made using atomic absorption spectrometry. Grace et al (1977) contributed to the understanding of the dynamics of Ca, Mg, P and K in rumen digesta and the factors that affect it. The authors used eight sheep cannulated in both rumen and duodenum/ileum and fed *Lolium perenne* to evaluate the extent of the association of Mg, Ca, P, and K with organic

constituents of the digesta in relation to different sites of the gastrointestinal tract and their apparent absorption. Rumen content was subdivided into different pools: water-soluble, diluted-alkali-insoluble, and dilute-alkali-soluble fractions.

Ca, Mg and P were 48.0, 57.4, 45.9 % water-soluble in the diet and 54.6, 56 and 50 % as water-soluble compounds in the rumen, respectively. The water-soluble fraction contained the ionic and bound forms of Ca, Mg, and P. The dilute-alkali-insoluble fraction contained $Ca^{+2}$, $Mg^{+2}$ and P: 37.9, 17.2 and 24%, respectively. The alkali-insoluble fraction from rumen digesta contained N, pectin, hemicellulose, cellulose, 72% $H_2SO_4$-insoluble and ash (Grace et al, 1977). Among those compounds pectin and lignin have the ability to bind $Ca^{+2}$ and $Mg^{+2}$(Molloy and Richards, 1971;, Somers 1973; Torre et al 1992).

When they evaluated the effect of changes in pH on binding capacity of the different minerals with the dilute-alkali-soluble and dilute-alkali-insoluble fractions, they found an increased Mg binding to the alkali-insoluble fraction when pH increased; as the pH decreased, more Mg and Ca became associated with the water-soluble fraction. Similar results occurred when the medium was diluted; at higher dilution, higher content of Ca and Mg was in the soluble fraction. When $Ca^{+2}$ was increased in the medium, it had a negative effect on $Mg^{+2}$ binding to the alkali-insoluble fraction, probably by competition at binding sites (Grace et al, 1977).

The results from Grace et al (1977) confirmed the earlier reported effect of pH on cell wall binding ions formulated by Storry (1961), but also clarify the issue related to the components of the water-soluble fraction, both ionic and bound

forms, and why total Ca of this fraction does not reflect the amount of ionized Ca, which is the interchangeable form and the one that is required by the microorganisms.

Ruminal volatile fatty acids (VFA) can bind calcium, forming Ca salts that are water soluble compounds, but not ionized (Windholz, 1976). These probably readily exchange with the ionized fraction as $Ca^{+2}$ becomes bound to fiber or long chain fatty acids, thus tending to maintain an equilibrium of the ionized form.

Ionized Ca concentrations in rumen fluid were monitored by Palmquist et al (1986) and Wagner et al (1993), who both measured $Ca^{+2}$ with ion-electrode. They demonstrated that rumen $Ca^{+2}$ concentrations are affected by the diet, source of Ca, and time after feeding, and the range varied widely between 0.25 mM to 7 mM.

Palmquist et al (1986) reported for cows supplemented with tallow and either $CaCl_2$ or $Ca_2PO_4$ + limestone, an average from different dietary treatments of 0.6 mM at 0 h and 1.5 mM of $Ca^{+2}$ at 10 h after feeding, when concentration reached a plateau. The mean rumen content of ionized calcium when $CaCl_2$ or dicalcium phosphate + limestone was supplemented were: 1.82 and 0.62 mM, respectively.

Wagner et al (1993) observed, in dairy cows, the peak concentration of $Ca^{+2}$ ocurred at 6 hours after feeding, independently of the type of diet evaluated, declining to basal levels at 12 hours after feeding. When high forage diets were supplemented with $CaCl_2$ or $CaCl_2$+ $NaHCO_3$ the values for $Ca^{+2}$ at 3, 6 and 12 hours after feeding were: 3.75, ~7, and 0.75 mM, respectively. When wheat

middlings with $CaCl_2$ were fed the $Ca^{+2}$ concentrations were: ~7, ~7 and 0.75 mM; whereas wheat middlings + $CaCl_2$ + $NaHCO_3$ were supplemented the concentrations were: 1.5, 3.75 and 0.25 mM. For wheat middlings+$NaHCO_3$, the values were: 1.5, 2.5 and 0.25 mM of $Ca^{+2}$.

Ferlay and Doreau (1995), feeding cows supplemented with rapeseed oil, evaluated the ultrafiltrable calcium concentrations from rumen fluid by AAS; observing a decreased concentration of calcium when fats/oil were fed to the cows; two diurnal peaks were observed related with time of feeding rather than the diet. Concentrations reported were: 77.6, 21.1, 25.7 mg/l ultrafiltrable calcium for control, and the two diets supplemented with oil, respectively.

**2.5. Bacterial cell walls:**

The cell walls of Gram (+) and Gram (-) bacteria differ in structure and composition. The Gram (+) bacteria have a cytoplasmic membrane and a thick peptidoglycan (also called mureine) layer. In contrast, Gram (-) bacteria have more complex cell walls: the cytoplasmic membrane, periplasmatic space, peptidoglycan, and outer membrane (bi-layer), which contains an inner layer of phospholipids, lipoproteins and proteins and an outer layer of lipopolysaccharide (40%) and proteins (60%) (http://www.ugr.es/~eianez/Microbiologia/05pared.htm 3/15/05). The cell walls of archea are the most simple, containing only the cytoplasmic membrane and S-layer (Mayer, 1999).

The peptidoglycan layer also contains a matrix of teichoic acid, teichuronic acid and lipoteichoic acid. The latter is present in all the Gram (+) bacteria, whereas the former is present in only some. Teichuronic acid is produced by certain Gram (+) bacteria when they are phosphate-restricted, and this acid contains anionic residues that are able to bind divalent cations, particularly $Mg^{+2}$ (http://www.ugr.es/~eianez/Microbiologia/05pared.htm 3/15/05).

The polyglycerol phosphate-teichoic acid, which is located in the periplasmic region of the cell, also can bind divalent cations and, together with teichoic, and teichuronic acids, form a cation-exchange system in the cell wall (Ghuysen and Shockman, 1973).

Bioynthesis of peptidoglycan occurs in 3 stages: "Peptidoglycan precursors made on a uridylic acid cytoplasmic carrier (stage 1) are transferred from uridylic acid to an undecaprenyl phosphate membrane carrier (stage 2) and to a final acceptor in the expanding wall peptidoglycan (stage 3). At some point during the later stages of this process, the nascent peptidoglycan undergoes cross-linking, which is required to make it insoluble, and covalently linked accessory wall polymers are attached." At the first stage the synthesis of the nucleotide precursors is catalyzed by enzymes that require ATP and either $Mg^{+2}$ or $Mn^{+2}$. At the second stage the translocation of N-acetyl-muramyl-pentapeptide requires $Mg^{+2}$ and is stimulated by $K^{+}$ and other monovalent cations (Ghuysen and Shockman, 1973).

Other structures are S-layers, and when present, they are the most external layer of the cell wall; they function as protective coats, for bacterial adhesion, as surface recognition, as ion traps, and as scaffolding for enzymes and virulence factors. They are ubiquitous, present in Gram (+) and (-) bacteria (Sleytr and Beveridge, 1999, Mayer, 1999). S-layers are self assembled protein/glycoprotein subunits, occurring in a monolayer, planar lattice (Sleytr and Beveridge, 1999,); $Ca^{+2}$ is required for the subunit attachment in *Caulobacter crescentus*, (Walker et al, 1994 cited by Sleytr and Beveridge, 1999), and *Campylobacter fetus* subspecies *fetus* (Dworkin et al, 1995 cited by Sleytr and Beveridge, 1999).

### 2.6. Interaction between cell wall and ions.

Among the factors that affect the initial nonspecific adhesion of bacteria to substrate are ionic or hydrophobic associations that involve van der Waals forces, $Ca^{+2}$, $Mg^{+2}$, or the double ion layer of K that can neutralize the repellent ionic negative charge between cell walls and bacteria (Van Soest, 1994, Miron et al, 2001); also temperature, pH, and age of the bacteria can affect this interaction (Miron et al, 2001).

Molloy and Richards (1971) reported the $Ca^{+2}$ and $Mg^{+2}$ binding capacities of different polymers from the cell wall of *Holcus lanatus*. They found that pectin and lignin had higher affinity for both cations, while hemicellulose and cellulose

had very low capacities to bind these cations. Increasing pH from 6.3 to 7.24 increased the $Ca^{+2}$ binding percentage from 28 to 31.7 % of pectin. Lignin bound 20.7% of $Ca^{+2}$ at pH 6.2. Pectin and lignin bound 3.8 and 12.5 % of $Mg^{+2}$, respectively.

Calcium ions have a particular affinity for plant cell walls, reacting with the carboxyl groups of the pectic components of cell walls; the adhesion is lost when tissues are washed with acid solutions (Somers, 1973).

Torre et al (1992) evaluated *in vitro* the $Ca^{+2}$ binding capacity of different components of the plant cell wall under different conditions of varying pH, $Ca^{+2}$ concentration, pectin, lignin and cellulose concentration. Binding by lignin appeared to be the strongest, due the proton-ionizable functional groups of lignin; binding was affected by pH (3.5-7.5, binding increased when pH increased), lignin and $Ca^{+2}$ concentrations. Apparently, $Ca^{+2}$ binds lignin according to the multiple equilibrium theory, with formation of a stoichiometric bond. The affinity of $Ca^{+2}$ with cellulose or pectin was very weak, and may occur only as $Ca^{+2}$ adsorption on the polysaccharide surface. The pH did not affect $Ca^{+2}$ binding by cellulose and pectin, suggesting that the binding capacity of cellulose is not associated with the hydroxyl residues of its polymer chains. By using low-methoxylated pectin, it was shown that pectin does not bind $Ca^{+2}$ through the carboxyl groups of the D-galacturonic acid. Based on these studies, cellulose does not bind $Ca^{+2}$, and lignin is the strongest compound in vegetable cell walls

that binds $Ca^{+2}$. With regard to pectin, the information is less conclusive, perhaps because of differences in chemical composition of pectin used in different studies or different methodology to establish binding.

Research from Van Soest's laboratory developed techniques to measure the cation exchange capacity (CEC) of feedstuffs (Allen et al, 1985). Among different resources evaluated, Whatman No 42 paper and solka floc showed the lowest CEC. A broad classification of feeds, from low to high CEC, was listed: corn silage, straws, brans, grass hays and legume hays. Among feedstuffs with intermediary NDF content, almond hulls and rapeseed showed the highest CEC values (Allen et al, 1985). McBurney et al (1986) reported a significant correlation between CEC and lignin content (0.693 to 0.843), lignin:ADF ratio (0.531 to 0.742) and nitrogen content (0.5 to 0.579), using the Pr III method under two pH: 3.5 and 7.0, respectively. CEC of feedstuffs increased when pH increased, in this form, the CEC from dietary fiber influences the rumen environment by buffering pH and affects the osmotic pressure because of the capacity to bind ions.

The affinity between bacterial cell walls and different ions also has been studied. Fitt et al (1974) reported that isolated rumen bacterial cell walls can take up $Ca^{+2}$ and $Mg^{+2}$; the binding increased with increased cation concentrations, and the presence of $Ca^{+2}$ reduced the binding of $Mg^{+2}$, and vice-versa. Fitt and Hutton (1974) reported that $K^{+}$ also interfered with $Mg^{+2}$ binding.

Beveridge and Murray (1980) reported that phosphodiester groups of teichoic acid in *Bacillus subtilis* bind $Mg^{+2}$ strongly (carboxyl groups from teichoic acid are the principal binding site for different ions); from the total mineral content of the native cell walls (21.108 umol of metal/mg) $Ca^{+2}$ represented 1.9%, while $Mg^{+2}$ was 39%, $Fe^{+3}$, 17% and $Cu^{+2}$, 14.2%.

Later, Beveridge et al (1982), studying *B. licheniformis*, found that the principal compounds in the cell wall able to bind metals were teichoic and teichuronic acids. On native cells walls $Ca^{+2}$ was 53%; $Mg^{+2}$, 24%; and $Na^{+}$, 11% of the total mineral content (0.208 umol of metal/mg).

Although both bacteria studied were Gram (+) and from the same genus, they had different cell wall composition with regard to the type, amount and structural disposition, thus affecting the capacity to bind different metals.

More evidence about the differences in cell wall composition among Gram (+) bacteria is the variability in response to Gram staining (Gram variable). Beveridge (1990) reported that the main reason for variability in Gram staining is aging of the bacteria, differences in composition (bacteria with higher percentage of peptidoglycan stain Gram (+)), thickness of the cell wall, and the presence of S-layers, as occur in *Bacillus*, *Butyrivibrio* and *Clostridium*.

When Beveridge et al (1982) evaluated the capacity to bind metals from the cell envelope of *E. coli*, they found different affinities among the 32 metals tested: $Ca^{+2}$, Cu, Na, K, $Fe^{+2}$ were bound in small amounts (<0.1umol/mg DM); $Mg^{+2}$, Zn, $Fe^{+3}$, Mn, were bound in intermediary amounts (0.1 to 0.4 umol/mg DM); and in large amounts were bound hafnium and osmium. The binding site

corresponds principally with the “polar head group regions of the constituent membranes or long peptidoglycan layer”. In Gram (-) bacteria the outer membrane contains $Mg^{+2}$ and sometimes $Ca^{+2}$ as integral compounds (Beveridge, 1981; cited by Beveridge et al, 1982) and they do not bind as much metal as Gram (+) bacteria do (Beveridge et al, 1982).

Durand and Kawashima (1980), considering the differences in cell wall/envelope composition between Gram (-) and Gram (+), indicated that the former must have lower requirements for $Ca^{+2}$ and $Mg^{+2}$ than do Gram (+) bacteria.

Well known is that pH affects the binding capacity of the walls, both plant and bacterial. Storry (1961) reported that the maximum adsorption of calcium to the cell wall occurs at neutral pH, but it is decreased as the pH decreases. This was supported by studies from Durand and Kawashima (1980) and McBurney et al (1986). This point is important to consider, because when pH conditions are maintained stable in a well buffered medium, normally close to neutral pH, the CEC is maximum and the minerals are bound to the cell wall and are not available to interchange with the media or with other bacteria. Under rumen conditions, pH decreases after feeding, principally because of increasing concentration of volatile fatty acids. This pH condition permits part of the minerals from the feed and bacteria to became ionized and available for the microflora and microfauna (Palmquist et al, 1986). Also the ions can form new

salts by interacting with other nutrients or metabolites (calcium soaps, VFA-salts, etc) (Sukhija and Palmquist, 1990) that can be soluble or insoluble in the ultrafiltrable rumen content (Grace et al, 1977).

### 2. 7. Calcium and Bacteria

The role of $Ca^{+2}$ in bacteria has been relegated to the cell wall and external environment of the bacterial cell, principally for activating external enzymes (Silver, 1977, Smith, 1995; Norris et al 1996). $Ca^{+2}$ is required for growth of photosynthetic bacteria and for *Azotobacter*; but routinely $Ca^{+2}$ is added to the culture media of all bacteria without knowing whether or not it was needed for growth (Silver, 1977). The active participation of $Ca^{+2}$ in cell wall synthesis is more related with sporulation and encystment processes (i.e. *A. vinelandii* require 0.4mM for optimum encystment), rather than with the synthesis of the cell wall (Silver, 1977); $Ca^{+2}$ can bind chemical compounds at the cell wall.

Lately, diverse roles have been identified for $Ca^{+2}$ in bacteria; it has been shown to participate in chemotaxis, thermostability, motility, activation of intracellular enzymes and cell cycle regulation processes (Ordal, 1977; Rampersaud et al, 1991; Tisa and Adler, 1995; Yu and Margolin, 1997; Herbaud et al, 1998; Sudom et al, 2003).

### 2.7.1. $Ca^{+2}$ homeostasis:

Intracellular $Ca^{+2}$ concentrations are maintained low in bacteria as in eukaryotes, due to the presence of specific $Ca^{+2}$ channels (Silver and Kralovic, 1969 cited by Gangola and Rosen 1987), which permit bacterial cells to export excess calcium (Deves and Brodie, 1981).

Intracellular concentrations of $Ca^{+2}$ in bacteria were measured first in *E. coli* by Rosen and Gangola (1987), who used a specific dye, fura-2 pentaacetoxymethyl ester, preceded by treatment with EDTA to facilitate permeation. The free-$Ca^{+2}$ concentrations were similar to those found in eukaryotic cells (90 ± 9 nM of $Ca^{+2}$ with extracellular range of 10uM – 10 mM). Total Ca concentrations increased with increasing extracellular $Ca^{+2}$ concentrations; however intracellular free-$Ca^{+2}$ was independent of the extracellular concentration, which they proposed could be due to a large pool of bound Ca and an efficient system to extrude $Ca^{+2}$ from the cell. When the energy status of the cells was normal, the intracellular concentration of $Ca^{+2}$ did not vary with extracellular $Ca^{+2}$ concentrations (10 uM to 10mM). When cells were energy depleted and extracellular $Ca^{+2}$ concentrations increased, the intracellular content of $Ca^{+2}$ in the cells increased until it became equilibrated with the extracellular concentrations, demonstrating energy dependence to extrude the $Ca^{+2}$ by $Ca^{+2}/H^{+}$ or $Ca^{+2}HPO^{-}_{4}/H^{+}$ antiporters.

Jones at al (1999) measured the intracellular free $Ca^{+2}$ in *E. coli* by using the bioluminescent protein aqueporin and demonstrated that internal $Ca^{+2}$ increases very rapidly in response to increased extracellular $Ca^{+2}$ concentrations.

Depending on the growth status when they were exposed to the increased $Ca^{+2}$, internal $Ca^{+2}$ concentration was normalized rapidly or slowly, without mediation of protein synthesis. When the bacteria were adapted to higher $Ca^{+2}$ concentrations, they were able to increase the intracellular $Ca^{+2}$ faster. Using different channel blockers it was found that apparently the bacteria have two $Ca^{+2}$ influx systems, one that is affected by $La^{+3}$ and the other that is not affected. The $La^{+3}$ sensitive channel corresponds with the voltage-activated poly-β-hydroxybutyrate/phosphate $Ca^{+2}$ channels described by Reusch et al (1995, cited by Jones et al, 1999).

Research from Campbell's laboratory (Jones et al, 1999) shows that the periplasmic space of *E. coli* (Gram-) can act as a "buffer" for $Ca^{+2}$ concentrations between the intracellular and the extracellular conditions. The highly anionic periplasmic membrane-derived oligosacharides are responsable for this $Ca^{+2}$ store; when these are modified the intracellular free-$Ca^{+2}$ concentration is predictable, because it is generated by a Donnan potential. They also showed that the periplasmic space can store three to sixfold the $Ca^{+2}$ concentration detected in the external medium when this is in micromolar concentrations. At millimolar concentration in the external medium, the periplasmic space can store $Ca^{+2}$ similar to concentrations in the external medium (Jones et al, 2002).

### 2.7.2. Calcium transport:

Channels are intramembrane proteins of the cytoplasmic membrane; also at the outer membrane there are proteins that can act as channels, called porins (Mayer, 1999). Deves and Brodie (1981) in their review on active transport of $Ca^{+2}$ in bacteria, describe three mechanisms for Ca transport in bacteria: $Ca^{+2}/H^{+}$ antiporter (*E. coli*, *A. vinelandii*, *M. phlei*), $Ca^{+2}/Na^{+}$ antiporter (*H. halobium*) and ATP (*S. faecalis*).

The presence of specific Ca channels, temperature-dependent in *E. coli*, was first described for bacteria by Silver and Kralovic1969 cited by Gangola and Rosen, 1987). Later, other channels were described for $Ca^{+2}$ efflux: a $Ca^{+2}$/H+ antiporter (Tsujibo and Rosen, 1983; cited by Gangola and Rosen, 1987) and a $Ca^{+2}HPO^{-}_{4}/H^{+}$ antiporter (Ambudkar, Zlotnick and Rosen, 1984, cited by Rosen and Gangola, 1987).

More recent information shows that polyphosphate / poly-(R) -3-hydroxybutyrate are homopolymers, ubiquitous in eukaryotic and prokaryotic cells; these are present at the bacterial plasma membranes, concentrations varying according to physiological stage and environmental conditions. They form ion channels in planar lipid bilayers that are highly selective for $Ca^{+2}$ over $Na^{+}$ at physiological pH. Apparently these complexes can “make” pores at the membrane, thus facilitating ion transport. These also can collaborate to regulate specific cation transfer systems where other molecules are involved (Reusch, 2000). All these mechanisms are used to maintain low intracellular $Ca^{+2}$ concentrations.

Ionophores: “An ionophore is a lipid-soluble small molecule that functions to transport an ion across otherwise impermeable membranes” (Silver 1977). One of the better known ionophores is monensin; the mechanism of action was described by Russell and Strobel (1987). Monenesin is an antiporter that facilitates the movement of monovalent cations across membranes: $K^+$(eflux)/$H^+$(influx) and $Na^+$(influx)/$H^+$(efflux).

There are a variety of other compounds that can act as ionophores for divalent cations; also, specific $Ca^{+2}$ ionophores are available: X537A (Lasalocid, Hoffman-La Roche), A23187 (Eli Lilly and Company), and beauvericin (Silver, 1977). The affinity for $Ca^{+2}$ or $Mg^{+2}$ or other divalent cations differ among ionophores, for X537A: $Ba^{+2}$>$Sr^{+2}$> $Ca^{+2}$>$Mg^{+2}$ (Pressman, 1973; cited by Silver 1977), whereas for A23187: $Mg^{+2}$> $Ca^{+2}$ (Reed and Lardy, 1972; cited by Silver 1977). Tetronasin is an ionophore that acts as an antiporter to interchange divalent cations with $H^+$, permitting the influx of two protons and the efflux of one $Ca^{+2}$ or $Mg^{+2}$ (Newbold et al, 1988).

### 2.7.3. Calcium as a signal:

Numerous papers show a signaling role for $Ca^{+2}$ in prokaryotes in the same fashion as this cation does in eukaryotic cells. The capacity of prokaryotes to regulate intracellular $Ca^{+2}$ concentrations (Rosen and Gangola, 1987; Tisa and Adler, 1995, Jones et al, 1999, Jones et al, 2002) supports this idea. $Ca^{+2}$ in prokaryotes has a role in cell division, chemotaxis, thermostability, cofactor-allosteric factor in enzymes etc, as will be described below.

In a review on the role of $Ca^{+2}$ in bacteria, Norris et al (1996) concluded that intracellular $Ca^{+2}$ concentrations and protein phosphorylation must be part of the structural change mediators in prokaryotes, just as they are in eukaryotes. Further, intracellular levels of $Ca^{+2}$ increase when *E. coli* is in cell division process and during adaptation to different environment; and $Ca^{+2}$ was suggested to participate in the regulation of the cell cycle via protein modifications that control the enzoskeleton, thus acting as a metabolic switch. Enzoskeleton was defined by Norris et al (1996) as "the ensemble of proteins that interact with one another and with membranes and nucleic acids to form extended estructures within the cell".

These ideas are supported by research from Yu and Margolin (1997), who found that $Ca^{+2}$ regulates GTP binding and FtsZ hydrolysis. FtsZ, a tubulin-like GTPase, forms a ring at the cell mid point before cytogenesis begins. FtsZ is essential to generate a cell division point and forms part of the enzoskeleton. It has the capacity to polymerize and depolymerize by adding or extracting $Ca^{+2}$ from the *in vitro* media. *E. coli* cells without FtsZ can grow without dividing, because no formation of the division septum, resulting in long multinucleated filaments.

L-forms of *E. coli* lack a normal peptidoglycan sacculus (wall-less); despite this, they can grow and divide. *E. coli* L-form NC-7 was used to demonstrate that the enzoskeleton is controlled by $Ca^{+2}$ (Onoda et al, 2000). When the medium lacked $Ca^{+2}$, the cells stopped division, become spherical, swelled, formed

vacuoles, and lysed. When $Ca^{+2}$ was reestablished in the medium (1.2 mM), cell growth was restarted (50 uM was not sufficient to restore growth). The L-form cells had FtsZ in lower concentrations than in the wild type (Onoda et al, 2000).

A role for intracellular $Ca^{+2}$ to activate intracellular enzymes was reported by Sudom et al (2003). They found under *in vitro* conditions in *E. coli* that phosphoenolpyruvate carboxykinase (PepCK), a key regulatory enzyme for gluconeogenesis, requires ATP, $Mg^{+2}$ and $Ca^{+2}$ as cofactors. $Ca^{+2}$ bound the enzyme at the active site in a different location than does $Mg^{+2}$; when trypsin was added the $Ca^{+2}$ activation of PEPCK was abolished. It is thought that PEPCK activation by $Ca^{+2}$ may play a role during glycogen synthesis at the stationary-phase of *E. coli* (Sudom et al, 2003).

Concerning a chemotactic role of $Ca^{+2}$, Ordal (1977), using *Bacillus subtilis*, demonstrated that changes in the intracellular $Ca^{+2}$ concentration affect the direction of flagellar rotation; high concentrations cause the bacteria to tumble, whereas at lower concentrations, they swim. Later, Tisa and Adler (1995), using wild-type and chemo-receptor mutants of *Escherichia coli*, tested the changes in intracellular $Ca^{+2}$, using the dye fura-2 as indicator. When attractans were included in the media, the intracellular $Ca^{+2}$ concentration increased briefly and the bacteria tumbled until $Ca^{+2}$ recovered its steady state condition. When repellents were added, intracellular $Ca^{+2}$ decreased briefly, and bacteria swam until $Ca^{+2}$ concentrations were reestablished to normal levels.

When neutral substances were added, no response was observed. When mutants with no chemoreceptors were tested, no response to the different chemo-stimulants/inhibitors was observed.

The tumbling and the swimming behaviour is regulated by the expression of CheY, a cell wall protein, in the phosphorylated or unphosphorylated state, respectively, responding to high or low levels of intracellular $Ca^{+2}$ (Lukat et al 1990 cited by Tisa and Adler (1995).

OmpC and OmpF are outer membrane porin proteins that change in response to changes in the osmolarity of the external environment. The transcriptional regulation of these proteins depends on EnvZ, a protein of the inner membrane with a periplasmic receptor domain and cytoplasmic signaling domain. EnvZ signals to OmpR and it regulates the OmpC and OmpF expression (Forst and Inouye, 1988; cited by Rampersaud et al, 1991). It was demonstrated that $Ca^{+2}$ stimulates EnvZ phosphorylation, followed by transfer of phosphate from EnvZ to OmpR, which activates the porin genes (Rampersaud et al 1991).

Van Asselt and Dijkstra (1999) reported the presence in *E. coli* of a lytic transglycosylase, Slt35, which has a motif similar to the EF-hand motif; this was able to bind $Ca^{+2}$, thus enhancing the thermostability of the enzyme. Slt35 catalyses the cleavage of β(1,4)-glycosidic bonds in peptidoglycan at the bacterial cell wall, allowing the insertion of new units into the growing cell wall, creating pores for proteins and transport, and acting as a cell wall zipper during cell division.

Outer membrane lipases, such as phospholipase A in *E. coli* and phospholipase C in *B. cereus* and *C. welchii*, are stimulated or inhibited by $Ca^{+2}$, depending on specificity of the respective phospholipase (Silver, 1977), thus affecting the permeability or fluidity of the membrane. Snijder and Dijkstra (2000) reported that outer membrane phopholipase A is encoded by the *pld*A gene, which is widespread among Gram (-) bacteria and associated with pathogenicity. Among rumen bacteria, *B. fibrisolvens* outer membrane phospholipases do not appear to be sensitive to $Ca^{+2}$ stimuli; rather, they are sensitive to the presence of cysteine, dithiothreitol and mercaptoethanol. No information about any other rumen Gram (-) bacteria was found (Hazlewood and Dawson, 1975 and 1976; cited by Harfoot, 1981).

Other $Ca^{+2}$ binding proteins in *B. subtilis* were reported by Herbaud et al (1998): flagellin, hydroperoxido reductase (AhpC), ribosomal protein (B-L9) and two major cold shock proteins (CspB), but in none of these was found EF-hand motifs in their amino acid sequences.

In eukaryotes, EF-hand proteins are ubiquitous and calmodulin is the representative proteins of this group; their regulatory function is well known for many different enzymes and ion channels (Michiels et al, 2002). Calerythrin was the first protein with EF-hand motifs found in bacteria (Leadly et al, 1984), from a Gram (+) bacterium *Saccharopolyspora erythrea*; later, calsymin from *Rhizobium etli*, Gram (-) bacterium was identified (Xi et al, 2000).

Michiels et al (2002) searched several databases and identified many EF-hand calcium-binding proteins in prokaryotes; among them a group of sixteen proteins with at least two EF-hand motifs were found, but they do not have a clear function, with the exception of calerythrin, calsymin (mentioned earlier), and Asp24 (an acid shock protein) from *Brucella abortus*, which play an infectious role. A second group of prokaryotic proteins with EF-hand motifs was represented by extracellular polysaccharide-degrading enzymes from *Clostridium*, *Ruminococcus* and *Bacteriodes*; a characteristic of this group of proteins is that only the second α–helix loop is present. A third group includes the periplasmic D-galactose-binding proteins that participate in glucose and galactose transport.

Earlier, the presence of EF-hand proteins belonging to this third group were described: periplasmic glucose/galactose-binding proteins from *E. coli* (Vyas et al, 1987 cited by Van Asselt and Dijkstra, 1999) and *S. typhimurium* (Zou et al, 1993 cited by Van Asselt and Dijkstra, 1999) membrane-bound lytic transglycosylase B (MltB) from Gram (-) bacteria (Van Asselt and Dijkstra, 1999). $Ca^{+2}$ depletion reduced 2 fold the sugar affinity of D-Galactose receptor in *E. coli*, due an allosteric structural change in the protein (Luck and Falke, 1991).

**2. 8. Magnesium and bacteria.**

Magnesium is the most abundant intracellular divalent cation and its essential role in all living cells is broadly recognized (Smith and Maguire, 1998).

In prokaryotes, Mg has many different cell functions: bacterial chemotaxis, enzyme cofactor, maintain integrity of the cell wall/cellular membrane and growth (Jasper and Silver, 1977; Smith and Maguire, 1998). The role may differ among bacterial species (Jasper and Silver, 1977).

Part of the Mg is present at the cell wall (Eagon et al, 1965; cited by Jasper and Silver, 1977, Durand and Kawashima, 1980), but most is bound to the ribosomes and is required to maintain their structure (Tissieres et al, 1959; cited by Jasper and Silver, 1977). The ribosomes of *E. coli* are highly sensitive to Mg starvation, and, when starvation occurs, bacterial growth stops because protein synthesis and DNA synthesis decrease. *E. coli* and many Gram (+) bacteria show morphological changes after short periods of Mg starvation, becoming filamentous. *A. aerogenes* is less sensitive than *E. coli* and can resist Mg starvation of 20 hr or longer, maintaining DNA synthesis. Salt tolerance of *S. aureus* is reduced when intracellular concentrations of Mg is decreased (Jasper and Silver, 1977).

The intracellular concentration of Mg appear to be similar between Gram (-) and Gram (+) bacteria; they have an absolute growth requirement for $Mg^{+2}$ that cannot be replaced by other ions (Webb, 1968 cited by Jasper and Silver, 1977).

Jasper and Silver (1977) reported that $Mg^{+2}$ is accumulated selectively by active transport across membranes; however, transport and homeostasis of Mg in prokaryotes remains poorly understand (Moncrief and Maguire, 1999). Three different transport systems are described for *Salmonella typhimurium* and *E. coli*: CorA, MgtA and MgtB and one regulatory system PhoP/PhoQ, which respond to extracellular $Mg^{+2}$ concentrations (Moncrief and Maguire, 1999; Chamnongpol and Groisman, 2002).

### 2.9. General Synthesis:

As mentioned in the introduction, the complex interaction among different nutrients (fatty acids, $Ca^{+2}$, structural carbohydrates) and bacteria determine a variable animal response. No conclusive information can explain why fiber digestion is higher when fats/oils are supplemented with a source of ionized calcium. The degradation of insoluble carbohydrate from cell walls is a subject of fundamental importance in ruminants, and amazing advances in the understanding of the process had been made; molecular biology without doubt had contributed to identifying the complex structures that participate in the attachment and fiber degradation by the different species of rumen cellulolytic bacteria; but the lack of information in other aspects related with the rumen bacteria function is evident. There is enough evidence concerning the participation of $Ca^{+2}$ in the different structures for cellulose attachment and its degradation by rumen cellulolytic bacteria. However, there is not available information that can demonstrate a specific requirement for function of rumen

cellulolytic bacteria, other than the information generated by Bryant et al (1959) for *B. succinogenes* S85 (actually *F. succinogenes*). The same is true for requirements for a numerous other mineral nutrients for bacterial growth. The results from *in vitro* and *vivo* experiments suggest a positive effect on cellulose or fiber degradation resulting from rumen cellulolytic bacteria increased calcium concentration in the media, but the experiments were made with a pool of rumen microorganisms, no species identification, and the calcium concentrations used were highly variable among experiments, and no ionized calcium was determined ($Ca^{+2}$).

Thus this study was undertaken with the purpose to explore the following hypotheses:

- *Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens*, predominant rumen cellulolytic bacteria, require ionized Ca for growth or cellulose degradation or both.
- *Fibrobacter succinogenes, Ruminococcus albus* and *R. flavefaciens*, predominant rumen cellulolytic bacteria, differ regarding their requirements of ionized Ca for growth or cellulose degradation or both.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1. Materials

3.1.1. Bacterial Species and Strains utilized:

*Fibrobacter succinogenes*, strains S85 (Bryant and Doetsch, 1954) and A3c (Dehority, 1963)

*Ruminococcus albus*, strains 7 (Bryant et al, 1958) and 8 (Hungate and Stack, 1982).

*Ruminococcus flavefaciens*, strains B34b (Dehority, 1963) and C94 (Bryant et al, 1958).

3.1.2. Media (Composition of all media utilized in the different experiments are described in Appendix A).

-Rumen-glucose-cellobiose-agar (RGCA) slants (Bryant and Burkey, 1953)

-Cellobiose complete liquid medium (Scott and Dehority, 1965)

-Cellulose complete liquid medium (modified from Scott and Dehority, 1965)

-Phosphate buffer

Media with varying concentrations of $Ca^{+2}$, Mg and other divalent cations were all prepared as modifications of the cellobiose and cellulose complete media.

## 3.2. General Procedure

Medium preparation, transfer of bacteria, inoculum preparation and inoculations were performed under a stream of $O_2$-free $CO_2$ (Hungate, 1950 modified by Dehority 1969).

Stock cultures: All cultures were obtained from RGCA slants stored at -60° C. Transfers were made to new slants, which were incubated at 39° C and transferred several times until the culture showed very active growth. In general, at the time that any strain was being used, it was transferred every day to a new slant. Those strains not in use were kept refrigerated at 5° C and transferred every 3 to 4 days.

Optical density (OD) was measured at 600 nm with a Spectronic® 20.

### 3.2.1. Protocol to determine ionized calcium requirements for growth in cellobiose liquid medium.

The different ionized Ca concentrations used were: 0, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mM for *F. succinogenes* and *R. albus*, whereas for *R. flavefaciens* 0, 0.02, 0.06, 0.09, 0.1 and 0.64 mM were used. Normal medium routinely used in the laboratory served as control (Scott and Dehority, 1965, Appendix A).

To prepare the pre-inoculum a complete cellobiose low $Ca^{+2}$ liquid medium (0.04 mM $Ca^{+2}$) was inoculated with a loop of bacteria from a RGCA slant. The culture was incubated overnight at 39° C, and the next morning 1 ml was transferred into a complete $Ca^{+2}$-free cellobiose liquid medium and incubated until it reached an OD between 0.6 and 0.7. Enough of this culture was added to cellobiose complete liquid $Ca^{+2}$-free medium to attain an OD of 0.1. Each experimental tube was then inoculated with 0.1 ml of the culture. Experimental tubes and non-inoculated tubes (blanks) were incubated at 39º C and after the lag period, the growth was monitored by OD at varying intervals.

The amount of 0.1 M $CaCl_2$ (Orion®) used to meet the different levels desired in the culture media were 0, 0.47, 0.93, 1.87, 3.73, 7.46 and 14.92 ml per liter of medium to obtain 0, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mM $Ca^{+2}$.

Table 3.1 lists the organisms, $Ca^{+2}$ concentrations, replicates and range of time over which the experiments were monitored.

| Species | Strain | $Ca^{+2}$ mM | | Range of monitoring time (hours) |
|---|---|---|---|---|
| *F. succinogenes* | A3c | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 24, 0 to 39 |
| *F. succinogenes* | S85 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 22, 0 to 36 |
| *R. albus* | 7 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 15, 0 to 13 |
| *R. albus* | 8 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 215, 0 to 219 |
| *R. flavefaciens* | B34b | 0, 0.04, 0.06, 0.08, 0.09, 0.1, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 24, 0 to 22 |
| *R. flavefaciens* | C94 | 0, 0.04, 0.06, 0.08, 0.09, 0.1, 0.64 and Control (0.36) | Duplicated, 1 replicate. | 0 to 47, 0 to 49 |

Table 3.1. Summary for growth experiments in cellobiose and different $Ca^{+2}$ concentrations.

### 3.2.2. Protocol to determine ionized calcium requirements for cellulose degradation.

The different ionized Ca concentrations used were: 0, 0.04, 0.06, 0.08, and 0.12 for *F. succinogenes* and 0, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 for *R. albus* and *R. flavefaciens*; The normal cellulose liquid medium routinely used in the laboratory served as control (Scott and Dehority, 1965).

Pre-inoculum, inoculum and inoculation of tubes were the same as previously described for $Ca^{+2}$ growth experiments.

Experimental tubes were incubated at 39º C. Five samples were taken at different times, i.e., 0 hour and 4 later times depending on extent of visible cellulose digestion (Table 3.2). *F. succinogenes* and *R. flavefaciens* strains were run in duplicate tubes (2 racks, each one containing a set of culture tubes with the various $Ca^{+2}$ concentrations, control and blanks), and the experiments were replicated 2 times. *R. albus* 7 and 8, were replicated 1 times. Blanks culture tubes, not-inoculated, were included and incubated.

Table 3.2 lists the organisms, $Ca^{+2}$ concentrations, replicates and sampling times for cellulose digestion.

| Species | Strain | $Ca^{+2}$ mM | | Sampling times (hours) |
|---|---|---|---|---|
| *F. succinogenes* | A3c | 0, 0.04, 0.06, 0.12, and Control (0.36) | Duplicated, 2 replicates | 0, 45, 55, 65, 80 |
| *F. succinogenes* | S85 | 0, 0.04, 0.06, 0.12, and Control (0.36) | Duplicated, 2 replicates | 0, 21, 30, 40, 58 |
| *R. albus* | 7 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicates | 0, 17, 27, 39, 54 |
| *R. albus* | 8 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 1 replicates | 0, 60, 132, 207, 275 |
| *R. flavefaciens* | B34b | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 2 replicates | 0, 21, 30, 40, 70, 142 |
| *R. flavefaciens* | C94 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and Control (0.36) | Duplicated, 2 replicates | 0, 30, 42, 66, 121 |

Table 3.2. Summary with cellulose degradation experiments and different $Ca^{+2}$ concentrations

### 3.2.3. Protocol to determine ionized calcium requirements for cellulose degradation in $NH_3$-free medium.

To eliminate the confunding growth effect on cellulose degradation a cellulose $NH_3$-free media was prepeared. Only *F. succinogenes*, strains S85 and A3c were used in this experiment, because these strains were the only one showing certain response to $Ca^{+2}$ concentrations in normal $NH_3$ media. The ionized Ca concentrations used were: 0, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mM.

The procedure to prepare the inoculum was similar to that for the $Ca^{+2}$ cellulose degradation experiments, but at least 8 culture tubes were grown in the low $Ca^{+2}$ liquid medium because a larger inoculum was used. The inoculum tubes were centrifuged at 900x*g* for 15 to 20 min, and the supernatant was decanted and discarded. Phosphate buffer was added to re-suspend the bacterial cells, the culture was mixed, OD determined and enough buffer was added to reach 0.7- 0.75 OD. One ml was used to inoculate each culture tube of $NH_3$-free cellulose media.

Experimental tubes were incubated at 39º C. A total of four samples were taken, at 0 hour and 3 later times depending on visual cellulose disappearance (Table 3.3). Each sample was taken in duplicate (2 racks, each containing a complete set of culture tubes with the different treatments, control and blanks). The experiments were replicated 1 time. Blanks (i.e., un-inoculated culture tubes) were included and incubated.

Table 3.3. lists the organisms, $Ca^{+2}$ concentrations, replicates and sampling times.

| Species | Strain | $Ca^{+2}$ (mM) | | Sampling times (hours) |
|---|---|---|---|---|
| *F. succinogenes* | A3c | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 | In duplicate, 1 replicate | R1: 0, 12, 24, 48<br>R2: 0, 18, 24, 48 |
| *F. succinogenes* | S85 | 0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 | In duplicate, 2 replicate | R1: 0, 12, 24, 48<br>R2: 0, 18, 24, 48 |

R1= Replicate 1, R2=Replicate 2

Table 3.3. Summary with $NH_3$-free cellulose degradation experiments and different $Ca^{+2}$ concentrations

### 3.2.4. Protocol to determine divalent-cation requirements for growth in cellobiose liquid medium.

*F. succinogenes*, strains S85 and A3c, *R. albus*, strain 7 , and *R. flavefaciens*, strain B34b were used in this experiment.

Using cellobiose as substrate, the treatments were complete media (Control), divalent-cation-free ($CaCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$, and $ZnSO_4$ were deleted), $Ca^{+2}$ and Mg free, $Ca^{+2}$-free, Mg-free, Fe-free, Mn-free, Zn-free, plus $Ca^{+2}$, plus Fe, plus Mg, plus Mn, plus Zn, plus $Ca^{+2}$ and Mg. Concentrations for each cation added were the same as those used by Scott and Dehority (1965).

The procedure was similar to other growth experiments. The inoculum preparation varied because the pre-inoculum was started with a loop full from a slant to a cellobiose liquid divalent-cation deficient medium (1/8 the concentration of normal medium), incubated overnight at 39° C and the next morning 1 ml was

transferred into complete cellobiose medium that was free of divalent cations and incubated until an OD between 0.6 to 0.7 was reached, depending on the strain involved.

The pre-inoculum (0.6 to 0.9 ml) was added into cellobiose complete liquid divalent-cation-free medium to attain 0.1 OD and from this 0.1 ml was used to inoculate each culture tube (containing 7 ml of liquid medium).

Experimental tubes were incubated at 39° C, and after the lag period the growth was monitored by optical density at 600 nm (Spectronic® 20). Readings were taken every 1 or 2 hours, depending on growth of the bacteria, until they reached maximum growth. Blank tubes (non-inoculated) were incubated simultaneously. Each experiment was run in duplicated and replicated 1 time.

### 3.2.5. Protocol to determine magnesium requirements for growth in cellobiose liquid medium.

The Mg concentrations evaluated were: 0, 0.05, 0.10, 0.20, and 0.82 mM. Normal medium routinely used in the laboratory served as Control (0.41 mM) (Scott and Dehority, 1965). Mg was supplied from $MgSO_4$.

Inoculum preparation was similar to the other experiments; however, in this experiment, cellobiose Mg-deficient liquid medium (0.05 mM Mg) was used to prepare the preinoculum and cellobiose Mg-free liquid medium to prepare the inoculum.

Incubation and monitoring of growth was similar to the other growth experiments. Each experiment was run in duplicated and replicated 1 time.

### 3.2.6. Protocol to determine ionized calcium and or magnesium requirements for cellulose degradation, $NH_3$-free cellulose medium.

*R. flavefaciens*, strains B34b and C94, were used in this experiment, with $Ca^{+2}$-free, Mg-free, and $Ca^{+2}$-Mg-free $NH_3$-free cellulose liquid medium. An $NH_3$-free cellulose liquid medium containing all divalent cations served as control.

The procedure to prepare the pre-inoculum was similar to the cellulose degradation experiment with *F. succinogenes* in $NH_3$-free medium, except $Ca^{+2}$, or Mg or $Ca^{+2}$ and Mg were the cations omitted.

Experimental tubes were incubated at 39º Cº. A total of four samples were taken, at 0 hour and 3 later times depending on visual cellulose disappearance. Each sample was taken in duplicate (2 racks, each one containing a complete set of culture tubes with the different treatments, control and blanks). The experiment was replicated 1 time. Blanks, culture tubes non-inoculated were included and incubated.

## 3.3. Analytical procedures:

**Cellulose residue:**

Following the procedure of Hiltner and Dehority (1983), the culture tubes with cellulose were centrifuged at ca. 900x*g* for 10 minutes, supernatant removed by suction, the residue was re-suspended in 5 ml of acid detergent fiber solution, tubes capped with aluminium caps, heated at 100° C in the autoclave for 60 minutes, the residue was washed twice with boiling deionized water; the suspension was transferred to a tared test tube, centrifuged, supernatant was removed by suction, and the tube was dried in an oven at 100° C until the weight was stable.

Weight of tube plus residue - weight of empty tube = cellulose residue.

cellulose residue at different sampling time (mg) - Cellulose residue of blank or 0 time (mg) - = mg degraded at each sampling time.

**Determination of calcium concentrations:**

Ionized Ca in the medium was supplied from $CaCl_2$ (0.1 M Calcium, Ionplus-Orion®), and different $Ca^{+2}$ concentrations in cellobiose and cellulose media were determined with an ion-meter (Accumet® AR25), using a specific calcium electrode (Thermo Orion®, Model 93-20) with a single junction reference electrode (Thermo Orion®, Model 90-01). To 30 ml of media, 0.6 ml of 4M KCl as an ionic strength adjustor (ISA) was added to determine $Ca^{+2}$ concentrations. Blanks and standards were treated in a similar manner.

Both cellobiose and cellulose media were analyzed to determine total calcium concentrations. The standard calcium solution and all samples were treated with 1N HCl; cellulose samples were lightly centrifuged to sediment the cellulose and an aliquot of supernatant was used for analysis; Standards and samples had $LaCl_3$ added to avoid interference from other minerals. Total Ca concentrations were determined by Atomic Absorption Spectrophotometer (Varian®) following standard analytical conditions for measuring calcium as established by the equipment manufacter.

**Determination of magnesium concentrations:**

Total content of Mg concentrations in cellobiose liquid media, used for Mg-requirement experiments were determined using an Atomic Absorption Spectrophotometer (Varian®); Samples and standard were treated with 1N HCl, similar to calcium samples; also, $LaCl_3$ was added to avoid interference with other minerals. Results confirmed the calculated concentrations (data not shown).

**Glassware**:

All the glassware used in the experiments was washed as normal for the laboratory. Subsequently it was soaked with 20% HCl for 24 hours and rinsed 3 times with deionized water. The objective of this treatment was to remove all possible mineral contamination.

### 3.4. Experimental design:

For growth and cellulose degradation $Ca^{+2}$ requirements, an incomplete factorial design was used, 2(3) x 6/8; factors were 2 strains whithin species (3), and 6 to 8 $Ca^{+2}$ levels.

The 2 strains of each species were run simultaneously, but each species was run at a different time; then the replications were considered as Block 1 and Block 2. Tube 1 and Tube 2 were the experimental units in growth experiments, because the OD was measured on the tube unit. Tube 1 and tube 2 were the experimental units for cellulose degradation, because at each sampling time one tube was taken from each of the duplicate racks.

For cellulose degradation in $NH_3$-free medium, a factorial 2x8 design was used, the two strains (A3c and S85) of *F. succinogenes*, were assigned to 8 different $Ca^{+2}$ concentrations. As in the other experiments, replications were considered as Block 1 and Block 2. Tube 1 and Tube 2 were the experimental units, because the cellulose degradation was measured in each.

For Mg growth requirements, a complete factorial design was used, 2(3)x6; factors were: strains (2) within species (3) and Mg concentrations (6).

The 2 strains of each species were run simultaneously, but each species was run at different times; then the replications were considered as Block 1 and Block 2. Tube 1 and Tube 2 were the experimental units, because the OD was measured on each tube.

For divalent-cation absolute requirements, an incomplete factorial design was used, 2(3)xn, where factors were strains (2) within species (3), but only 1 for *R. albus*), and n treatments. The experiment was run in duplicate and repeated twice. The experimental unit was the culture tube, because OD was measured for each.

The experimental design for ionized calcium and or magnesium absolute requirements for cellulose degradation in $NH_3$-free cellulose media for *R. flavefaciens* was a factorial design 2x4, 2 strains and 4 treatments. The experiment was run in duplicate and replicated 1 time.

### 3.5. Statistical analysis:

As general statement, statistical significances will be accepted at P≤0.05; and a tendency will be accepted when 0.05 < P ≤ 0.15.

### Growth experiments, response to different $Ca^{+2}$ and Mg concentrations:

Due to the form of the growth curve, a non-linear regression analysis was used; the data were evaluated using the model from Zwietering et al (1990) that describe a sigmoid curve similar to bacterial growth. The model is

$$OD = \frac{A}{1 + \exp^{4*B / A * (C - time) + 2}}$$

where, A, B and C parameters, respectively, represent:

A: the maximum growth, in this case maximum OD (units OD)

B: the rate of growth, (units OD/h)

C: lag time (hours)

using NLIN PROC from SAS. Due to the different number of observations and the different times when the OD were registered in each experiment, and the requirement from the analytical program, it was not possible use NLIN MIXED for the analysis.

All the species and strains evaluated, with the exception of *R. albus* 8 for $Ca^{+2}$ growth, fitted the Zwietering model (P<0.05).

Parameters A, B and C, and their respective standard errors for each tube were obtained; the parameters were weighted by the reciprocal of the standard error; these were analyzed by variance analysis (ANOVA) using PROC MIXED (SAS). Fixed effects were: $Ca^{+2}$ concentrations, species, strain within species; the interactions: strain within species * $Ca^{+2}$ concentrations; random effects were: block within species and tube within block. The extended and reduced models were compared; because these were different (P<0.05), the extended model was

used. The homogeneity of the variance was evaluated, and for $Ca^{+2}$ growth response A, B, C, parameters were analyzed with an unstructured variance test. Least squares means (LSM) were obtained and compared with linear and quadratic contrasts ( PROC MIXED, SAS).

To identify the requirement of $Ca^{+2}$ or Mg for growth, was used the maximum growth, rate of growth and lag time as criteria. When linear effect was obtained as statistically significant, no estimation of requirement can be made, because end point was not attained. When quadratic effect was obtained, non linear analysis was made to find the break point and the plateau of the function (PROC NLIN, SAS). When this method did not solve, maximum of the first derivative of the quadratic function (PROC REG, SAS) was used to estimate the requirements.

For Mg growth response an extended model with unstructured variance test was used for A and B, and a reduced model for C.

Data for RA-8 growth curve did not fit the logistic model, and in this case, the data were not included in the overall analysis. It was analyzed with linear regression to determine the rate of growth (PROC REG, SAS); then the “b” coefficients or slopes obtained were evaluated by ANOVA. If significant differences were found, the least means differences test was applied (GLM, SAS) to identify effects of $Ca^{+2}$ concentrations on rate of growth. Maximum growth obtained from raw data, were analyzed by ANOVA, and no differences were found ($P>0.05$).

Concepts related to enzyme function and substrate affinity were applied to the data, using the Lineweaver-Burk method of plotting enzyme kinetics (Price and Dwek, 1984). Assuming that the bacteria correspond to the “enzyme” and $Ca^{+2}$ the “substrate”, and rate of growth to the “velocity of the reaction”, this approach appears be useful as a tool to study the bacterial affinity for $Ca^{+2}$ under the experimental conditions of the study. The affinity constant (Ks) corresponds to the amount of substrate necessary to attain ½ of the μmax (maximum rate of growth). Using the reciprocal of the least squares means of rate of growth (1/B)

of each treatment (as Y) plotted vs. the reciprocal of $Ca^{+2}$ concentrations ($1/[Ca^{+2}]$) (as X), a linear function was estimated (PROC REG, SAS), thus 1/μmax (when X = 0) and -1/Ks (when Y = 0) were identified to obtain the μmax and Ks for each strain. Ranking the Ks values, it is possible to establish the tendency in the affinity of different strains for $Ca^{+2}$.

**Extended Statistical model used to study the effect of $Ca^{+2}$ or Mg on bacterial growth on cellobiose liquid media:**

$Y = [Ca^{+2}]$ + species + strain (species)+ block + tube(block) + $[Ca^{+2}]$*strain (species) + error

$Y_{ijklm} = C_i + Sp_j + St(sp)_{k:j} + B_{l:k} + T(B)_{m:l} + C*Sp_{ij} + C*St(sp)_{i\ k:j} + \varepsilon_{ijklm}$

Where: C ($[Ca^{+2}]$), i = 1, 2, 3, 4, 5, 6, 7, 8 or

C ([Mg]), i = 1, 2, 3, 4, 5, 6

Sp (Species), j = 1, 2, 3

St(sp) (Strain (species)), k = 1, 2

B (Block), l = 1, 2

T(B) (Tube(Block)), m = 1, 2

C*Sp, interaction $Ca^{+2}$ with species

C*St(sp), interaction $Ca^{+2}$ with Strain within Species

ε (Error), i,j,k,l,m ( 0, $\sigma^2_e$ )

**Cellulose degradation experiments:**

For cellulose degradation, where different tubes were sampled in different chronological times, the duplicate rack was the experimental unit. Two racks were incubated in each replicate. Each rack contained tubes to establish the basal cellulose content at time 0 for each treatment, and 4 tubes for each $Ca^{+2}$ concentration at each of the 4 sampling times. Control tubes, uninoculated tubes were incubated simultaneously.

The profile of the data from cellulose degradation was similar to a growth curve. Because the inoculum was small the bacteria multiplied using the energy derived from degrading the cellulose. The function to characterize and study the bacterial response to $Ca^{+2}$ concentrations on cellulose was also the logistic model (Zwietering et al, 1990). Other models were discarded because the substrate was only crystalline cellulose, whereby no rapid degradation or disappearance occurs. Functions used normally to study nutrient degradation in the rumen are first-order degradation kinetics Orskov and McDonald (1979) or the model which includes a lag time, Orskov and McDonald (1979), modified by Dhanoa (1988) (both cited by Nozziere and Michalet-Doreau, 2000 in their review), but the data did not fit these models.

Thus, a non-linear regression analysis was used; the data were studied using the model from Zwietering et al (1990) that describe a sigmoid curve. The model is the same used for bacterial growth, the A, B and C parameters, respectively, represent:

A: the maximum cellulose degradation, (mg cellulose)

B: the rate of cellulose degradation, (mg/h)

C: lag time (hours)

Also NLIN PROC from SAS was used for the analysis. Due to the different times when samples were taken because of the characteristic behaviors of the different bacterial species, and the requirement from the analytical program, it was not possible use NLIN MIXED for the analysis. All the species and strains evaluated fitted the Zwietering model ($P<0.05$), with the exception of *R. albus* 8.

Parameters A, B and C, and their respective standard errors, for each tube were obtained, parameters were weighted by the reciprocal of the standard error and analyzed by variance analysis (ANOVA) using PROC MIXED (SAS).

In the statistical model, $Ca^{+2}$ concentrations, species, strain within species; and the interaction: strain within species * $Ca^{+2}$ concentrations were considered as fixed effects; block and tube as random effects.

The extended model and the reduced model were compared. Since there were no differences (P>0.05), the reduced model was use for ANOVA analysis. LSM were compared by linear and quadratic contrasts (PROC MIXED, SAS). Quadratic functions were estimated with PROC REG (SAS). Break point was searched by maximum of the first derivate of quadratic function.

RA-8 did not fit the logistic model, in this case, linear regression was used, at least to establish the rate of degradation (PROC REG, SAS); then the slopes obtained were studied by ANOVA, but no significant differences were found (P>0.05) among $Ca^{+2}$ concentrations (GLM, SAS). The extent od degrdation obtained at the last sampling was used to evaluated maximum degradation, the data was analyzed by ANOVA and no differences were found (P>0.05).

The substrate affinity concept from Lineweaver-Burk method was also applied to the rate of cellulose degradation (Price and Dwek, 1984), as was made for growth experiments with $Ca^{+2}$ and Mg (PROC REG, SAS).

**Reduced model:**

$Y = [Ca^{+2}] + species + strain\ (species) + [Ca^{+2}]*strain\ (species) + error$

$Y_{ijkl} = C_i + Sp_j + St(sp)_{k:j} + C*Sp_{ij} + C*St(sp)_{i\ k:j} + \varepsilon_{ijkl}$

Where: C ($[Ca^{+2}]$), $i = 1, \ldots.10$

Sp (Species), $j = 1, 2, 3$

St(sp) (Strain (species)), $k = 1, 2$

C*Sp, interaction $Ca^{+2}$ with species

C*St(sp), interaction $Ca^{+2}$ with Strain within Species

ε (Error), $i,j,k,l\ (0, \sigma^2_e)$

**Statistical analysis for cellulose degradation in $NH_3$-free media with F. succinogenes:**

Similar to the other cellulose degradation experiments, the data were studied using the model from Zwietering et al (1990), using NLIN PROC from SAS. A, B and C parameters, respectively, represent:

A: the maximum cellulose degradation, (mg cellulose)

B: the rate of cellulose degradation, (mg/h)

C: lag time (hours)

Thus parameters A, B and C, and their respective standard errors, for each tube were obtained, these were analyzed similarly to the other cellulose experiment described above. The statistical model included as fixed effects: $Ca^{+2}$ concentrations, strain; the interaction strain * $Ca^{+2}$ concentrations and as random effects: block and tube. The extended model and the reduced model were compared; because no difference was found ($P>0.05$), the reduced model was use for ANOVA analysis. Then LSM were analyzed by linear and quadratic contrasts (PROC MIXED, SAS). Quadratic functions were estimated with PROC REG (SAS). Break point was searched by maximum of the first derivate of quadratic function.

In the case of FS-A3c NH3-free experiments the data from 0.02 mM $Ca^{+2}$ did not fit the logistic model or the linear regression. Then LSM of cellulose degraded per each sampling time were calculated and separated (DIFF option), to establish the existence of differences in degradation among sampling times.

The substrate affinity concept from Lineweaver-Burk method was also applied to the rate of cellulose degradation (Price and Dwek, 1984), as was described earlier for growth experiments with $Ca^{+2}$ and Mg and cellulose degradation with different $Ca^{+2}$ concentrations with normal N content.

**Reduced statistical model:**

$Y = [Ca^{+2}] + strain + [Ca^{+2}]*strain + error$

$Y_{ijkl} = C_i + St_j + B_k + T(B)_{l:k} + C*St_{ij} + \varepsilon_{ijkl}$

Where: C ($[Ca^{+2}]$), $i = 1, \ldots.7$

St (Strain, $k = 1, 2$

C*St, interaction $Ca^{+2}$ with Strain

ε (Error), $i,j,k,l,m\ (0, \sigma^2_e)$

**Statistical analysis of Ionized calcium and or magnesium requirements for cellulose degradation, $NH_3$-free cellulose media for *R. flavefaciens* experiments.**

In this experiment no degradation occurred with $Ca^{+2}$-Mg free and Mg-free and these treatmet were not included in the overall analysis; thus only the treatments with $Ca^{+2}$ + Mg (control) and $Ca^{+2}$-free, were analyzed; then the extent of degradation was estimated and the data for this parameter were analyzed by ANOVA, obtaining LSM per strain*cation treatment interaction (PROC GLM, SAS). No mean separation was made, because no differences were found between treatments within strain ($P>0.05$).

The statistical model is:

$Y = cation\ treatment + strain + strain*cation\ treatment + error$

$Y_{ijk} = T_i + St_j + St*T_{ij} + \varepsilon_{ijkl}$

Where: T (presence or absence of action), $i = 1, 2$

St (Strain, $j = 1, 2$

St*T (Strain*cation treatment interaction)

ε (Error), $i,j,k\ (0, \sigma^2_e)$

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1. Ionized calcium concentrations in cellobiose and cellulose media:

Ionized calcium concentrations in the media were regularly checked to confirm that calculated and actual levels were similar (Table 4.1). Later the media were randomly analyzed, either to verify the concentrations or when was necessary because experimental observations were markedly different than expected (Figure 4.1).

Total Ca concentrations were higher than ionized calcium, as was expected (Figure 4.2); Thus the regression $Ca^{+2}$ = 0.5012*(total Ca) - 0.0237 ($r^2$= 0.89) can be used to calculated $Ca^{+2}$ concentrations from total Ca, and about 47% of total Ca is ionized Ca.

| Calculated $Ca^{+2}$ (mM) | Measured $Ca^{+2}$ (mM)* | Measured total Ca (mM)** | Calculated total Ca (mM) | 0.1M $CaCl_2$ (ml added/ liter medium) |
|---|---|---|---|---|
| 0 | 0.0032-0.014 | 0.056 | 0 | 0 |
| 0.02 | 0.029 | 0.10 | 0.047 | 0.47 |
| 0.04 | 0.045 | 0.16 | 0.10 | 0.93 |
| 0.08 | 0.078 | 0.26 | 0.19 | 1.87 |
| 0.16 | 0.178 | 0.45 | 0.37 | 3.73 |
| 0.32 | 0.35 | 0.83 | 0.75 | 7.46 |
| 0.64 | 0.63 | 1.6 | 1.49 | 14.92 |
| Normal | 0.36 | 0.515 | 0.45 | *** |

* Measured with an ion-electrode

** Measured by atomic absorption spectrophotometry

***0.33 g $CaCl_2$/liter of mineral solution B (Appendix A)

Table 4.1. Ionized Ca concentration calculated and measured by ion-electrode, total Ca concentrations calculated and measured by atomic absorption spectrophotometry.

Possible explainations for the differences between total Ca and $Ca^{+2}$, even though the source of calcium was $CaCl_2$, which has 100% solubility in water (Hodgman et al, 1959), are related to dilution and the presence of elements in the anaerobic media that can precipitate or form complexes with Ca. These affect the electrode response to the free calcium in solution, i.e., to ionized calcium ($Ca^{+2}$). Calcium can form soluble complexes with hydroxide, bicarbonate, polyphosphates, citrate, tartrate and EDTA (ThermoOrion, 2001) and the extent of this association of calcium is pH dependent: greater at higher calcium concentrations and at higher pH values (pH:10-11) (ThermoOrion, 2001). NaOH is used to adjust the medium pH to 6.7 to 6.9, and since the pH is neutral, the tendency of hydroxide to form complexes with $Ca^{+2}$ can be eliminated. Also $Ca^{+2}$ can form relatively insoluble compound with oxalate, fluoride, phosphate and sulfate and their solubility is increased at lower pH (ThermoOrion, 2001). Many of these ions are present in the experimental anaerobic media (sulfate and phosphate), and can potentially bind $Ca^{+2}$. Due to the addition of a volatile fatty acids (VFA) mix to the medium, its initial pH is aproximate 3.5, for this reason, to avoid the possible complex formation at this pH, the $CaCl_2$ was added to the medium after pH was adjusted to 6.7 to 6.9 and after cysteine·HCl was added.

A background level of calcium in the medium was impossible to avoid, since almost all reagent grade minerals contain trace amounts of calcium (sodium carbonate, sodium chloride, magnesium sulfate). Also the vitamin Pantothenic acid, was only available as calcium pantothenate. In general the quality of water was very good, with a few exceptions that were detected and

identified easily. Desionized water was obtained from desionizer water equipment (Nanopure Diamond$^{TM}$), and quality was very similar to HPLC water normally used as a reference.

Temperature is a factor affecting the solubility of different compounds (Hodgman et al, 1959), and it is necessary to mention that the anaerobic media was sterilized at 120º C, which could affect the solubilization of different compounds and later the reconstitution of the different salts.

The pH can also affect the solubility of calcium salts in the media. As a result of the fermentation that occurs in the culture tube, VFA are produced and pH is decreased. For this reason, $Ca^{+2}$ concentrations were verified under lower pH attained by the addition of VFA (the same mix of VFA used to prepare the media). Different amounts of VFA were added, pH was determined and $Ca^{+2}$ measured, with no change in $Ca^{+2}$ observed. The pH was decreased to 4.0, lower than any culture condition, but still within the range of pH for normal function of the ion electrode (as recommended by the manufacter, ThermoOrion, 2001).

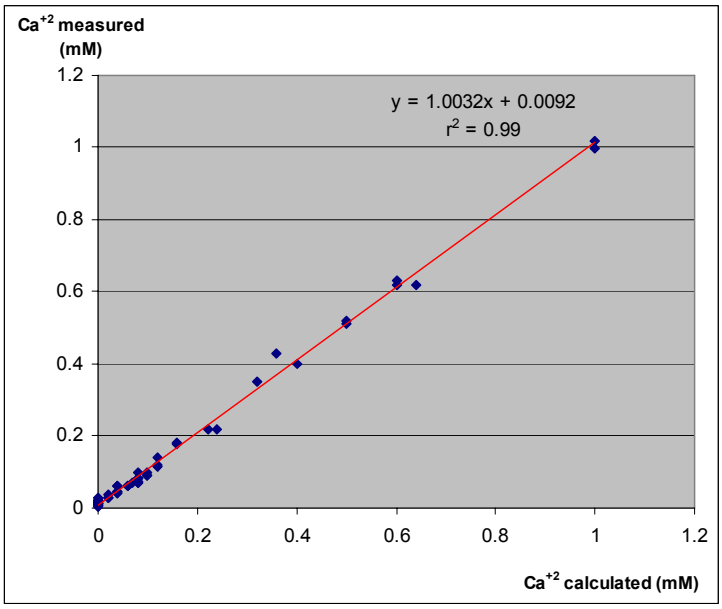


Figure 4. 1. $Ca^{+2}$ concentrations in anaerobic medium measured by ion-electrode vs. calculated (mM).

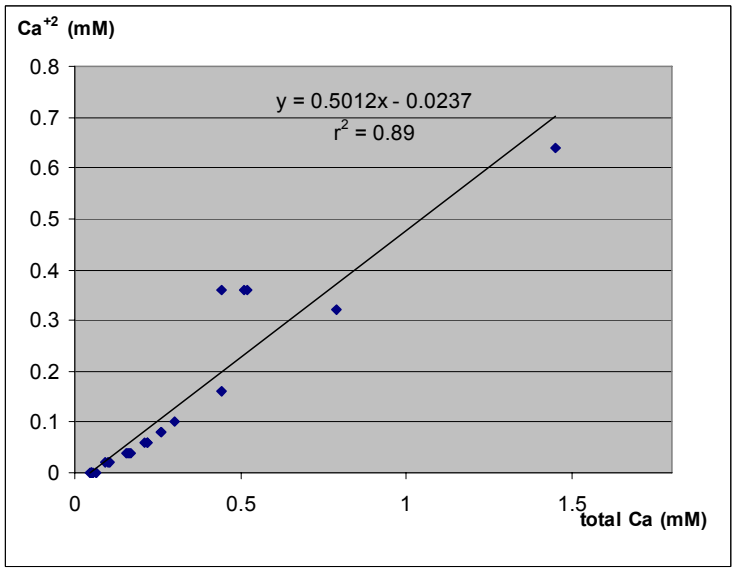


Figure 4. 2. $Ca^{+2}$ concentrations (measured with ion-electrode) vs. total Ca concentrations (measured with AAS).

It is necessary to keep in mind the sensitivity and the limitations of the determination of $Ca^{+2}$ i.e., range of $10^{-5}$ to $10^{-1}$ M $Ca^{+2}$ concentrations are needed for a linear response. In addition, as mentioned earlier, the proper conditions, such as temperature, pH, and lack of chemical interference from other ions, can influence the proper analysis (ThermoOrion, 2001). Thus the determinations of $Ca^{+2}$ from $Ca^{+2}$-free media, water, $Ca^{+2}$-free mineral solution B and mineral solution A can have more error than solutions or media with higher $Ca^{+2}$ concentrations.

**4.2. Growth of predominant rumen cellulolytic bacteria on cellobiose media with different $Ca^{+2}$ concentrations.**

In tables 4.2, 4.3 and 4.4 are presented the least squares means (LSM) of the parameters used to characterize the growth curves of the bacteria grown with different concentrations of $Ca^{+2}$. These curves shown in figures 4.3, 4.4, 4.5, and 4.7, are described by a logistic function with three parameters: maximum growth defined as maximum optical density (max OD), rate of growth (OD unit/hour) and lag time (hours). The statistical analysis by ANOVA identified as significant sources of variation the main effects of species, strains within species, $Ca^{+2}$ concentrations, and the interactions of species*concentrations, and concentrations*strain within species (all $P<0.05$) for max growth, rate of growth and lag time. LSM of different $Ca^{+2}$ concentrations were compared by linear and quadratic contrasts.

Maximum growth for *Fibrobacter succinogenes* strain A3c (FS-A3c) did not show neither linear or quadratic response to increased $Ca^{+2}$ concentrations (Table 4.2); rate of growth increased in a linear (P=0.0027) and quadratic (P=0.0075) manner to increased $Ca^{+2}$ concentrations; and lag time had a linear response (P=0.0084) to $Ca^{+2}$ concentrations, decreasing the length of the lag period when $Ca^{+2}$ increased (Table 4.2, Figure 4.3). Maximum growth of *F. succinogenes* strain S85 (FS-S85) showed a response increasing linearly (P=0.0012) and quadratically (P=0.0316) as $Ca^{+2}$ concentrations increased; and rate of growth increased linearly to increased $Ca^{+2}$ concentrations (Table 4.2, Figure 4.4). Neither a linear (P=0.1065) or quadratic (P=0.145) response to $Ca^{+2}$ concentrations for lag time was observed for FS-S85.

For FS-A3c, $Ca^{+2}$ requirements for growth cannot be estimated from the actual results; the linear effect on rate of growth and lag time does not permit reaching an end point, requiring the use of higher concentrations in future experiments. Rate of growth had a quadratic response, and in this case, $Ca^{+2}$ requirement was estimated as 0.43 mM, using the maximum of the first derivative of the quadratic function (P=0.0001, $r^2$=0.72). FS-S85 responded linear and quadratic to $Ca^{+2}$ concentrations for max growth; then from the quadratic response a requirement was estimated on 0.47 mM (using a quadratic function, P=0.0205; $r^2$=0.27). From rate of growth, no requirement can be estimated because the linear response observed. Similar to FS-A3c higher $Ca^{+2}$ concentrations will be needed in future research.

| | Max growth** | Rate of growth*** | Lag time**** |
|---|---|---|---|
| ***F. succinogenes* A3c** | | | |
| 0* | 1.43 ± 0.06 | 0.14 ± 0.018 | 17.2 ± 1.2 |
| 0.02 | 1.39 ± 0.04 | 0.15 ± 0.016 | 17.0 ± 1.1 |
| 0.08 | 1.37 ± 0.03 | 0.18 ± 0.016 | 15.0 ± 0.9 |
| 0.16 | 1.50 ± 0.04 | 0.18 ± 0.015 | 11.5 ± 0.9 |
| 0.32 | 1.40 ± 0.07 | 0.20 ± 0.025 | 17.4 ± 1.2 |
| 0.36 | 1.46 ± 0.03 | 0.20 ± 0.016 | 14.0 ± 0.9 |
| 0.64 | 1.51 ± 0.03 | 0.19 ± 0.016 | 11.1 ± 0.9 |
| Linear1 | 0.1608 | 0.0027 | 0.0084 |
| Quadratic | 0.7988 | 0.0075 | 0.5403 |
| ***F. succinogenes S*85** | | | |
| 0* | 1.27 ± 0.06 | 0.11 ± 0.017 | 14.3 ± 1.1 |
| 0.02 | 1.27 ± 0.06 | 0.11 ± 0.016 | 15.2 ± 1.3 |
| 0.08 | 1.26 ± 0.04 | 0.13 ± 0.016 | 14.4 ± 0.9 |
| 0.16 | 1.37 ± 0.05 | 0.14 ± 0.015 | 13.9 ± 0.9 |
| 0.32 | 1.44 ± 0.08 | 0.13 ± 0.021 | 16.9 ± 1.2 |
| 0.36 | 1.34 ± 0.03 | 0.15 ± 0.016 | 13.3 ± 0.9 |
| 0.64 | 1.37 ± 0.04 | 0.14 ± 0.016 | 13.5 ± 0.9 |
| Linear1 | 0.0012 | 0.0039 | 0.1065 |
| Quadratic | 0.0316 | 0.2221 | 0.1455 |

* $Ca^{+2}$, mM. ** OD units *** OD units/hour **** hours

**1**: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.2. Effect of $Ca^{+2}$ (mM) concentrations on growth of *Fibrobacter succinogenes* strains A3c and S85 in liquid media with cellobiose as source of carbohydrate.

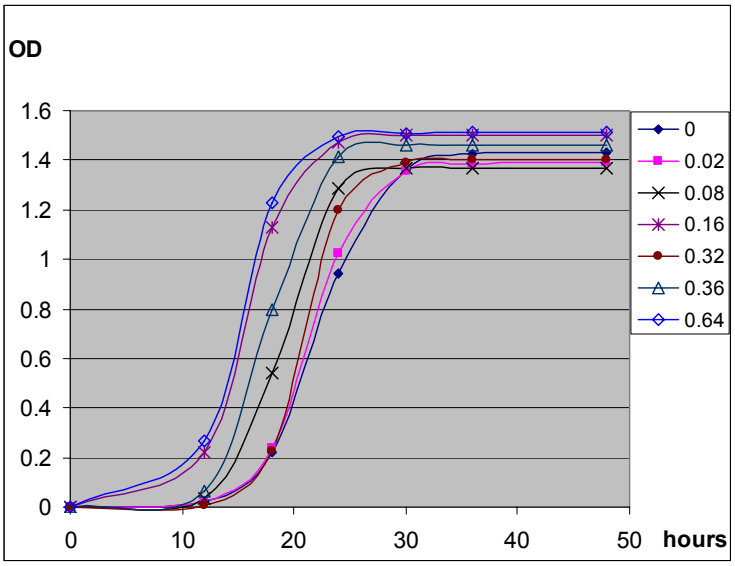


Figure 4.3. Predicted growth curves of *F. succinogenes* A3c with different $Ca^{+2}$ concentrations in cellobiose media, using the parameters obtained from the logistic function.

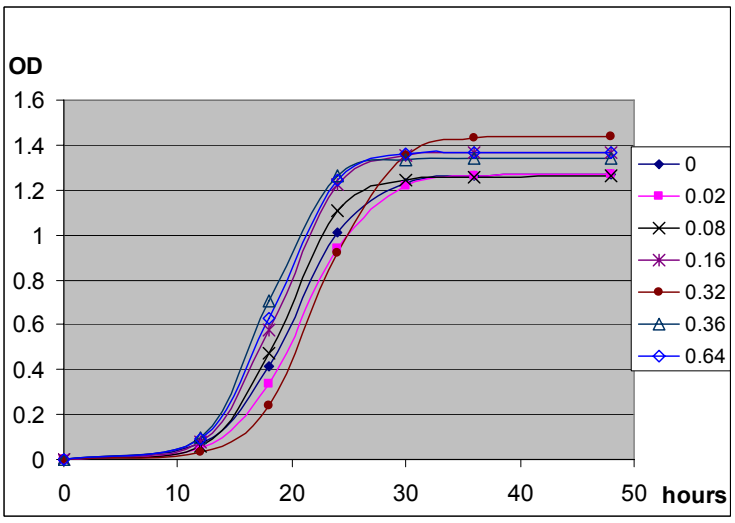


Figure 4.4. Predicted growth curves of *F. succinogenes* S85 with different $Ca^{+2}$ concentrations in cellobiose media, using the parameters obtained from the logistic function.

Results on the effect of $Ca^{+2}$ concentrations for *Ruminococcus albus* strains 7 (RA-7) and 8 (RA-8) are shown in Table 4.3. For RA-7, maximum growth did not show a linear or quadratic response to increased $Ca^{+2}$ concentrations; however, there was a tendency for a linear response (P=0.0864). Rate of growth of RA-7 had a tendency for a quadratic response (P=0.0565) to increased $Ca^{+2}$ concentrations, thus, RA-7 could reach the maximum rate of growth at 0.36 mM $Ca^{+2}$, according to the quadratic function estimation; but the quadratic equation was not significant (P=0.2131, $r^2$=0.12), then this estimation lack precision. In table 4.3, the $Ca^{+2}$ concentration of 0.36 mM shows the lower rate of growth. Lag times had a linear decrease with increasing $Ca^{+2}$ concentrations (P=0.0065). The lack of response from RA-7 to increased $Ca^{+2}$ concentrations is graphically shown in Figure 4.5, 4.8, and 4.9 where are shown the predictive growth curves from logistic model, the curves of maximum growth vs. $Ca^{+2}$ concentrations, and the curves of rate of growth vs. $Ca^{+2}$ concentrations, respectively.

RA-8 grew very slowly and did not reach a max OD higher than 0.56, the lowest among all the strains studied. In addition, the growth curve showed characteristics different from all other strains (Figure 4.6). This strain did not fit the logistic function; therefore it was evaluated separately. Values for RA-8 were adjusted to a linear function (P<0.05); the model and the slope were statistically significant (P<0.05), but the intercept was not (P>0.05). When the rate of growth represented by the slope of the linear functions, were compared among $Ca^{+2}$ concentrations, RA-8 showed the highest rate of growth at 0.32 and 0.36 mM

$Ca^{+2}$, with intermediary values for 0.08 and 0.64 mM $Ca^{+2}$, while bacteria growing with 0 and 0.02 mM $Ca^{+2}$ showed the lowest rate of growth. Max OD reported in table 4.3 corresponds to the observed values; lag time observed values had a length of approximated to 20-22 hours, and time to reach the max OD ranged 118 to 211 hours.

| | **Max growth**** | **Rate of growth***** | **Lag time****** |
|---|---|---|---|
| ***R. albus* 7** | | | |
| 0* | 1.21 ± 0.06 | 0.27 ± 0.021 | 7.5 ± 1.1 |
| 0.02 | 1.12 ± 0.04 | 0.25 ± 0.018 | 7.4 ± 1.2 |
| 0.08 | 1.11 ± 0.04 | 0.25 ± 0.016 | 7.1 ± 0.9 |
| 0.16 | 1.20 ± 0.06 | 0.26 ± 0.016 | 7.1 ± 0.9 |
| 0.32 | 1.24 ± 0.07 | 0.24 ± 0.028 | 7.1 ± 1.2 |
| 0.36 | 1.16 ± 0.03 | 0.23 ± 0.018 | 7.1 ± 0.9 |
| 0.64 | 1.23 ± 0.05 | 0.26 ± 0.017 | 6.7 ± 0.9 |
| | | | |
| Linear[1] | 0.0864 | 0.1501 | 0.0065 |
| Quadratic | 0.9869 | 0.0565 | 0.9654 |
| | | | |
| ***R. albus* 8** | | | |
| 0* | 0.46 (211)[2] | 0.0020 ± 0.00018 **a** | 20-22[3] |
| 0.02 | 0.48 (199) | 0.0022 ± 0.00014 **a** | |
| 0.08 | 0.51 (159) | 0.0031 ± 0.00023 **b** | |
| 0.16 | 0.50 (206) | 0.0023 ± 0.00019 **a** | |
| 0.32 | 0.55 (118) | 0.0044 ± 0.00029 **c** | |
| 0.36 | 0.55 (127) | 0.0045 ± 0.00032 **c** | |
| 0.64 | 0.53 (173) | 0.0029 ± 0.00024 **b** | |

* $Ca^{+2}$, mM. ** OD units *** OD units/hour **** hours

**1**: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented.

**2**: max OD observed and time to attain it (hours)

**3**: lag time observed (hours)

**a,b,c**: indicate differences (P<0.05) among $Ca^{+2}$ concentrations for *R. albus* 8

Table 4.3. Effect of $Ca^{+2}$ (mM) concentrations on growth of *Ruminococcus albus* strains 7 and 8 in liquid media with cellobiose as source of carbohydrate.

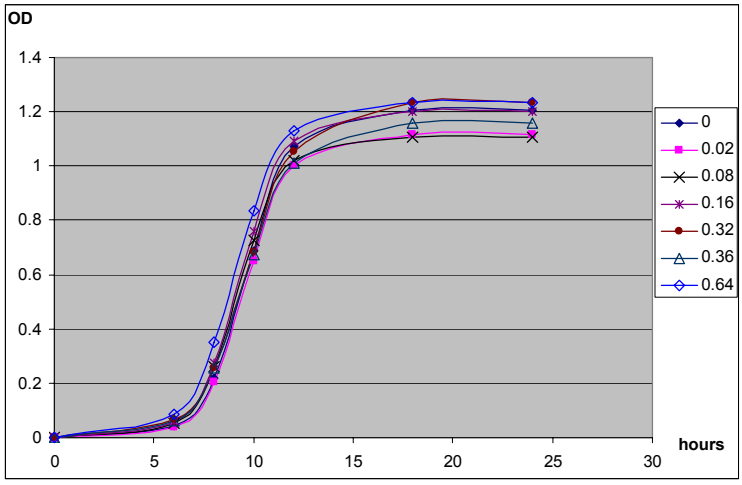


Figure 4.5. Predicted growth curves of *R. albus* 7 with different $Ca^{+2}$ concentrations in cellobiose media, using the parameters obtained from the logistic function.

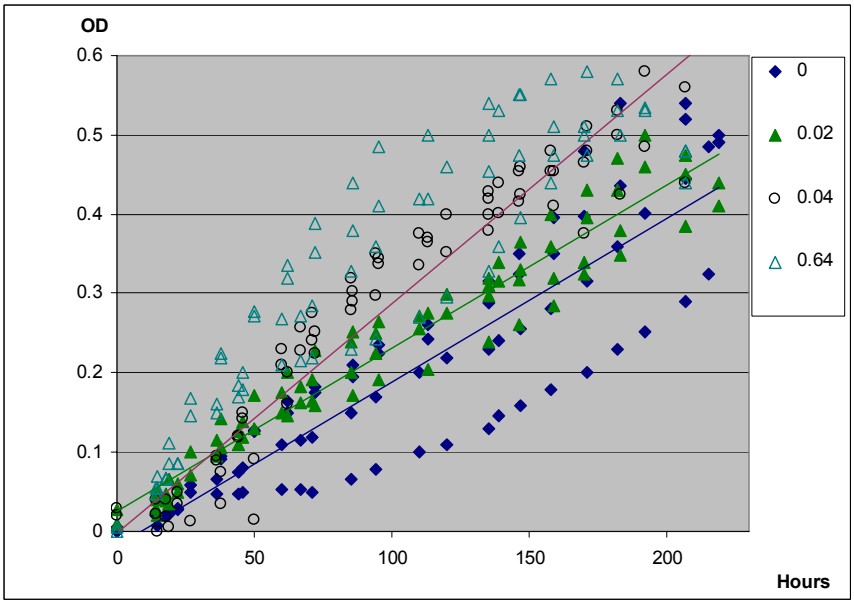


Figure 4.6. Growth of *R. albus* 8 with different $Ca^{+2}$ concentrations in cellobiose media (max OD vs. time, raw data).

*R. flavefaciens* strains B34b (RF-B34b) and C94 (RF-C94) did not respond to increased $Ca^{+2}$ concentrations, in maximum growth, rate of growth or lag time. Linear and quadratic contrast could not be calculated ($P>0.05$). The results are presented in Table 4.4., and Figure 4.7 for RF-C94 due to the particular high growth in $Ca^{+2}$-free. Consistently *R. flavefaciens* showed a maximum growth with 0 mM $Ca^{+2}$, and this response was confirmed after successive transfers from $Ca^{+2}$-free media to $Ca^{+2}$-free without affecting the growth response (Appendix B).

| | Max growth** | Rate of growth*** | Lag time**** |
|---|---|---|---|
| ***R. flavefaciens* B34b** | | | |
| 0* | 0.89 ± 0.06 | 0.082 ± 0.017 | 9.1 ± 1.3 |
| 0.02 | 0.88 ± 0.05 | 0.077 ± 0.016 | 7.3 ± 1.5 |
| 0.04 | 0.82 ± 0.19 | 0.083 ± 0.045 | 10.0 ± 1.2 |
| 0.06 | 0.86 ± 0.04 | 0.080 ± 0.017 | 8.0 ± 1.0 |
| 0.09 | 0.81 ± 0.04 | 0.122 ± 0.016 | 8.4 ± 1.0 |
| 0.10 | 0.88 ± 0.03 | 0.096 ± 0.016 | 7.4 ± 0.8 |
| 0.36 | 0.90 ± 0.03 | 0.106 ± 0.017 | 8.1 ± 1.0 |
| 0.64 | 0.85 ± 0.04 | 0.093 ± 0.016 | 6.8 ± 1.0 |
| Linear[1] | NS | NS | NS |
| Quadratic | NS | NS | NS |
| ***R. flavefaciens* C94** | | | |
| 0* | 0.86 ± 0.04 | 0.162 ± 0.018 | 18.9 ± 1.2 |
| 0.02 | 0.89 ± 0.05 | 0.033 ± 0.016 | 12.4 ± 1.7 |
| 0.04 | 0.63 ± 0.16 | 0.024 ± 0.027 | 12.2 ± 1.6 |
| 0.06 | 0.65 ± 0.04 | 0.018 ± 0.016 | 6.6 ± 1.2 |
| 0.09 | 0.69 ± 0.04 | 0.022 ± 0.015 | 5.3 ± 1.3 |
| 0.10 | 0.85 ± 0.04 | 0.023 ± 0.015 | 10.5 ± 0.9 |
| 0.36 | 0.82 ± 0.03 | 0.054 ± 0.016 | 10.7 ± 1.0 |
| 0.64 | 0.80 ± 0.05 | 0.020 ± 0.015 | 9.2 ± 1.1 |
| Linear[1] | NS | NS | NS |
| Quadratic | NS | NS | NS |

* $Ca^{+2}$, mM. ** OD units *** OD units/hour **** hours

1: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented. NS= no solution.

Table 4.4. Effect of $Ca^{+2}$ (mM) concentrations on growth of *Ruminococcus flavefaciens* strains B34b and C94 in liquid media with cellobiose as source of carbohydrate.

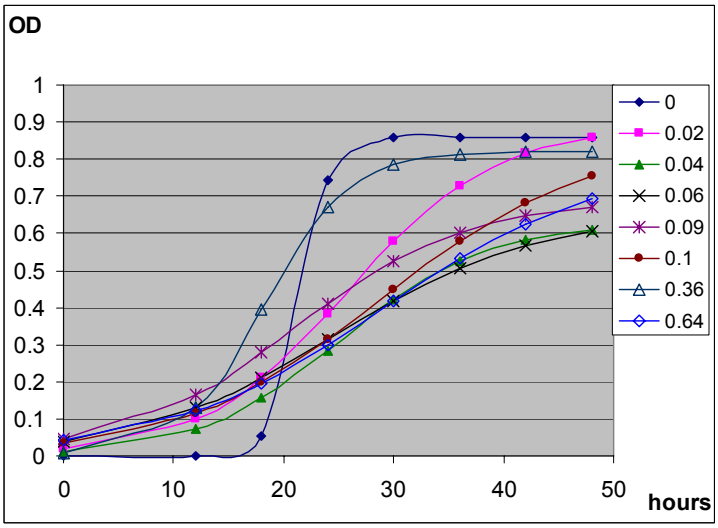


Figure 4.7. Predicted growth curves of *R. flavefaciens* C94 with different $Ca^{+2}$ concentrations in cellobiose media, using the parameters obtained from the logistic function.

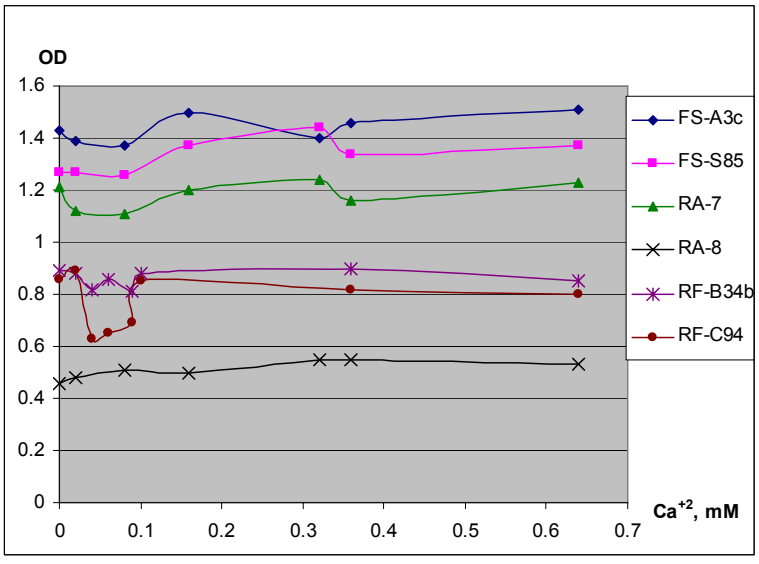


Figure 4.8. Curves of response to $Ca^{+2}$ concentrations on maximum growth (OD) by the different strains of rumen cellulolytic bacteria.

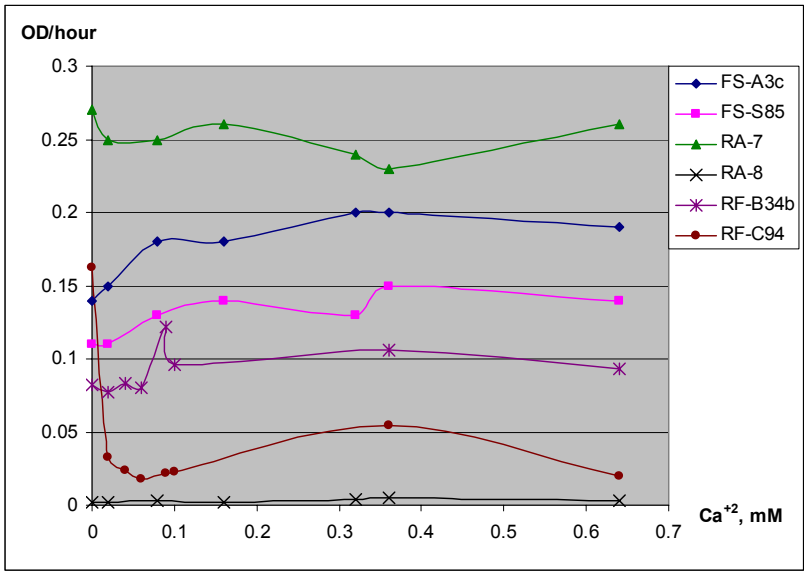


Figure 4.9. Curves of response to $Ca^{+2}$ concentrations on rate of growth (OD units/hour) by the different strains of rumen cellulolytic bacteria.

Considering the differences in response of the different species and strains to increasing $Ca^{+2}$ concentrations, it is important to evaluate the true mineral requirements of these bacteria. Previous results on mineral requirement reported by Bryant et al (1959) for FS-S85, have been considered to be universal for all the rumen bacteria. They defined the requirements of FS-S85 for several different minerals; among these, Ca and Mg, which were determined as a function of max OD. They observed an increase in max OD with increasing calcium concentrations. Similarly, the present results indicate a quadratic response for max OD by FS-S85. Bryan et al (1959) reported a requirement of

0.25 mM total Ca, which is lower than the requirement that was possible estimate at the present study as 0.47 mM $Ca^{+2}$, although this value may not very precise, because the low $r^2$ obtained (0.27). *R. albus* 7 showed a tendency for linear response and for *F. succinogenes* A3c was not possible to establish a requirement, due the lack of fit with different functions (linear, quadratic), because the oscillatory response to different $Ca^{+2}$ concentrations. Similarly for *R. flavefaciens*, under the actual conditions no $Ca^{+2}$ requirements for growth can be determined.

When FS-A3c attained a max growth near to 1.4 OD in $Ca^{+2}$-free media, this result was unexpected, due to the observations from earlier experiments where this bacterium did not growth in the $Ca^{+2}$-free medium. The cultures were examined microscopically and extremely pleomorphic cells were observed. On this basis the $Ca^{+2}$-free media used in this experiment was analyzed after the experiment was completed, and found to contain 0.019 mM $Ca^{+2}$. The other $Ca^{+2}$ media also had higher concentrations than the estimated, probably because of water contamination since the increase in concentration was similar.

In many experiments conducted during this study (not all reported), with different objectives, (pre-experimental tests, response to different divalent cations, consecutive bacterial transfers, etc), FS-A3c did not always grow in $Ca^{+2}$-free media (only 3 from a total 14 experiments). In those experiments where FS-A3c did not grow $Ca^{+2}$ concentrations were determined as: 0.0138 mM for $Ca^{+2}$, and 0.053 mM for total Ca. When a consecutive bacterial transfer experiment was performed, transferring all the strains, from $Ca^{+2}$-free to $Ca^{+2}$-

free media, when $Ca^{+2}$ was 0.014 mM and total Ca was 0.046 mM, FS-A3c did not grow (Appendix B). However, when $Ca^{+2}$ concentrations, from $Ca^{+2}$-free media, were 0.02 mM, FS-A3c grew normally until the $7^{th}$ - $8^{th}$ transfers, when growth ceased. Obviously, this information confirms the small $Ca^{+2}$ requirement for FS-A3c. FS-S85, as well as all other organisms in this study always grew under all the conditions described above. (Appendix B).

Using the Lineweaver-Burk method of plotting enzyme kinetic data (Price and Dwek, 1984) to find the bacterial affinity for $Ca^{+2}$ the reciprocal of the least squares means of rate of growth of each treatment (Y) was plotted vs. the reciprocal of $Ca^{+2}$ concentrations (X), and a linear function was calculated. Only data for FS-A3c and FS-S85 fit the linear functiona (Table 4.5). Then μmax (maximum rate of growth) and Ks for each strain were calculated: 0.20 unit OD/h and 0.0057 mM $Ca^{+2}$, respectively, for FS-A3c, and 0.15 unit OD/h and 0.005 mM $Ca^{+2}$, respectively, for FS-S85 ($P<0.05$). For all the other strains, no relationship between rate of growth with $Ca^{+2}$ concentrations was found ($P>0.05$).

| Strain | Linear function | | P = | $r^2$ | μmax | Ks |
|---|---|---|---|---|---|---|
| | Intercept | Slope | | | (OD/h) | (mM) |
| **FS-A3c** | 5.11±0.09 | 0.029±0.004 | 0.003 | 0.92 | 0.20 | 0.0057 |
| **FS-S85** | 7.04±0.22 | 0.036±0.01 | 0.03 | 0.75 | 0.15 | 0.005 |

Table 4.5. Linear regression parameters from reciprocal of rate of growth vs. reciprocal of $Ca^{+2}$ concentrations, and μmax and Ks coefficients derivated from the linear function.

Thus, it can be concluded that FS-S85 and FS-A3c, Gram (-) bacteria, have a specific affinity with $Ca^{+2}$, for growth process, and this is very low (0.005 and 0.0057 mM $Ca^{+2}$). These concentrations may not be possible to attain under our experimental conditions. *R. albus* strains 7 and 8 and *R. flavefaciens* strains B34b and C94, (Gram (+)) did not show any substrate or $Ca^{+2}$ affinity.

Bryant et al, (1959) demonstrated the essentiality of Ca for bacterial growth in *B. succinogenes* S85, (later *B. succinogenes* was reclassified as *F. succinogenes*) reporting that 0.05 mg /5 ml of media (0.25 mM Ca) was optimum for growth. The authors did not confirm their Ca concentrations by mineral analysis, assuming that the concentration was the amount added to the medium. The present data suggest that the calcium requirements, for FS-S85 and FS-A3c

maybe higher than this concentration, and requirements for *R. albus* 7 may be higher also; whereas no requirement could be demonstrated for *R. flavefaciens*. Other studies that evaluated mineral requirements for bacteria from the *Bacteroides* genus had shown different response to Ca concentrations. Thus, Caldwell et al (1973) found no effect of Ca on growth of *B. amylophilus* (now *Ruminobacter amylophilus)*, and Caldwell and Arcand (1974) observed no effect on *B. ruminicola* (now *Prevotella ruminicola)*, but *B. fragilis* 2044 and *B. oralis* J1 were sensitive to the deletion of Ca from the growth media. The addition of Ca (28 uM) to a cation-depleted media did not reestablish the normal growth of *B. ruminicola* subsp. *ruminicola* B18, *B. fragilis* subsp. *vulgatus* 8483 and *B. succinogenes* S85; but if Mg (10uM) was also added to the media, normal growth was observed. These results suggest differences among species in the requirement for Ca. (Caldwell and Arcand, 1974) confirming the different response from different strains to increased $Ca^{+2}$ concentrations; although due to the linear response it is not possible to determine the different requirements.

For a long time it was considered that $Ca^{+2}$ did not play a metabolic role in microbial metabolism (Silver, 1977) and that its function was relegated to the outer (cell wall) environment of the bacteria. Durand and Kawashima (1980) mention in their review that Ca is involved in the synthesis and stability of the cell walls, and for this reason Gram (+) bacteria may have higher Ca and Mg requirements for cell wall synthesis than Gram (-) bacteria. However, no clear results were observed in the present study, where *F. succinogenes* (Gram (-)) and *R. albus* (Gram (+)) responded to $Ca^{+2}$ concentrations, whereas *R.*

*flavefaciens* (Gram (+)) did not, at least not in the same manner as the former did. However, Beveridge et al (1982) showed that Gram (-) bacteria have lower capacity to bind $Ca^{+2}$ in the cell envelope, and thus they have a lower reservoir of $Ca^{+2}$ than Gram (+) bacteria. This could explain why Gram (-) bacteria are more sensitive to $Ca^{+2}$ concentrations in the media. The different response from *R. albus* and *R. flavefaciens* may be due to the cell wall composition of these bacteria and their different capacity to bind different ions, such as calcium (Beveridge, 1990, and Beveridge et al, 1985, 1986).

There is current evidence that $Ca^{+2}$ acts as a signal under certain environmental conditions that subsequently modify the internal concentration of $Ca^{+2}$ (Jones et al, 1999). Thus, $Ca^{+2}$ can mediate the bacterial response as a consequence of changes in osmotic or thermic conditions that drive different processes such as sporulation, encapsulation, motility and reproduction (Ordal, 1977; Silver, 1977; Rampersaud et al, 1991; Tisa and Adler, 1995; Herbaud et al, 1998). It has been demonstrated that *E. coli*, a Gram (-) bacteria, requires $Ca^{+2}$ to signal initiation of the reproduction process, activating the expression of a specific protein, FtsZ, that signals the equator for cell division and allows division to occur (Yu and Margolin, 1997; Onoda et al, 2000). For other bacteria, such as *Bacillus subtilis* (Herbaud et al, 1998), the presence of $Ca^{+2}$, affects both lag time and activation of the reproduction process. When $Ca^{+2}$ was present in the media, the bacteria grew normally, whereas, in the absence of $Ca^{+2}$, no growth was observed.

*E. coli* and *F. succinogenes* share the characteristics of being Gram (-) bacteria and perhaps the evidence available for *E. coli* is a key to explain the observed $Ca^{+2}$ response by *F. succinogenes*. As was mentioned earlier, in several experiments, FS-A3c grew in $Ca^{+2}$-free medium. When the culture was examined microscopically, (with the phase contrast microscope, immersion oil objective) marked pleomorphism was observed. This medium was later analyzed for $Ca^{+2}$ and found to contain 0.019 mM $Ca^{+2}$. This observation was found repeatedly when FS-A3c grew in low $Ca^{+2}$ concentrations and is consistent with concepts discussed above. Durand and Kawashima (1980) reported that Ca deficiency in bacteria "can cause growth defects and the formation of pleomorphic forms", without any reference to the type, genus or species of bacteria. When $Ca^{+2}$ is deficient or absent, pleomorphism can be the result of the abnormal function of the FtsZ protein that is responsible for the polymerization of the enzoskeleton, which in turn signals the equator to unsergo cell division. In the absence of $Ca^{+2}$, *E. coli* can grow but does not divide and forms long multinucleated filaments (Yu and Margolin, 1997; Onoda et al, 2000). Onoda et al (2000) reported that 1.2 mM of $Ca^{+2}$ was necessary to reestablish normal growth.

### 4.3. Cations required for bacterial growth with specific attention to magnesium requirements.

Since *R. flavefaciens* did not require $Ca^{+2}$ at a concentration above the background in the basal medium and sequential transferring from $Ca^{+2}$-free media to $Ca^{+2}$-free media did not decrease max OD, the absolute requirements

for other specific divalent cations (Fe, Mg, Mn and Zn) were investigated. Both strains of *R. flavefaciens* had an absolute requirement for Mg (Table 4.6.), since any combinations of divalent cations without Mg, did not support growth. *R. albus* 7 had a decrease in max growth when Mg was not present in the media, alltough it was able to growth in divalent cation free media (morphological changes were observed under the microscope i.e., agglutination of cells and the loss of coccus form). *F. succinogenes* A3c did not grow when $Ca^{+2}$ was omitted from the different combinations of divalent cation; however, the absence of either Mg, Zn, Fe, or Mn permitted a normal growth. In contrast, FS-S85 was not affected by the individual absence of $Ca^{+2}$ or any divalent cation tested, but did not grow or only grew very slowly when $Ca^{+2}$ and Mg were both omitted from the medium.

| Medium | Organism | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FS-A3c | | FS-S85 | | RF-B34b | | RA-7 | |
| Normal | N | N | N | N | N | N | N | N |
| Divalent cation free | - | - | - | - | - | - | L | L |
| $Ca^{+2}$-Mg free | -/L | - | -/L | -/L | - | - | L | L |
| $Ca^{+2}$-free | - | - | N | N | N | N | N | N |
| Mg-free | -/L | L | L | L | - | - | L | L |
| Zn-free | N | N | N | N | N | N | N | N |
| Mn-free | -/N | N | N | N | | | | |
| Fe-free | N | N | N | N | L | L | | |
| $+Ca^{+2}$ | - | L | N | N | - | - | L | L |
| +Mg | L/- | -/N | -/L | L | L | L | N | N |
| +Zn | - | - | - | - | - | - | L | L |
| +Mn | - | - | - | - | - | - | L | L |
| +Fe | - | - | - | - | | | L | L |
| $+Ca^{+2}$+Mg | N | N | N | N | L | L | N | N |
| $+Ca^{+2+}$Zn | | | | | - | - | L | L |
| $+Ca^{+2}$+Mn | | | | | - | - | L | L |
| $+Ca^{+2}$+Fe | | | | | - | - | L | L |
| +Mg+Mn | | | | | L | L | N | N |
| +Mg+Zn | | | | | L | L | N | N |
| +Mg+Fe | | | | | L | L | L | L |
| +Zn+Mn | | | | | - | - | L | L |
| L+Zn+Fe | | | | | - | - | L | L |
| $+Ca^{+2}$+Mg+Zn | | | | | L | L | N | N |

(-) : negative, no growth;
N: normal growth and L: lower growth than N (75% or lower than max OD of N). Each column represents an experiment in duplicate, when a different response was observed between duplicates the response for each one is indicated (i.e., -/N or -/L).

Table 4.6. Growth response to divalent cations in cellobiose liquid medium by predominant rumen cellulolytic bacteria.

Since all the bacterial species used in the $Ca^{+2}$ experiments were affected by omission of Mg from the medium their requirement for Mg was investigated. Treatments evaluated only total concentration of Mg, because no methods were available to measure ionized Mg concentrations.

Growth, measured as OD, was characterized by a logistic function, which is similar to that for the results from $Ca^{+2}$ treatments. The statistical analysis of the parameters of the growth curves showed significant main effects for species, strain within species, Mg concentrations and for the interactions: species*concentration and strain within species*concentrations ($P< 0.05$). Since RF-B34b and RF-C94 did not grow when they were cultured in Mg-free media, the data from 0 Mg concentration was not included in the statistical analysis.

As can be seen in Table 4.7 and Figure 4.10 and 4.11, neither RF-B34b or RF-C94 grew in Mg-free medium. Maximum growth for RF-B34b responded to both linear and quadratic contrasts ($P= 0.0009$ and 0.0138, respectively), and RF-C94 responded linearly increasing max OD ($P=0.0010$) to increased Mg concentrations. Rate of growth differed with Mg concentrations; RF-B34b responded to quadratic contrast (0.0020) whereas RF-C94 had a linear response (0.0532). Lag time of *R. flavefaciens* was sensitive to Mg concentrations, thus longer lag times were observed for both strains at lower Mg concentrations, showing a linear and quadratic response ($P<0.05$). Thus, using the maximum of the quadratic function (Appendix C), Mg requirements can be estimated for RF-B34b as ~0.5 mM of Mg, considering max growth/max OD or rate of growth as

parameters. RF-C94 showed a increased linear response to increased Mg concentrations, therefore requirement could not be determined because an end point or plateau was not attained in the present study.

| | Max growth** | Rate of growth*** | Lag time**** |
|---|---|---|---|
| ***R. flavefaciens* B34b** | | | |
| 0* | No growth | | |
| 0.05 | 0.50 ± 0.07 | 0.045 ± 0.002 | 16.5 ± 1.00 |
| 0.1 | 0.62 ± 0.03 | 0.073 ± 0.005 | 12.4 ± 0.75 |
| 0.2 | 0.76 ± 0.04 | 0.099 ± 0.006 | 10.4 ± 0.68 |
| 0.41 | 0.88 ± 0.05 | 0.085 ± 0.006 | 11.4 ± 0.67 |
| 0.82 | 0.80 ± 0.04 | 0.089 ± 0.009 | 9.3 ± 0.71 |
| | | | |
| Linear[1] | 0.0009 | 0.4341 | 0.0004 |
| Quadratic | 0.0138 | 0.0020 | 0.0030 |
| | | | |
| ***R. flavefaciens* C94** | | | |
| 0* | No growth | | |
| 0.05 | 0.25 ± 0.03 | 0.011 ± 0.001 | 25.0 ± 1.45 |
| 0.1 | 0.51 ± 0.04 | 0.025 ± 0.003 | 15.4 ± 0.93 |
| 0.2 | 0.52 ± 0.03 | 0.024 ± 0.003 | 12.8 ± 0.98 |
| 0.41 | 0.53 ± 0.03 | 0.028 ± 0.004 | 10.2 ± 1.02 |
| 0.82 | 0.62 ± 0.03 | 0.029 ± 0.004 | 11.6 ± 0.92 |
| | | | |
| Linear[1] | 0.0010 | 0.0532 | 0.0014 |
| Quadratic | 0.1631 | 0.1403 | 0.0042 |

*Mg, mM. ** OD units *** OD units/hour **** hours
[1]: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.7. Growth of *Ruminococcus flavefaciens* strains B34b and C94 with different concentrations of Mg in liquid media.

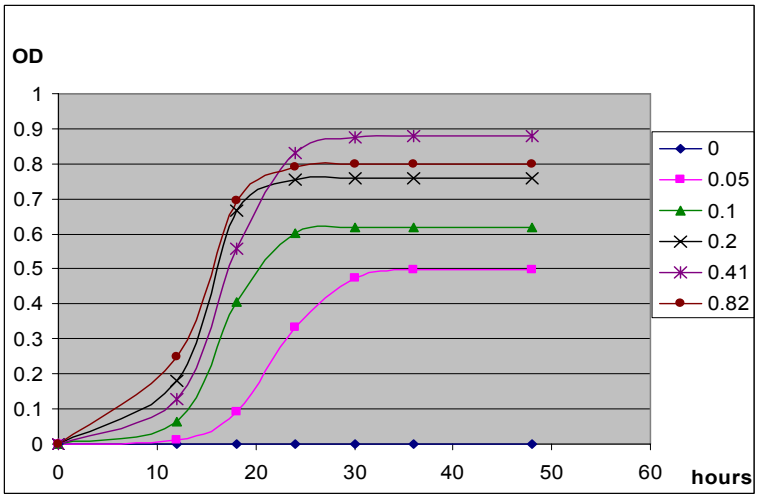


Figure 4.10. Predicted growth curves of *R. flavefaciens* B34b with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

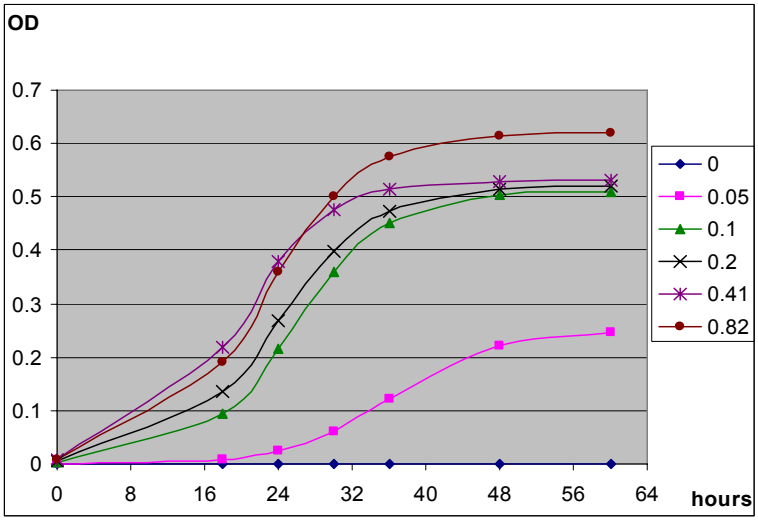


Figure 4.11. Predicted growth curves of *R. flavefaciens* C94 with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

Maximum growth of *R. albus* 7 was reduced when Mg was not present in the media (Table 4.8. Figure 4.12). This strain was also able to growth in divalent cation free media (although morphological changes were observed). Max growth for *R. albus* 7 responded linearly (P=0.0568) to increasing Mg concentrations, with a tendency for a quadratic response (P=0.0744). No effect on rate of growth or lag time was observed for RA-7 with increasing Mg concentrations (linear and quadratic contrasts were not solved).

*R. albus* 8 (Table 4.8; Figure 4.13), showed a quadratic response (P=0.0154) for max growth (max OD) when Mg concentrations increased, estimating the Mg requirement for maximum growth as 0.5 mM Mg (using the maximum of the quadratic function, appendix C). Data for RA-8 for rate of growth and lag time did not show structure for either a linear or quadratic contrasts.

Then, RA-7 showed, apparently, to have higher Mg requirements for maximum growth than RA-8 (estimated as 0.5 mM Mg). Thus, differences between the two *R. albus* strains for Mg requirements are evident from the present results.

| | Max growth** | Rate of growth*** | Lag time**** |
|---|---|---|---|
| ***R. albus* 7** | | | |
| 0* | 0.80 ± 0.06 | 0.16 ± 0.027 | 6.5 ± 0.83 |
| 0.05 | 1.05 ± 0.06 | 0.22 ± 0.005 | 6.9 ± 0.72 |
| 0.1 | 1.19 ± 0.06 | 0.24 ± 0.010 | 7.1 ± 0.68 |
| 0.2 | 1.21 ± 0.06 | 0.25 ± 0.012 | 6.8 ± 0.68 |
| 0.41 | 1.19 ± 0.06 | 0.24 ± 0.014 | 7.0 ± 0.68 |
| 0.82 | 1.19 ± 0.04 | 0.28 ± 0.019 | 6.8 ± 0.77 |
| | | | |
| Linear[1] | 0.0568 | NS | NS |
| Quadratic | 0.0744 | NS | NS |
| | | | |
| ***R. albus* 8** | | | |
| 0* | 0.53 ± 0.04 | 0.003 ± 0.002 | 12.5 ± 2.44 |
| 0.05 | 0.47 ± 0.04 | 0.004 ± 0.001 | 15.5 ± 2.41 |
| 0.1 | 0.49 ± 0.04 | 0.004 ± 0.001 | 13.8 ± 2.27 |
| 0.2 | 0.58 ± 0.04 | 0.005 ± 0.001 | 23.0 ± 2.03 |
| 0.41 | 0.59 ± 0.03 | 0.006 ± 0.002 | 15.5 ± 1.95 |
| 0.82 | 0.55 ± 0.04 | 0.005 ± 0.002 | 36.1 ± 2.03 |
| | | | |
| Linear[1] | 0.1588 | NS | NS |
| Quadratic | 0.0154 | NS | NS |

*Mg, mM. ** OD units *** OD units/hour **** hours

[1]: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.8. Growth of *Ruminococcus albus* strains 7 and 8 with different concentrations of Mg in liquid media

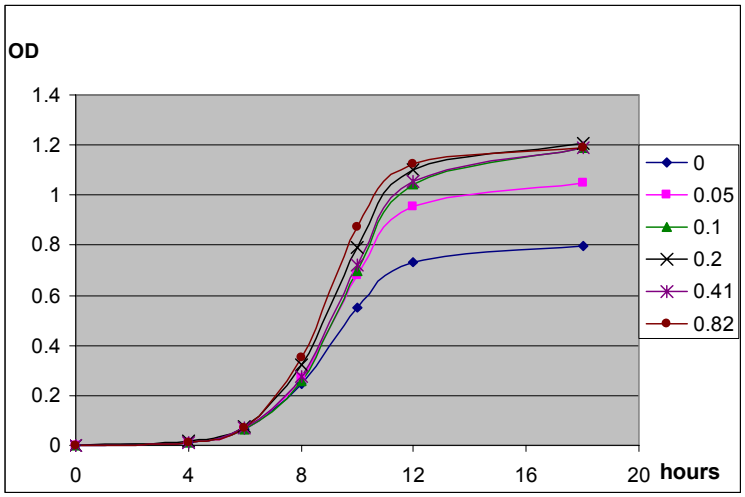


Figure 4.12. Predicted growth curves of *R. albus* 7 with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

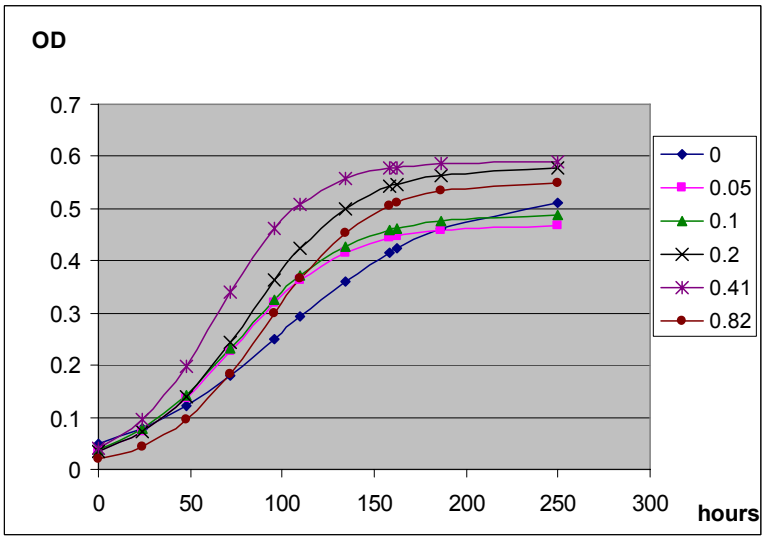


Figure 4.13. Predicted growth curves of *R. albus* 8 with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

Both strains of *F. succinogenes* responded in max growth to increased Mg concentrations (Table 4.9., Figures 4.14 and 4.15). FS-A3c had a positive linear response ($P=0.0366$) to increased Mg concentrations, whereas FS-S85 showed, both, increasing max growth in linear and quadratic way ($P<0.0001$, respectively) to increased Mg concentrations. Thus, for both strains the lower maximum growth was obtained with the lower Mg concentration. FS-A3c also had a positive linear response to Mg concentrations for rate of growth ($P=0.0027$) and only a tendency to decrease the lag time linearly ($P=0.0725$) as Mg concentration increased. The lower rate of growth and the longer lag time was observed with 0 Mg for FS-A3c. FS-S85 did not response to increased Mg concentrations, neither linearly or quadratic, for rate of growth and lag time. Thus, Mg requirement for FS-A3c cannot be defined from the data available and the method used for it estimation (Figure 4.16 and 4.17). For FS-S85, Mg requirements for maximum growth can be estimated as 0.56 mM of Mg from the quadratic function (Appendix C). Apparently, FS-A3c has a higher requirement for Mg than FS-S85, considering that FS-A3c had a linear response to Mg concentrations. The requirement for FS-S85 attained in this study is higher than that reported by Bryant et al (1959) for the same strain (0.1 mM Mg).

| | Max growth** | Rate of growth*** | Lag time**** |
|---|---|---|---|
| ***F. succinogenes* A3c** | | | |
| 0* | 0.99 ± 0.14 | 0.12 ± 0.026 | 15.3 ± 1.11 |
| 0.05 | 1.43 ± 0.11 | 0.17 ± 0.007 | 11.5 ± 1.29 |
| 0.1 | 1.54 ± 0.06 | 0.18 ± 0.006 | 12.4 ± 0.64 |
| 0.2 | 1.54 ± 0.07 | 0.18 ± 0.009 | 12.0 ± 0.73 |
| 0.41 | 1.53 ± 0.05 | 0.20 ± 0.010 | 11.9 ± 0.62 |
| 0.82 | 1.63 ± 0.07 | 0.17 ± 0.010 | 12.7 ± 0.63 |
| | | | |
| Linear[1] | 0.0366 | 0.0027 | 0.0725 |
| Quadratic | NS | NS | NS |
| | | | |
| ***F. succinogenes* S85** | | | |
| 0* | 0.86 ± 0.06 | 0.05 ± 0.017 | 17.1 ± 0.94 |
| 0.05 | 1.06 ± 0.05 | 0.14 ± 0.004 | 15.3 ± 0.77 |
| 0.1 | 1.24 ± 0.05 | 0.14 ± 0.007 | 14.8 ± 0.75 |
| 0.2 | 1.34 ± 0.05 | 0.15 ± 0.008 | 14.7 ± 0.72 |
| 0.41 | 1.29 ± 0.05 | 0.15 ± 0.009 | 15.0 ± 0.72 |
| 0.82 | 1.35 ± 0.05 | 0.15 ± 0.011 | 14.3 ± 0.72 |
| | | | |
| Linear[1] | <0.0001 | NS | NS |
| Quadratic | <0.0001 | NS | NS |

* Mg, mM. ** OD units *** OD units/hour **** hours

[1]: for each strain, max growth, rate of growth and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.9. Growth of *Fibrobacter succinogenes* strains A3c and S85 with different concentrations of Mg in liquid media.

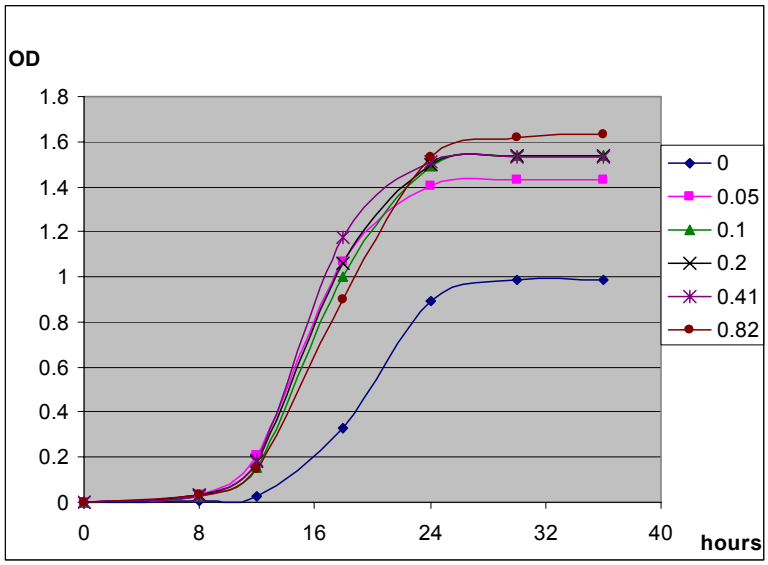


Figure 4.14. Predicted growth curves of F. *succinogenes* A3c with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

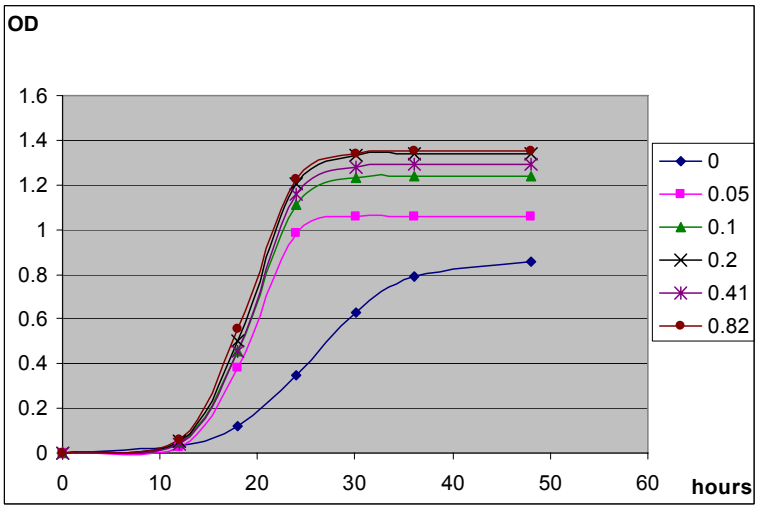


Figure 4.15. Predicted growth curves of F. succinogenes S85 with different Mg concentrations in cellobiose media, using the parameters obtained from the logistic function.

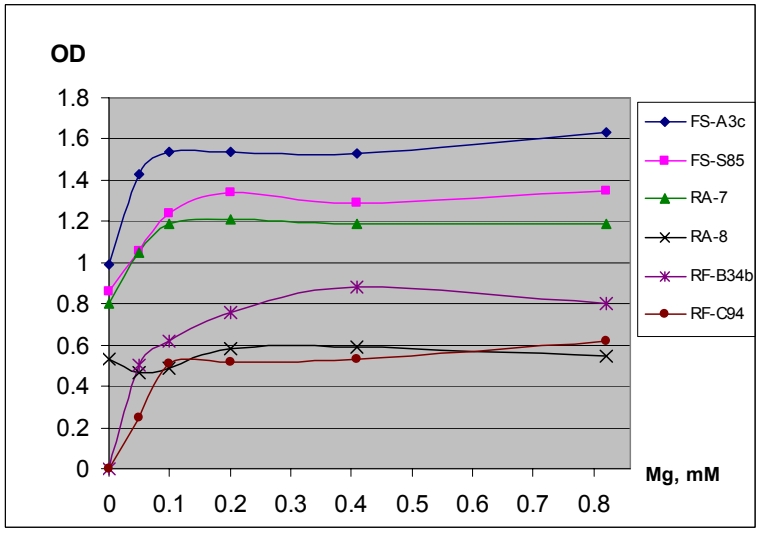


Figure 4.16. Curves of response to Mg concentrations on maximum growth (OD) by the different strains of rumen cellulolytic bacteria.

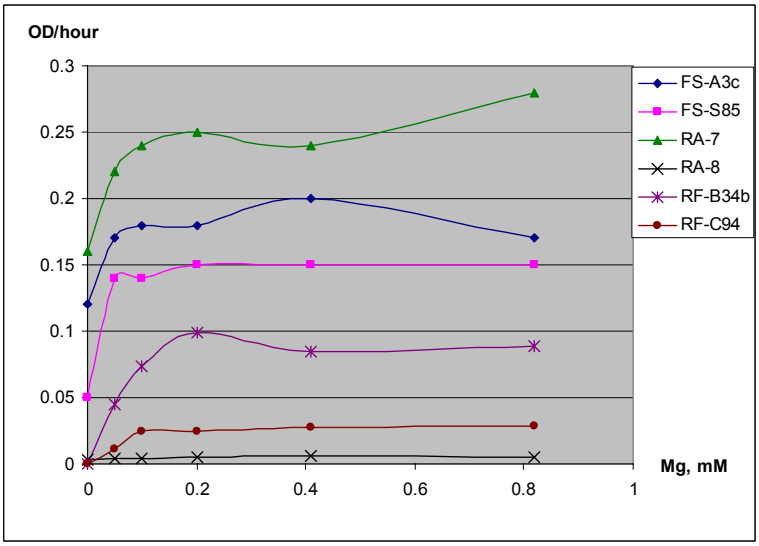


Figure 4.17. Curves of response to Mg concentrations on rate of (OD units/hour) by the different strains of rumen cellulolytic bacteria.

The same approach used with $Ca^{+2}$ and substrate affinity was applied to evaluate Mg affinity by the bacteria used in the present study. Linear functions were obtained for all the strains (Table 4.10) with the exception of FS-A3c, which did not fit the linear function (P>0.05). Values calculated, μmax (units OD/h) and Ks (mM Mg), from these functions were respectively, for RF-B34b: 0.11 and 0.067; RF-C94: 0.038 and 0.104; RA-7: 0.27 and 0.01; RA-8: 0.0056 and 0.031; FS-S85: 0.15 and 0.from highest to lowest: FS-S85 > RA-7 > RA-8 > RF-B34b > RF-C94. This is consistent with the higher Mg requirements observed for *R. flavefaciens*.

| Strain | Linear function | | P = | $r^2$ | μmax (OD/h) | Ks (mM) |
|---|---|---|---|---|---|---|
| | Intercept | Slope | | | | |
| **RF-B34b** | 9.12± 1.28* | 0.61± 0.12* | 0.016 | 0.89 | 0.11 | 0.067 |
| **RF-C94** | 26.69± 6.61* | 2.77± 0.64* | 0.023 | 0.86 | 0.038 | 0.104 |
| **RA-7** | 3.76 ± 0.15* | 0.038± 0.014** | 0.079 | 0.70 | 0.27 | 0.01 |
| **RA-8** | 177.3± 14.3* | 5.44 ± 1.39* | 0.029 | 0.84 | 0.0056 | 0.031 |
| **FS-S85** | 6.58± 0.1* | 0.039± 0.01* | 0.027 | 0.85 | 0.15 | 0.006 |

***** P<0.05 ****** 0.05<P>0.10

Table 4.10. Linear regression parameters from reciprocal of rate of growth vs. reciprocal of Mg concentrations, and μmax and Ks coefficients derivated from the linear function.

Thus, only *R. flavefaciens* has an absolute requirement for Mg, while the remaining strains have different Mg requirements for optimum growth.

Several different authors (Bryant et al, 1959; Scott and Dehority, 1965, Caldwell et al, 1973; Caldwell and Arcand, 1974) have used maximum growth (max OD) as the indicator for determining nutrient requirements. Max OD was very useful in determining Mg requirement in the present study, whereas this was not the case with $Ca^{+2}$. Possibly, a consideration of the different roles of these two cations in bacterial metabolism may provide clues as how to requirements are influenced and how they can be measured. Because Mg functions in so many different processes in the cell, i.e., associated with ribosomes, as coenzyme in CHO metabolism, synthesis and integrity of cell walls (Jasper and Silver, 1977; Durand and Kawashima, 1980; Smith and Maguire, 1998). On the other hand, $Ca^{+2}$ appears more like a signaling molecule (Herbaud et al, 1998). Thus max growth may be a better reflection of Mg requirements, because the limiting nutrient is affecting both rate and extent of bacterial growth.

Considering the role of Mg in cell wall synthesis, it could be logical to expect a high Mg requirement for Gram (+) bacteria, as was reported by Durand and Kawashima (1980); and observed for *R. flavefaciens*, which has an absolute requirement for Mg. Apparently *R. albus* has lower requirements than *R. flavefaciens*; at least *R. albus* can growth without Mg in the medium. This may be due to differences in metabolic demands, different capacity to control intracellular concentrations or Mg-channels may be different between the two species. Unfortunately, all of these explanations are only speculations, due to the

lack of information related to this topic in rumen bacteria. Information from *Salmonella, E. coli* and other pathogens shows differences in resistance to Mg starvation, which may be due to different channel systems and variations in how different species manage their Mg status (Jasper and Silver, 1977; Smith and Maguire, 1998).

Beveridge (1990) and Beveridge et al (1985, 1986) reported differences in cell wall composition between two species of *Bacillus*, *B. subtilis*, and *B. licheniformis*. These differences consisted of changes in the amount and structural disposition of teichoic, lipoteichoic and teicuronic acid in the cell wall matrix, which altered the capacity of these bacteria to bind different ions and in different proportions. The differences in Mg affinity observed for the predominant rumen cellulolytic bacteria may be related with differences in their cell wall composition, but the information available about cell wall composition for these bacteria is not enough to establish this different capacity to bind Mg (Dehority, 1977; Vinogradov et al, 2001).

**4.4. Cellulose degradation and $Ca^{+2}$ concentrations**:

The results for cellulose degradation with varying $Ca^{+2}$ concentrations are presented in Tables 4.11, 4.12 and 4.13 as LSM of the parameters: maximum extent of degradation, rate of degradation and lag time. The statistical analysis showed significant ($P<0.05$) main effects for species, strain within species, $Ca^{+2}$ concentration and for interactions of species*$Ca^{+2}$ concentration and strain within

species*$Ca^{+2}$ concentration, for maximum degradation and lag time. For rate of degradation all main effects ($P<0.05$), with the exception of $Ca^{+2}$ concentrations ($P>0.05$), and all measured interactions were significant ($P<0.05$).

*R. albus* 8 did not fit the logistic function. Maximum degradation was estimated from the data of degradation at last sampling time and arithmetic average is reported for each $Ca^{+2}$ concentration, and analyzed by ANOVA (PROC GLM, SAS). To estimate rate of degradation the data were analyzed by linear regression ($P<0.05$); the slopes obtained later were analyzed by ANOVA (PROC GLM, SAS) and LSM per $Ca^{+2}$ concentration were obtained. However no effect of $Ca^{+2}$ concentrations on rate of cellulose degradation by RA-8 was found ($P>0.05$).

The maximum cellulose degradation and lag time were affected by $Ca^{+2}$ concentrations in all the strains, except for RA-8. Rate of degradation was not affected by $Ca^{+2}$ concentrations ($P>0.05$). Interactions of species*treatment and strain within species*treatment were detected significant ($P<0.05$); thus, only *F. succinogenes* A3c and S85, showed that rate of cellulose degradation was affected by the $Ca^{+2}$ concentrations ($P<0.05$).

Neither strain of *F. succinogenes* degraded cellulose in the absence of supplemental $Ca^{+2}$; however, they both responded to increased $Ca^{+2}$ concentrations with an irregular pattern of maximum cellulose degradation (table 4.11, figure 4.18, 4.19, and 4.24). FS-A3c had a linear response for maximum degradation and rate of degradation ($P<0.0001$), meaning that higher $Ca^{+2}$ concentrations need be tested to establish their requirement. Although there is

an effect of $Ca^{+2}$ concentrations on maximum degradation for FS-S85, it cannot be represented by a linear or quadratic adjustment, the contrasts analysis had no solution ($P>0.05$). In figure 4.24, for both strains, maximum cellulose degradation plotted vs. $Ca^{+2}$ concentrations shows the incapacity to reach a break point and then define a requirement. It would be necessary to test higher concentrations than those used in this experiment. Although, lag time was significant affected by $Ca^{+2}$ concentrations, it did not fit the linear and quadratic contrasts for FS-A3c ($P=0.6165$, NS) and FS-S85 (no solution for contrasts).

| | Max degradation** | Rate of degradation*** | Lag time**** |
|---|---|---|---|
| ***F. succinogenes* A3c** | | | |
| 0* | No degradation | | |
| 0.04 | 21.0 ± 0.26 | 0.33 ± 0.18 | 49.0 ± 6.24 |
| 0.06 | 14.0 ± 0.07 | 0.99 ± 0.11 | 37.5 ± 2.78 |
| 0.08 | 21.5 ± 0.09 | 0.69 ± 0.29 | 35.5 ± 2.87 |
| 0.12 | 20.6 ± 0.11 | 0.51 ± 0.20 | 16.4 ± 4.03 |
| 0.36 | 32.0 ± 0.07 | 2.00 ± 0.08 | 28.8 ± 2.72 |
| Linear[1] | <0.0001 | <0.0001 | 0.6165 |
| Quadratic | NS | NS | NS |
| ***F. succinogenes* S85** | | | |
| 0* | No degradation | | |
| 0.04 | 25.0 ± 0.09 | 1.11 ± 0.18 | 20.4 ± 2.92 |
| 0.06 | 22.2 ± 0.09 | 1.24 ± 0.37 | 21.0 ± 2.82 |
| 0.08 | 28.0 ± 0.08 | 2.48 ± 0.28 | 22.2 ± 2.75 |
| 0.12 | 25.8 ± 0.09 | 1.25 ± 0.46 | 23.4 ± 2.81 |
| 0.36 | 31.8 ± 0.11 | 1.45 ± 0.25 | 21.2 ± 3.05 |
| Linear[1] | NS | NS | NS |
| Quadratic | NS | NS | NS |

*$Ca^{+2}$ mM **mg cellulose ***mg cellulose/hour ****hours
[1]: for each strain, maximum degradation, rate of degradation and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.11. Cellulose degradation by *Fibrobacter succinogenes* strains A3c and S85 incubated with different concentrations of ionized $Ca^{+2}$.

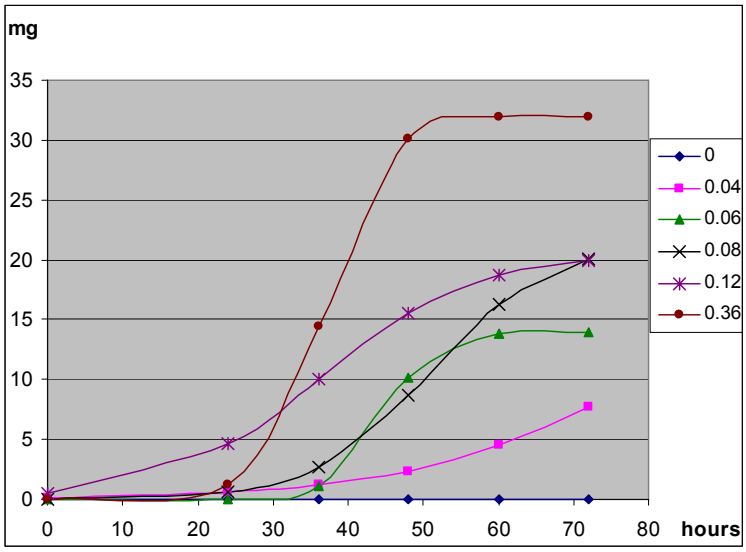


Figure 4. 18. Predicted cellulose degradation curves of *F. succinogenes* A3c with different $Ca^{+2}$ concentrations in cellulose media, using the parameters obtained from the logistic function.

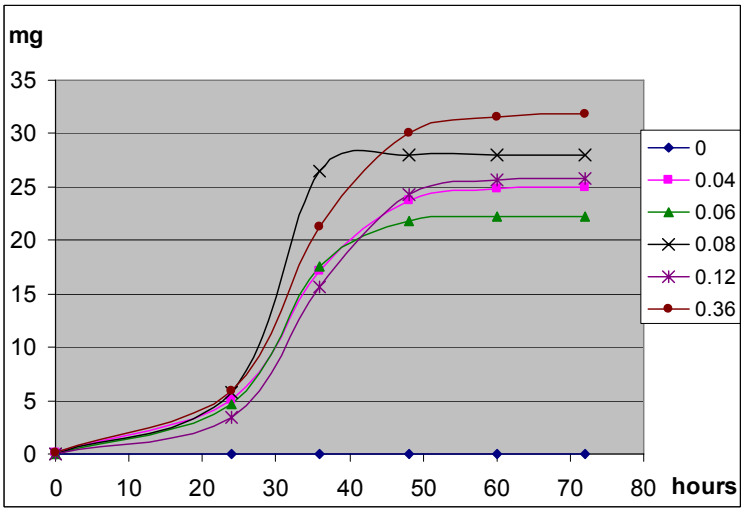


Figure 4. 19. Predicted cellulose degradation curves of *F. succinogenes* S85 with different $Ca^{+2}$ concentrations in cellulose media, using the parameters obtained from the logistic function.

The highest maximum degradation among all the strains studied was obtained with RA-7, which appears to be the most aggressive cellulolytic strain (Table 4.12 and Figure 4.20). $Ca^{+2}$ concentrations had a significant effect on cellulose degradation; RA-7 responded with increased maximum degradation either linearly (P=0.0027) or quadratic (P=0.0372) to increased $Ca^{+2}$ concentrations. The lowest maximum degradation was obtained with 0 $Ca^{+2}$, increasing when $Ca^{+2}$ increased, showing a tendency to reach a plateau with higher concentrations (Figure 4.24); RA-7 responded decreasing lag time, linearly or quadratic, to increased $Ca^{+2}$ concentrations. The requirement for maximum degradation and lag time were estimated as 0.28 and 0.34 mM $Ca^{+2}$, respectively. The quadratic functions used to estimate the requirement for maximum degradation, lack of significance (P=0.1440; $r^2$=0.12); only the function for lag time was significant (P<0.0001; $r^2$=0.54), then be the one useful for estimation; although lag time is not a parameter commonly used to evaluate requirements.

RA-8 was analyzed independently, since its values did not fit the logistic function (Figure 4.21). The maximum degradation, averages of the extent of degradation obtained at the last sampling, were analyzed by ANOVA, but no effect of $Ca^{+2}$ concentrations was detected (P>0.05). The slope of the linear function for each $Ca^{+2}$ concentration, representing the rate of cellulose degradation, was analyzed by ANOVA and did not show differences due the effect of $Ca^{+2}$ concentration, thus no further analysis was made (P>0.05) (Table 4.12.). Because of the lack of fit with the logistic function, the parameter “C”,

representing lag time, was not obtained. In table 4.12 the time of the first sampling was reported as lag time, only as a reference for the time delay until evident cellulose degradation was observed and samples were taken.

| | **Max Cellulose degradation**** | **Rate of degradation***** | **Lag time******** |
|---|---|---|---|
| ***R. albus* 7** | | | |
| 0* | 31.34 ± 1.37 | 1.08 ± 0.19 | 15.62 ± 4.02 |
| 0.02 | 38.32 ± 1.45 | 1.41 ± 0.26 | 10.90 ± 4.11 |
| 0.04 | 36.59 ± 1.46 | 1.48 ± 0.28 | 12.11 ± 4.13 |
| 0.08 | 34.30 ± 1.59 | 1.49 ± 0.35 | 10.61 ± 4.29 |
| 0.16 | 37.88 ± 1.53 | 1.50 ± 0.31 | 9.36 ± 4.22 |
| 0.32 | 36.94 ± 1.46 | 1.47 ± 0.29 | 9.84 ± 4.16 |
| 0.36 | 33.46 ± 1.74 | 1.23 ± 0.36 | 7.09 ± 4.55 |
| 0.64 | 33.96 ± 1.76 | 1.55 ± 0.41 | 12.57 ± 4.42 |
| Linear[1] | 0.0027 | N.C. | <0.0001 |
| Quadratic | 0.0372 | | 0.0009 |
| ***R. albus* 8** | | | |
| 0* | 9.80[2] | 0.0285 ± 0.016[3] | 60[4] |
| 0.02 | 15.10 | 0.0418 ± 0.017 | 60 |
| 0.04 | 13.55 | 0.0589 ± 0.043 | 60 |
| 0.08 | 19.68 | 0.0395 ± 0.028 | 60 |
| 0.16 | 18.83 | 0.0574 ± 0.021 | 60 |
| 0.32 | 26.00 | 0.0854 ± 0.026 | 60 |
| 0.36 | 21.96 | 0.0659 ± 0.021 | 60 |
| 0.64 | 21.10 | 0.0649 ± 0.022 | 60 |

* $Ca^{+2}$ mM **mg of cellulose ***mg cellulose/hour ****hours

[1]: for *R. albus* 7, max degradation and lag time were evaluated by linear and quadratic contrasts, P values are presented. **N.C.** : No contratsts analysis, no effect of $Ca^{+2}$ concentrations (P>0.05).

For *R. albus* 8, for max cellulose degradation ([2]) extent of degradation obtained at the last sampling time (275 hours), for rate of degradation ([3]) the slope of the linear regression (P=0.064), for lag time ([4]) the time for first sampling determined visually.

Table 4.12. Cellulose degradation by *Ruminococcus albus* strains 7 and 8 incubated with different concentrations of $Ca^{+2}$.

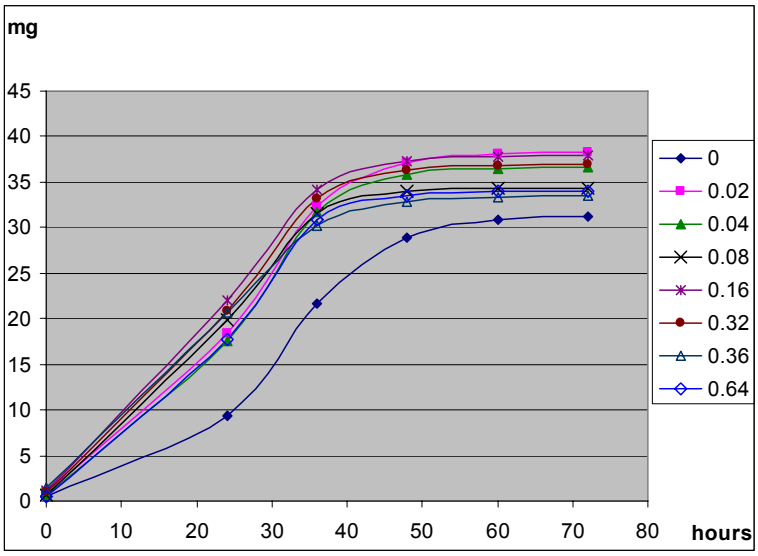


Figure 4. 20. Predicted cellulose degradation curves of *R. albus* 7 with different $Ca^{+2}$ concentrations in cellulose media, using the parameters obtained from the logistic function.

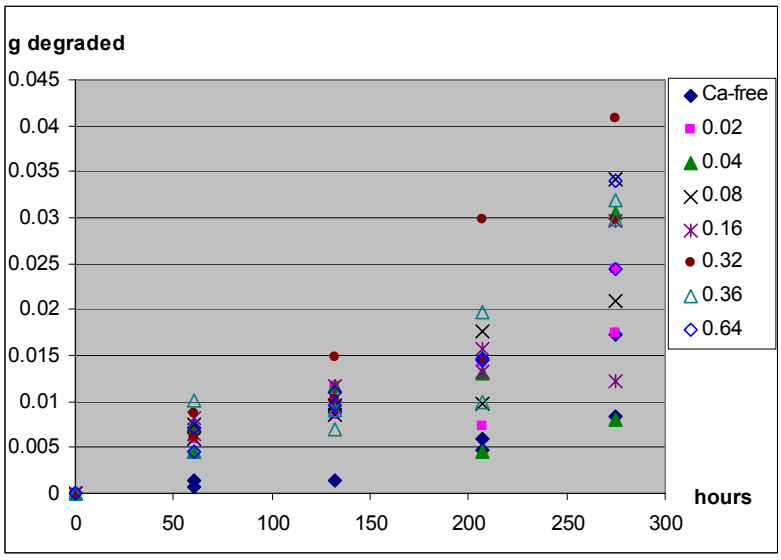


Figure 4. 21. Cellulose degradation curves of *R. albus* 8 with different $Ca^{+2}$ concentrations in cellulose media (raw data data from replicate 1).

Extent of cellulose degradation and lag time for *R. flavefaciens* B34b and C94 were both affected by $Ca^{+2}$ concentrations ($P<0.05$) (Table 4.13 and Figure 4.22 and 4.23). Rate of degradation was not affected by $Ca^{+2}$ concentrations ($P>0.05$). For RF-B34b and RF-C94, maximum degradation increased linearly ($P<0.0001$) as $Ca^{+2}$ concentrations increased. However, this response should be taken with care, since it may be an artifact of the adjustment Figure 4.24 graphically shows the tendency to increase degradation, but with variations that do not permit identifying a break point. The linear function fitted the adjustment, although it does not reflect the biology of the response. For both strains, lag time was affected by $Ca^{+2}$ concentrations, but only RF-C94 showed a quadratic response ($P=0.0007$) to the contrasts evaluation. The estimated quadratic function lack of significance ($P=0.2659$; $r^2=0.06$), then the calculated $Ca^{+2}$ requirement 0.1 mM is not correct. However, lag time is not a common parameter used to measure requirements.

| | Max Cellulose degradation** | Rate of degradation*** | Lag time**** |
|---|---|---|---|
| ***R. flavefaciens* B34b** | | | |
| 0* | 18.24 ± 1.10 | 0.885 ± 0.30 | 15.94 ± 3.78 |
| 0.02 | 16.87 ± 1.22 | 0.479 ± 0.20 | 9.14 ± 4.05 |
| 0.04 | 17.25 ± 1.19 | 0.542 ± 0.18 | 11.02 ± 3.75 |
| 0.08 | 19.24 ± 0.94 | 0.729 ± 0.16 | 12.61 ± 3.40 |
| 0.16 | 19.27 ± 0.95 | 0.776 ± 0.18 | 12.87 ± 3.42 |
| 0.32 | 20.83 ± 1.01 | 0.743 ± 0.17 | 11.90 ± 3.45 |
| 0.36 | 18.90 ± 1.23 | 0.734 ± 0.23 | 15.89 ± 3.67 |
| 0.64 | 21.44 ± 1.17 | 0.708 ± 0.21 | 11.42 ± 3.69 |
| Linear[1] | <0.0001 | **N.C.** | 0.9585 |
| Quadratic | 0.3507 | | 0.9850 |
| ***R. flavefaciens* C94** | | | |
| 0* | 26.43 ± 1.58 | 0.994 ± 0.23 | 15.51 ± 4.57 |
| 0.02 | 28.58 ± 1.13 | 0.742 ± 0.19 | 23.62 ± 3.59 |
| 0.04 | 28.78 ± 1.60 | 0.462 ± 0.25 | 8.83 ± 4.86 |
| 0.08 | 30.23 ± 1.47 | 0.742 ± 0.29 | 8.46 ± 4.67 |
| 0.16 | 28.51 ± 1.49 | 0.579 ± 0.25 | 8.83 ± 4.79 |
| 0.32 | 35.64 ± 1.53 | 1.006 ± 0.35 | 14.36 ± 4.37 |
| 0.36 | 26.83 ± 1.74 | 0.534 ± 0.26 | 24.33 ± 4.60 |
| 0.64 | 27.74 ± 1.64 | 0.464 ± 0.23 | 6.74 ± 4.95 |
| Linear[1] | <0.0001 | **N.C.** | 0.1955 |
| Quadratic | 0.1222 | | 0.0007 |

* $Ca^{+2}$ mM **mg of cellulose ***mg cellulose/hour ****hours

**N.C.** : No contratsts analysis, no effect of $Ca^{+2}$ concentrations (P>0.05).

Table 4.13. Cellulose degradation by *Ruminococcus flavefaciens* strains B34b and C94 incubated with different concentrations of $Ca^{+2}$.

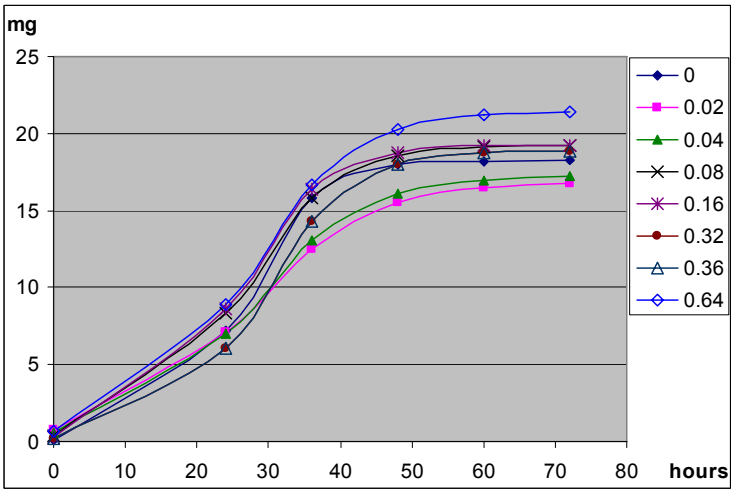


Figure 4. 22. Predicted cellulose degradation curves of *R. flavefaciens* B34b with different $Ca^{+2}$ concentrations in cellulose media, using the parameters obtained from the logistic function.

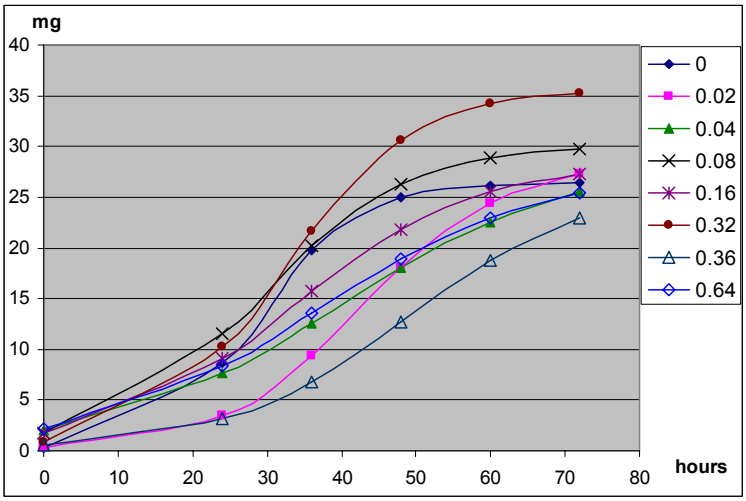


Figure 4. 23. Predicted cellulose degradation curves of *R. flavefaciens* C94 with different $Ca^{+2}$ concentrations in cellulose media, using the parameters obtained from the logistic function.

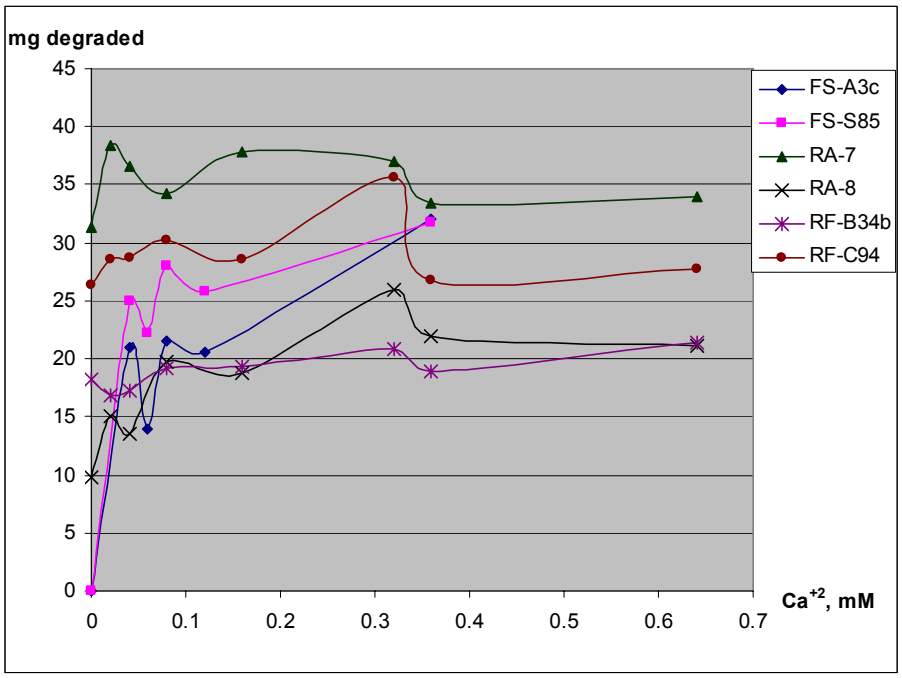


Figure 4.24. Curves of response to $Ca^{+2}$ concentrations on maximum cellulose degradation (mg degraded) by the different strains of rumen cellulolytic bacteria.

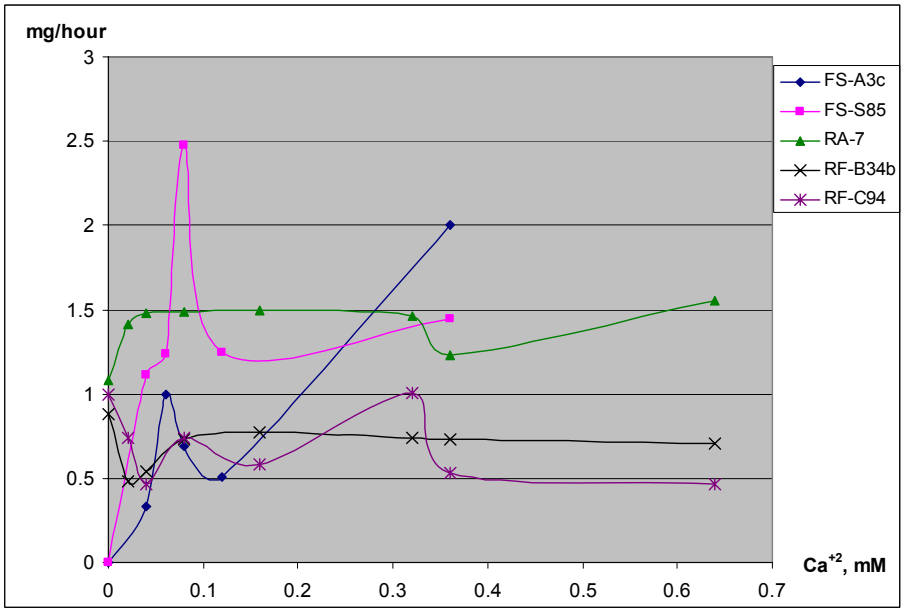


Figure 4.25. Curves of response to $Ca^{+2}$ concentrations on rate of degradation (mg degraded/hour) by the different strains of rumen cellulolytic bacteria.

Similar to earlier experiments, the kinetic approach was used to study the response from FS-A3c and FS-S85 to $Ca^{+2}$ treatments on cellulose degradation; Only *F. succinogenes* strains were included since only they presented different rates of degradation due to different $Ca^{+2}$ concentrations (identified from the significant interaction ($P<0.05$), although, they are not protective by the effect of $Ca^{+2}$ concentrations). Linear functions were obtained, but the statistical significance showed only a tendency (Table 4.14).

| Strains | Linear function | | P = | $r^2$ | μmax | Ks |
|---|---|---|---|---|---|---|
| | intercept | slope | | | (mg/h) | (mM) |
| **FS-A3c** | 0.50± 0.47 | 0.102±0.03 | 0.088 | 0.83 | 2.01 | 0.204 |
| **FS-S85** | 0.69± 0.03 | 0.083±0.002 | 0.062 | 0.88 | 1.45 | 0.012 |

Table 4.14. Linear regression parameters from reciprocal of rate of cellulose degradation vs. reciprocal of $Ca^{+2}$ concentrations, and μmax and Ks coefficients derivated from the linear function of the reciprocals.

The Ks for FS-A3c was 0.204 mM $Ca^{+2}$ and for FS-S85 was 0.012 mM $Ca^{+2}$, and the μMax was 2.01 mg cellulose/h for FS-A3c, and 1.45 mg cellulose/h for FS-S85. These results indicate a tendency for higher affinity for $Ca^{+2}$ by FS-S85 than FS-A3c, thus confirming the apparently higher $Ca^{+2}$ requirement of FS-A3c for cellulose degradation.

### 4.5. Cellulose degradation by *F. succinogenes* with different $Ca^{+2}$ concentrations in $NH_3$-free media.

Because *F. succinogenes* was found to have an absolute requirement for $Ca^{+2}$ with cellulose as a substrate and because degradation also responded to $Ca^{+2}$ concentrations with considerable variation, a study was designed to measure cellulose degradation in $NH_3$-free media. This eliminates growth as a confounding factor in the determination of $Ca^{+2}$ requirement for cellulose degradation. The effects of strain, $Ca^{+2}$ concentration and the interaction of strain*treatment on cellulose degradation in $NH_3$-free media were evaluated. Maximum degradation was affected for all the effects tested ($P<0.05$), and rate of degradation was significantly affected by $Ca^{+2}$ concentration ($P<0.05$); the effect of strain and the interaction of strain*treatment showed only a tendency ($P= 0.13$ and 0.08, respectively). Lag time was affected only by strain ($P<0.05$) and FS-S85 showed longer lag times than FS-A3c.

For both strains of FS with the $Ca^{+2}$-free medium, no cellulose degradation occurred (Table 4.15); therefore there was no data to include in the overall analysis. Very low degradation by FS-A3c was obtained with 0.02mM $Ca^{+2}$, and

the data from this treatment fit neither the logistic nor linear model. Therefore the least squares means were estimated for each sampling time and comparisons among them were made to determine if degradation along time was different or not. Differences were found between the two later sampling times 24 and 48 hours, and this difference was used to estimate the rate of degradation. The LSM for 48 hours is reported as the extent of cellulose degradation: 3.9 mg.

Thus, cellulose degradation in both strains of *F. succinogenes* responded to the $Ca^{+2}$ concentrations following a definite pattern. With 0 $Ca^{+2}$ no degradation occurred; as $Ca^{+2}$ concentrations increase, cellulose degradation also increased, both in extent and the rate of degradation (Table 4.15., Figure 4.26 and 4.27). FS-A3c showed a linear and quadratic response for maximum cellulose degradation ($P<0.0001$), whereas FS-S85 has a linear ($P=0.0029$) increasing in maximum degradation as $Ca^{+2}$ concentration increased (Table 4.15 and Figure 4.28). Considering the significance of the contrasts and the curve of maximum degradation vs. $Ca^{+2}$ concentrations for A3c, the quadratic adjustment is more likely to define the $Ca^{+2}$ requirement for maximum degradation. Thus by calculation of maximum from the quadratic function, $Ca^{+2}$ requirement is 0.47 mM. It is not possible to calculate a requirement for FS-S85 because the linear contrasts, although the curve in the figure 4.28 shows a tendency to be quadratic. This may be due to the high variation of the measurements. Rate of degradation responded to increased $Ca^{+2}$ concentrations in a quadratic fashion (Figure 4.29), statistically significant for FS-A3c ($P=0.0126$) and only as a

tendency for FS-S85 (P=0.0805). In this case, the requirement can be defined as 0.4 mM for rate of degradation for FS-A3c. For both strains, lag time was not affected by $Ca^{+2}$ concentrations (P>0.05).

| | **Max of degradation**** | **Rate of degradation***** | **Lag time******** |
|---|---|---|---|
| ***F. succinogenes* A3c** | | | |
| 0* | No degradation | | |
| 0.02 | ***3.90 (at 48 h)*** | ***0.11 (estimated)*** | ***< 12 (estimated)*** |
| 0.04 | 7.14 ± 1.22 | 0.30 ± 0.12 | 10.42 ± 3.45 |
| 0.08 | 11.23 ± 1.07 | 0.42 ± 0.09 | 9.95 ± 2.29 |
| 0.16 | 12.17 ± 1.21 | 0.41 ± 0.09 | 6.53 ± 2.41 |
| 0.32 | 11.32 ± 0.83 | 0.67 ± 0.09 | 9.15 ± 1.39 |
| 0.64 | 12.08 ± 1.15 | 0.50 ± 0.10 | 6.92 ± 2.40 |
| | | | |
| Linear[1] | <0.0001 | 0.0560 | 0.2030 |
| Quadratic | <0.0001 | 0.0126 | 0.6559 |
| | | | |
| ***F. succinogenes* S85** | | | |
| 0* | No degradation | | |
| 0.02 | 6.67 ± 1.36 | 0.18 ± 0.08 | 11.48 ± 6.88 |
| 0.04 | 8.73 ± 1.45 | 0.39 ± 0.14 | 11.07 ± 3.28 |
| 0.08 | 10.05 ± 0.11 | 0.55 ± 0.06 | 14.97 ± 0.16 |
| 0.16 | 11.26 ± 1.18 | 0.53 ± 0.11 | 8.62 ± 2.47 |
| 0.32 | 12.71 ± 0.59 | 0.51 ± 0.05 | 12.11 ± 1.10 |
| 0.64 | 11.03 ± 0.27 | 0.80 ± 0.03 | 15.95 ± 0.44 |
| | | | |
| Linear[1] | 0.0029 | 0.0521 | 0.9901 |
| Quadratic | 0.2737 | 0.0805 | 0.8366 |

* $Ca^{+2}$ mM **mg of cellulose ***mg cellulose/hour ****hours
[1]: for each strain, max degradation, rate of degradation and lag time were evaluated by linear and quadratic contrasts, P values are presented.

Table 4.15. Cellulose degradation by *Fibrobacter succinogenes* strains A3c and S85 incubated with different concentrations of $Ca^{+2}$ and $NH_3$-free media.

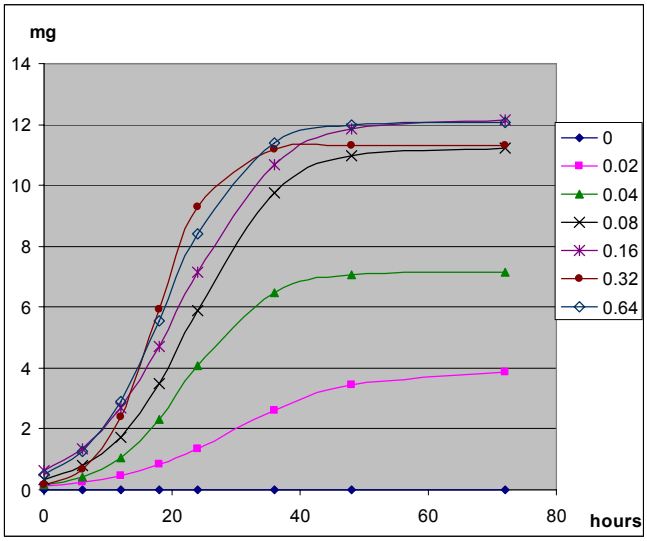


Figure 4.26. Predicted cellulose degradation curves of *F. succinogenes* A3c with different $Ca^{+2}$ concentrations in $NH_3$-free cellulose media, using the parameters obtained from the logistic function.

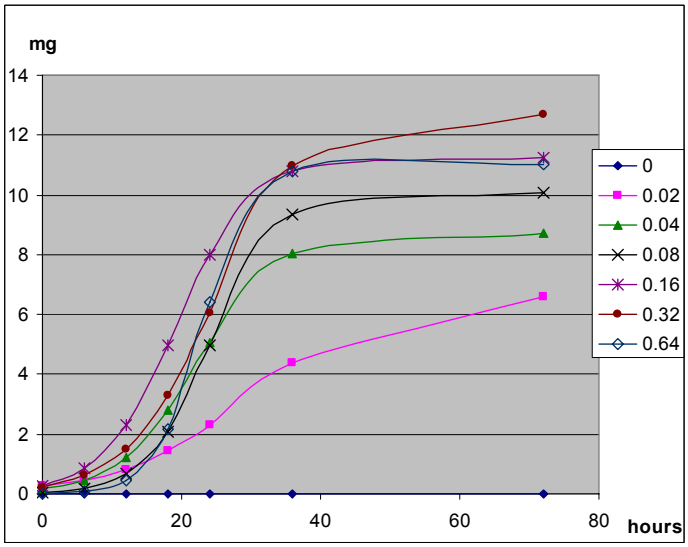


Figure 4. 27. Predicted cellulose degradation curves of *F. succinogenes* S85 with different $Ca^{+2}$ concentrations in $NH_3$-free cellulose media, using the parameters obtained from the logistic function.

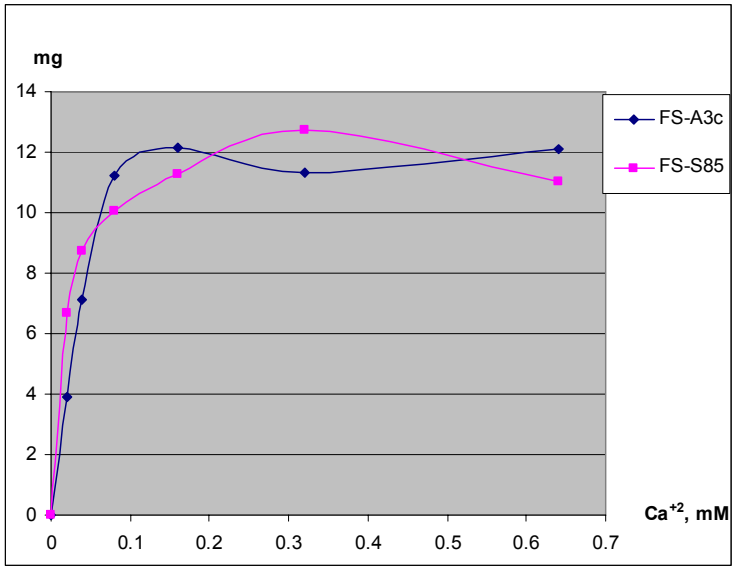


Figure 4. 28. Maximum cellulose degradation vs. $Ca^{+2}$ concentrations for *F. succinogenes* strains A3c and S85, from $NH_3$-free medium.

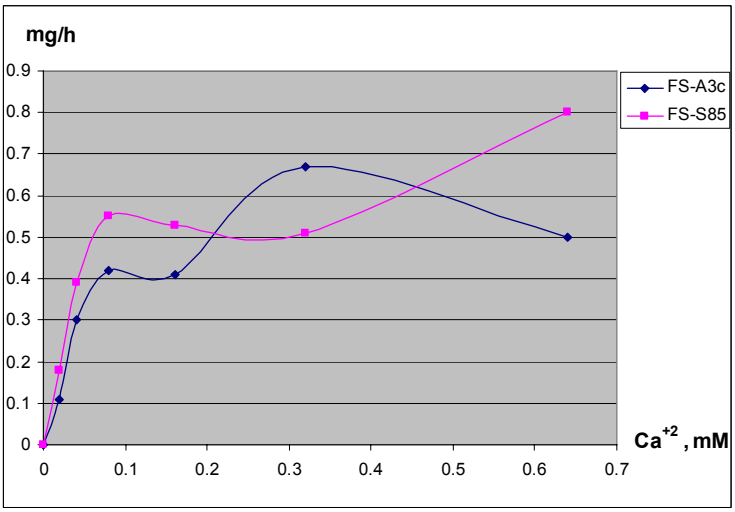


Figure 4.29. Rate of cellulose degradation vs. $Ca^{+2}$ concentrations for *F. succinogenes* strains A3c and S85, from $NH_3$-free medium.

Examining the data by the kinetic approach used previously, linear functions of the reciprocals were obtained (Table 4.16. and Figure 4 24). μVmax and Ks calculated were, respectively: 0.61 mg/h and 0.041 mM $Ca^{+2}$ for FS-A3c, and 0.68 mg/h and 0.056 mM $Ca^{+2}$ for FS-S85.

| Strain | Coefficients of linear function | | P = | $r^2$ | μmax (mg/h) | Ks (mM) |
|---|---|---|---|---|---|---|
| | Intercept | Slope | | | | |
| **FS-A3c*** | 1.66±0.22 | 0.068±0.02 | 0.029 | 0.84 | 0.61 | 0.041 |
| **FS-S85** | 1.47±0.15 | 0.08±0.006 | 0.0002 | 0.98 | 0.68 | 0.056 |

*without data from 0.02 mM

Table 4.16. Linear regression parameters from reciprocal of rate of cellulose degradation vs. reciprocal of $Ca^{+2}$ concentrations, and μmax and Ks coefficients derivated from the linear function.

Attachment is an important issue for cellulose degradation, and many authors have studied this process with different experimental methods. Akin (1976, 1980) did morphological studies using the electron microscope, Minato

and Suto (1978, 1981) studied the mechanisms of how the bacterium attaches to the fiber (cellulose binding proteins). Gong and Forsberg (1989) and Roger et al (1990) evaluated the effect of different factors in this process among them, different cations.

Gong and Forsberg (1989), using *F. succinogenes* S85, evaluated different concentrations of Ca and Mg on cellulose adhesion. They found that the percentage of adhesion increased abruptly with 27.3 mM Ca or 20.2 mM Mg, probably due to the ionic strength positively improving the bacterial adhesion, rather than the effect of the cations per se. When the same authors evaluated the effect of heat, trypsin, glutaraldehyde or pronase, bacterial adhesion to cellulose was markedly reduced, suggesting that a protein and not cations was involved in adhesion to cellulose of *F. succinogenes.*

Roger et al (1990) evaluated the lack of Ca or Mg, or both on adhesion of *R. flavefaciens* (007) and *F. succinogenes* (S85) on cellulose. No changes in adhesion for *F. succinogenes* were observed, and a reduced adhesion was observed in *R. flavefaciens* when either cation was absent; the effect was greater with the lack of Mg or Ca+Mg.

Concerning the interaction between different ions and plant cell walls, Molloy and Richards (1971) reported that lignin and pectin can bind $Ca^{+2}$ and $Mg^{+2}$, and Somers (1973) demonstrated that pectin in plant cell walls bind $Ca^{+2}$. Torre et al (1992) found that cellulose does not have the capacity to bind $Ca^{+2}$, while pectin has a large capacity to bind $Ca^{+2}$. Consistent with this information; results of cation exchange capacity (CEC) obtained by Allen et al (1985) showed

a very low CEC for Whatman paper or solka floc; and McBurney et al (1986) found that CEC is correlated with lignin, lignin:ADF ratio, and nitrogen content, but not with cellulose.

Considering the information from Gong and Forsberg (1989), Roger et al (1990), who did not find a relation between the presence of Ca or Mg and attachment of *F. succinogenes* to cellulose, and the findings from Molloy and Richards (1971), Somers (1973), Allen et al (1985), McBuerney et al (1986) and Torre et al (1992), who agreed that pectin and lignin, but not cellulose, are the cell wall components responsible for the capacity to bind divalent cations; it is possible to assume that $Ca^{+2}$ did not have a direct effect on binding for *F. succinogenes* to purified cellulose. The response in cellulose degradation must be related either with the secretion of enzymes from the bacterial cell, or as an intermediate in the attachment between the enzyme(s) and cellulose, or to the amount and activity of the enzymes. Our experimental evidence is insufficient to elucidate which of these mechanisms might be involved.

Numerous experiments have observed an increased in cellulose degradation when Ca was added to the medium, either using whole bacteria or isolated enzymes from cellulolytic bacteria. Using washed rumen bacteria Hubbert et al (1958a) suggested the optimal range for Ca supplementation was 0.34 – 2.04 mM. With isolated endoglucanase-1 from *F. succinogenes*, increasing the concentration from 1 to 10mM of $CaCl_2$ (0.27 to 2.7mM Ca) increased rate of enzyme activity, whereas endoglucanase-2 did not change its activity (McGavin and Forsberg, 1988). Taylor et al (1987) using a Cel-encoded

endoglucanase expressed in *E. coli*, obtained an increased enzymatic activity when Ca (10mM) was added to the media; however, no response was observed using the native cellulolytic enzyme(s) from *F. succinogenes*. Tsai et al (2003) characterizing the Fsβ-glucanase from *F. succinogenes,* observed that the addition of 1 mM $Ca^{+2}$ increased the thermal stability of the enzyme.

Phospholipase A (PLA) is an enzyme present in the outer membrane of Gram (-) bacteria which is sensitive to $Ca^{+2}$ for its activation. This enzyme causes changes in fatty acid composition, permeability and fluidity of the membrane (Snijder and Dijkstra, 2000). It has been suggested that this is the mechanism used by the bacteria to allow secretion of different substances involved in pathogenicity of the bacteria (Snijder and Dijkstra, 2000). Christie (1981) reported that PLA of *Butyrivibrio fibrisolvens* is not sensitive to $Ca^{+2}$, but no information about other rumen Gram (-) bacteria apparently is available. Could this be a mechanism used for *F. succinogenes* to secret the enzymes complex across the membrane?

### 4.6. Cellulose degradation by *R. flavefaciens* in $NH_3$-free media, when the medium is also $Ca^{+2}$ and Mg-free, $Ca^{+2}$-free, or Mg-free.

When growth experiments were analyzed, *R. flavefaciens* showed a high max growth and rate of growth in $Ca^{+2}$ free and low $Ca^{+2}$ media. Later an absolute growth requirement of *R. flavefaciens* for Mg was observed. When cellulose degradation was studied, the increased $Ca^{+2}$ concentration was

reflected in a small increment of cellulose degradation, characterized as a linear response ($P<0.0001$). To separate the potentially confounding effect of growth on cellulose degradation, due to $Ca^{+2}$ or Mg, an experiment was carried out with *R. flavefaciens* in $NH_3$-free media. Treatments were: $Ca^{+2}$ and Mg at normal concentrations (control), $Ca^{+2}$-free, Mg-free, and $Ca^{+2}$ and Mg-free. The results are shown in Table 4.17. For both strains the extent of degradation at 60 hours incubation with $Ca^{+2}$ and Mg was similar to $Ca^{+2}$-free ($P>0.05$). Neither Mg-free nor $Ca^{+2}$ and Mg-free, showed any cellulose degradation. Thus when Mg was not present in the $NH_3$-free medium, no cellulose degradation occurred. No interaction between strain and cation treatment was observed ($P>0.05$). Strain effect was observed ($P<0.05$), where RF-C94 showed lower cellulose degradation. This suggests that *R. flavefaciens* requires Mg to degrade cellulose, and may has no requirement or requires very low concentrations of $Ca^{+2}$ for cellulose degradation; if not why no degradation occurred when the media Mg-free+$Ca^{+2}$ had 0.36 mM of $Ca^{+2}$.

| Strains | Cellulose media $NH_3$-free | | | |
|---|---|---|---|---|
| | $Ca^{+2}$+Mg | $Ca^{+2}$-free + Mg | ($Ca^{+2}$+Mg)-free | Mg-free +$Ca^{+2}$ |
| **RF-B34b** | 14.38 ± 1.93 **a** | 13.55 ± 1.93 **a** | ND | ND |
| **RF-C94** | 10.35 ± 1.93 **b** | 7.50 ± 1.93 **b** | ND | ND |

mg of cellulose degraded
ND: no degradation
**a, b**: different letters indicate a strain effect on extent of degradation ($P<0.05$)

Table 4.17. Extent of cellulose degradation (mg degraded) at 60 hours incubation for *Ruminococcus flavefaciens* (B34b and C94) in the presence or absence of $Ca^{+2}$ and/or Mg in $NH_3$-free cellulose liquid medium.

### 4.7. General discussion.

The results from the present study show different responses for $Ca^{+2}$ and Mg among species and strains of the predominant rumen cellulolytic bacteria: *Fibrobacter suucinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. These three species are the predominant cultured cellulolytic bacteria found in the rumen (Miron et al, 2001; Russell, 2002), and among these the mechanisms for cellulose degradation have been investigated. Studies have been conducted on nutrient requirements, type of enzymes produced, and mechanisms of attachment to the substrate.

With the discovery of the cellulosome in *Clostridium thermocelum* by Bayer and coworkers, in 1983, the enzymes and attachment to the substrate were understood to be part of an integrated system secreted by the bacterium. This provided a new approach to study the mechanisms that rumen bacteria can use to degrade the insoluble carbohydrates. Since then, different groups of researchers have advanced the characterization of the molecular structure from different systems of attachment and substrate degradation. Also the genome sequence has been characterizated for different species. Cellulosome, fimbria or pili and glycocalix are the different systems found in rumen cellulolytic bacteria (Miron et al, 2001). *Ruminoccocus albus* has cellulosome and fimbria as attachment systems, *R. flavefaciens* has cellulosome and glycocalyx, and *F. succinogenes* apparently has only glycocalyx (Chesson and Forsberg, 1997; Ohara et al, 2001, Rincon et al, 2001, Bayer et al, 2004). Evidence from molecular studies shows that many of the structures from cellulosome, fimbria, and glycocalyx have proteins with peptide sequences and amino acid residues which form an EF-hand motif that has the capacity to bind $Ca^{+2}$, thus affecting the molecular stability or structure and perhaps also the activity of many enzymes (Chavaux et al, 1990; Chavaux et al, 1995; Chio and Ljungdahl, 1996a,b; Kirby et al, 1997; Ohara et al, 2000; Mitsumori and Minato, 2000; Lyttle et al, 2001, Rincon et al, 2001).

Due to the evidence that cellulosome, fimbria, and individual enzymes, all involved in cellulose digestion, all requiring $Ca^{+2}$; it is possible that the three cellulolytic rumen bacterial species may have a specific requirement for this

divalent cation. There is no information related with $Ca^{+2}$ requirements for either *R. albus* or *R. flavefaciens*. For *F. succinogenes,* only total calcium requirement for growth is available (Bryant et al, 1959), whereas, no information for $Ca^{+2}$ requirement for cellulose degradation is available. Thus, this study explored the $Ca^{+2}$ requirements for the three predominant rumen cultured cellulolytic bacteria, for growth as well as for cellulose degradation.

For maximum growth a statistically significant response was found with increasing $Ca^{+2}$ concentrations. FS-S85 responded in quadratic form, while RA-7 had a tendency for linear response. For FS-A3c, RF-B34b and RF-C94, the contrasts analysis, to evaluated the form of the response, did not give a solution; maybe due the special response from these bacteria to increased $Ca^{+2}$ concentrations (Figure 4.8). Although, when rate of growth was evaluated, FS-A3c showed a quadratic response to the increased $Ca^{+2}$ concentrations (Figure 4.9).

An especial situation was observed with FS-A3c, in that under certain conditions it did not grow in $Ca^{+2}$-free medium. Further, when FS-A3c grew in apparently $Ca^{+2}$-free media, a small amount of $Ca^{+2}$ was found in the medium, apparently from contamination in the water supply. Thus a very small amount of $Ca^{+2}$ triggered a growth response in this bacterium. However, this growth was not normal from the reproduction aspect, since FS-A3c showed an increased pleomorphism under these conditions (≤ 0.02 mM $Ca^{+2}$).

Although relatively normal growth was achieved with the very low $Ca^{+2}$ concentrations, the $Ca^{+2}$ requirement for maximum growth was higher than expected. The biological importance of this response is may be questionable in the majority of cases. The incremental response to increasing $Ca^{+2}$ concentration is small and in many cases highly variable (Figure 4.8). Relatively low $Ca^{+2}$ concentrations which occur from contamination in reagent grade minerals, permit normal growth, and for example, satisfy the growth of *R. albus* 7.

There is no logical explanation for the inconsistent results from this study for FS-S85 compared with those obtained for the same strain by Bryant et al (1959). In this study, FS-S85, reached maximum growth under all experimental $Ca^{+2}$ concentrations. Although FS-S85 had a quadratic response to increasing $Ca^{+2}$ concentrations, the incremental response was very small. In contrast, Bryant et al (1959) observed increasing growth response to increased Ca concentrations and defined a requirement for this strain as 0.05 mg / 5 ml (calculated as 0.25 mM total Ca), which is lower than this estimated from the present results (0.47 mM as $Ca^{+2}$).

The special response to increasing $Ca^{+2}$ may be related to the function of $Ca^{+2}$ in bacterial cells. The evidence from aerobic pathogenic bacteria shows $Ca^{+2}$ has a role as a signaling molecule (Herbaud et al, 1998). Bacteria, similar to eukaryotic cells, can regulate intracellular $Ca^{+2}$ concentrations. They respond to changes of $Ca^{+2}$ concentrations in the external medium, depending on a normal energy status, because the function of $Ca^{+2}$ channels is energy dependent (Rosen and Gangola, 1987). Changes in $Ca^{+2}$ concentrations in the

external media are reflected by transient modification in intracellular $Ca^{+2}$ concentrations; recovery to the steady state depends on the adaptation to concentrations in the medium (Jones et al, 1999). Thus if the media $Ca^{+2}$ concentration is in the micromolar range they are able to internally accumulate 3 to 6 fold the $Ca^{+2}$ concentration that is in the medium. However, if the medium concentrations are in millimolar order, they tend to accumulate similar concentrations as in the medium (Jones et al, 2002). Perhaps, *F. succinogenes*, has regulatory mechanisms similar to *E. coli*, and can accumulated $Ca^{+2}$ in the cell.

Durand and Kawashima (1980), reported an increases in bacterial pleomorphism when calcium was deficient in the culture medium. However, the authors did not mention which species of bacteria were affected and gave no explanation for this effect. Although, FS-A3c naturally has a tendency to have some pleomorphic rods in older cultures (Dehority, 2003), this was not evident when the bacterium was grown in normal medium. When FS-A3c was grown with low $Ca^{+2}$ concentrations (0.02 mM $Ca^{+2}$), pleomorphic forms were observed and the bacteria grew in size but did not divide. Evidence to explain this effect from $Ca^{+2}$ deficiency came from studies with *E.coli.* It was found that $Ca^{+2}$ plays a regulatory role in cell division through FtsZ, a protein that require $Ca^{+2}$ to became polymerized, and is responsible to signal the cell equator and form the division ring for cell division (Yu and Margolin, 1997). When $Ca^{+2}$ is depleted in the medium cells became elongated, forming filamentous multinucleated cells.

Unfortunately, in the present study no observations were made at other $Ca^{+2}$ concentrations to determine what concentration is the threshold for this phenomenon.

The lack of response from *R. flavefaciens* to increasing $Ca^{+2}$ concentrations was not expected, based on the information reported by Durand and Kawashima (1980), who suggested that these organisms have a higher requirement for Ca and Mg because these elements are needed for synthesis of the cell walls by Gram (+) bacteria. In general, Mg, as a cofactor, is involved in many different steps in the synthesis of the cell walls (Ghuysen and Shockman, 1973), while no role has been identified in this process for $Ca^{+2}$ , other than its role in sporulation process (Silver, 1977). Both $Ca^{+2}$ and Mg are part of the cell wall structure, due to chemical interactions between compounds at the bacterial cell wall and divalent cations in the medium. Thus, teichoic and/or teichuronic acids present at the matrix of the peptidoglycan layer of Gram (+) bacteria can bind $Ca^{+2}$ and Mg from the medium and the amount bound will vary as a function of the cell wall composition (Beveridge and Murray, 1980; Beveridge et al, 1982; Beveridge, 1990). It is necessary to consider that these cations, while they are bound to the cell wall, are not available for exchange with the medium or other cells. If the bacteria die or the pH conditions became acidic, these cations become available in the medium (Durand and Kawashima, 1980). Thus, a kind of mineral recycling can happen. But in the present study, this option may not apply, since the size of the inoculum utilized was very small (0.1 ml of 0.1 OD culture).

Other structures of the cell envelope are the S-layers that are ubiquitous in Gram (+) and Gram (-) bacteria. A role for $Ca^{+2}$ in the assembly of the units of the S-layer in *Caulobacter crescentus* and *Campylobacter fetus fetus* was described by Sleytr and Beveridge (1999). Although no information is available for S-layers in rumen bacteria, in theory they must be related with secretory processes, possibly the cellulosome or extracellular enzymes.

For cellulose degradation there is without doubt an absolute $Ca^{+2}$ requirement by *F. succinogenes*, because neither strain degraded cellulose at 0-added $Ca^{+2}$ concentration. Extent of degradation and lag time were affected by the $Ca^{+2}$ concentrations to different degrees. For maximum degradation, FS-A3c responded linearly and FS-S85 did not reach solution for contrasts analysis. This may be due to variation within strain, because the form of the curves described for both are very similar (Figure 4,18). When growth was excluded from the process by deleting N from the medium, the results confirmed the specific requirement of $Ca^{+2}$ for cellulose degradation. Both extent and rate of degradation were significantly affected by $Ca^{+2}$ concentrations (Figure 4.28 and 4.29). The highest extent of degradation was attained with 0.42 mM, whereas the highest rate of degradation was with 0.39 mM, respectively, for FS-A3c, values that are very close. For FS-S85 a linear contrast was significant for both maximum degradation and rate of degradation. When rate of degradation was plotted vs $Ca^{+2}$ concentrations (Figure 4.29), it was readily apparent that maximum rate of degradation was attained when $Ca^{+2}$ was around 0.32 and 0.64 mM of $Ca^{+2}$, for FS-A3c and FS-S85, respectively, observation that is close to the

statistical estimation. The affinity (Ks) values for $Ca^{+2}$ were estimated to be 0.041 and 0.056 mM $Ca^{+2}$ for FS-A3c and FS-S85, respectively, which are very low values compared with the concentrations at which what maximum rate of degradation was obtained.

McGavin and Forsberg (1988) found that endoglucanase-1 from *F. succinogenes* S85 increased in activity when $CaCl_2$ was added to the medium (1 to 10 mM), whereas endoglucanase-2 did not respond; Mitsumori et al (2002) reported that recombinant endoglucanase-F had an improved binding of the enzyme to carboxymethylcellulose when $Ca^{+2}$ was supplemented to the medium, although no $Ca^{+2}$ binding motif was identified in this enzyme. These inconclusive data correspond to the varied responses observed for $Ca^{+2}$ requirements in the present studies.

As expected, cellulose degradation by *R. albus* and *R. flavefaciens* increased with increased $Ca^{+2}$ concentrations. Both species have cellulosome structures which have amino acid sequences that bind $Ca^{+2}$. Although RA-7 showed a response for maximum degradation, rate of degradation, and lag time, RA-8 did not response to $Ca^{+2}$ concentrations ($P>0.05$). The very slow degradation observed for that this bacterium may have affected its response. All factors known to be needed for *R.albus* 8 to degrade cellulose were included in the medium (Stack et al, 1983).

*F. succinogenes* has a very unstable bacterial membrane that apparently permits the secretion of the enzyme as blebs on the cell envelope (Huang and Forsberg, 1988); this could be related to the chemical structure of the cell envelope, as well to the presence of phospholipases A and C on the bacterial wall, but there is no available information to confirm it. As mention earlier, *E. coli*, *B. cereus* and *C. welchii*, have phospholipase A and C, which are activated by $Ca^{+2}$, modifying its lipid composition, and the membrane become more fluid; permiting the secretion of proteins involved in their pathogenic capacity (Silver, 1977). Perhaps similar mechanisms can occur with *F. succinogenes* to secrete their enzymes; since was observed a positive response to $Ca^{+2}$ for cellulose degradation.

Succinic acid, a normal metabolic end product for *F. succinogenes* (Russell, 2002, Dehority, 2003), can chelate $Ca^{+2}$ to form calcium succinate, a substance that is slightly soluble in water (Windholz, 1976). Under rumen conditions, succinic acid is rapidly used by other bacteria and is not freely available. However, in this study working with individual strains, *F. succinogenes* could have produced succinic acid that would have accumulated and formed calcium salts, thus chelating part of the available $Ca^{+2}$. Because of the neutral pH of the medium and the pKa of succinic acid (2.5), chelation would be strongly in the salt form. However, if this happens, it occurs after the energy source becomes available (cellobiose or cellulose). The most likely situation for this to occur would have been when bacteria were grown on cellobiose, a soluble carbohydrate; but in general the response to $Ca^{+2}$ observed with *F. succinogenes*

growing with cellobiose, suggest that if succinic acid was chelating $Ca^{+2}$ it would have resulted in decreasing $Ca^{+2}$ concentration that did not affect the growth of the bacterium.

The present results are supported by the evidence available in the literature. It is reported that $Ca^{+2}$ binding improves the stability of the structures involved, or permits the interaction between dockerin domains and catalytic domains, or improves enzymatic activity, as was demonstrated for *C. thermocellum* (Chavaux et al, 1990 and 1995; Choi and Ljungdahl,1996, 1996b; Bayer et al, 1998; Shoham et al, 1999; Lytle et al, 2001). The evidence for *R. flavefaciens* came studies from Kirby et al (1997), Aurilia et al, (2000), Ding et al (2001), and Rincon et al (2001); and for *R. albus*, from Ohara et al, (2000).

Also, the information available, from washed rumen bacteria in response to Ca for cellulose degradation, is supporting the positive results obtained at the present study. Although, the concentrations used for different authors are high and very variable among studies; Hubbert et al (1958a) suggest as optimal concentration a wide range of 50 - 300 ug/ml of $CaCl_2$, (0.34 to 2.04 mM total Ca) with a toxic level of 450 ug/ml of $CaCl_2$ (11.23 mM total Ca). Uesaka et al (1967), cited by Durand and Kawashima (1980), reported a positive response by increasing 10 to 100 ug/ml of $CaCl_2$ (0.068 to 0.68 mM total Ca) and Bales et al (1978) reporting an increased DM digestion of corn stalk when Ca increased from 10 to 190 ppm (0.068 to 1.29 mM total Ca).

If the response from the present experiments is extrapolated to the general rumen ecosystem, considering the $Ca^{+2}$ concentrations reported by Palmquist et al (1986) and Wagner et al (1993), who are the only authors that measured ionized calcium in rumen fluid, it is not possible to envision a deficiency. Reported $Ca^{+2}$ concentrations were 0.6 mM at 0 h, and 1.5 mM at 10 h (Palmquist et al, 1986); and 1.75, 7, and 0.25 mM for 3, 6, and 12 h post prandial (Wagner et al, 1993); all higher than the apparent requirements observed in this study. Fluctuations in $Ca^{+2}$ concentration observed as a consequence of the feeding time apparently have no consequences on bacterial activity, although the $Ca^{+2}$ released from different forages or diets and the solubility of the supplemental sources could have different time patterns after feeding.

When no response to $Ca^{+2}$ for growth was observed with *R. flavefaciens,* possible divalent cation requirements for growth were investigated. Magnesium was the only cation identified as an absolute requirement and only for *R. flavefaciens*. Growth requirements for Mg were then measured in all species, and all responded to increased Mg concentrations, either in maximum growth (Figure 4.16), rate of growth (Figure 4.17) or lag time. *R. flavefaciens* has highest requirement. FS-A3c did not fit with linear or quadratic response, although Mg concentrations affected the growth of the strain (Table 4.9, and Figure 4.16). The results obtained for Mg requirement for growth with FS-S85 are higher than the results obtained by Bryant et al (1959) for the same strain (0.56 mM *vs.* 0.1 mM Mg).

The high requirements for Mg by *R. flavefaciens* and *R. albus* are somewhat logical since both are Gram (+) bacteria and they have higher Mg requirements for cell wall synthesis (Ghuysen and Shockman, 1973). The higher requirements by *R. flavefaciens* than *R. albus* may be due to differences in the composition of the cell wall (Beveridge and Murray, 1980; Beveridge et al, 1982; Beveridge, 1990), or to the mechanisms by which Mg is interchanged between the intracellular and extracellular environments (Moncrief and Maguire et al, 1999; Chamnongpol and Groisman, 2002). Unfortunately, no specific information about this subject is available for rumen bacteria.

Because *R. flavefaciens* C94 tended to have a high max growth and rate of growth at 0 mM $Ca^{+2}$, and had a small, linear, response to increased $Ca^{+2}$ concentration in cellulose degradation; and also has a high Mg requirement for growth; $NH_3$-free medium was used to evaluate a possible role of Mg for *R. flavefaciens* in cellulose degradation. Using a large inoculum of bacteria it was observed that RF-B34b and RF-C94 did not degrade cellulose when Mg was deleted from the medium. No effect on cellulose degradation was observed when $Ca^{+2}$ was omitted.

The summary of results for $Ca^{+2}$ and Mg requirements for cellulolytic rumen bacteria are presented in table 4.18. The criteria used were maximum growth and extent of cellulose degradation.

For growth and $Ca^{+2}$, FS-S85 and RA-7 appear to have 0.47 mM and >0.64 mM $Ca^{+2}$ as requirement for growth, respectively. While no requirement estimations for FS-A3c, RF-B34b and RF-C94 were made, because the lack of solution on contrasts analysis. RA-8 did not respond to $Ca^{+2}$ concentrations on maximum growth, and no requirements can be establish for this cation.

For Mg, only *R. flavefaciens* has an absolute requirement for growth. For FS-A3c no estimation can be made. Because RF-C94 responded linearly to Mg, the requirement may be higher than 0.82 mM Mg. For FS-S85, RA-7, RA-8, and RF-B34b Mg requirements were estimated trough max growth as 0.56, 0.52, 0.51 and 0.54 mM, respectively.

For cellulose degradation only *F. succinogenes* has an absolute $Ca^{+2}$ requirement, either in $NH_3$-normal or $NH_3$-free medium; requirements for FS-A3c are higher than 0.36 mM of $Ca^{+2}$ in $NH_3$-normal cellulose medium and 0.42 mM of $Ca^{+2}$ in $NH_3$-free cellulose medium. Despite the absolute $Ca^{+2}$ requirement for cellulose degradation for FS-S85 no estimation can be made from $NH_3$-normal cellulose medium. FS-S85 in $NH_3$-free medium showed a linear response and then requirement may be higher than 0.64 mM $Ca^{+2}$. When $NH_3$-free medium was used, and rate of degradation was the criterium, with 0.39 and 0.44 mM $Ca^{+2}$ maximum rate of degradation was attained, for FS-A3c and FS-S85, respectively (Tables 4.11 and 4.15).

For cellulose degradation, *R. albus* 7, and *R. flavefaciens,* B34b and C94, responded to increased $Ca^{+2}$ concentrations, apparently RA-7 reach the maximum degradation at 0.28 mM; and RF-B34b and RF-C94 had a significant

linear response, although this can be a mathematical adjustment to the more complex behavior as is evident in the chart, and may not represent the biology of the response (Figure 4.24).

| | Maximum growth | | Maximum degradation | |
|---|---|---|---|---|
| | Cellobiose | | Cellulose | Cellulose $NH_3$-free |
| | [1]$Ca^{+2}$ | [1]Mg | [1]$Ca^{+2}$ | [1]$Ca^{+2}$ |
| ***F. succinogenes* A3c** | n.e. | n.e. | >0.36* | 0.42* |
| ***F. succinogenes* S85** | 0.47 | 0.56 | n.e.* | >0.64* |
| ***R. albus* 7** | >0.64** | 0.52 | 0.28 | - |
| ***R. albus* 8** | n.r. | 0.51 | n.r. | - |
| ***R. flavefaciens* B34b** | n.e. | 0.54* | >0.64 | - |
| ***R. flavefaciens* C94** | n.e. | >0.82* | >0.64 | - |

[1] $Ca^{+2}$ and Mg concentrations as mM.
* For the bacterium and respective substrate there is an absolute cation requirement.
** statistical tendency, (0.05<P>0.10).
**n.e.** no estimable, because no solution for contrasts.
**n.r.** no requirement can be established, because no response to the specific cation concentrations.

Table 4. 18. Summary of $Ca^{+2}$ and Mg requirements for cellulolytic rumen bacteria using maximum growth and extent of cellulose degradation as criteria; $Ca^{+2}$ requirements for growth and cellulose degradation, and Mg requirements for growth.

I have concerns related with the response in cellulose degradation and possible $Ca^{+2}$ requirements for FS-S85, RF-B34b, and RF-C94. FS-S85 look very alike with FS-A3c response, but the lack of solution from the statistical analysis limit the estimation under these conditions. The linear response of *R. flavefaciens* strains does not appear so clear, as was mention before. The possible the role that Mg has in cellulose degradation for *R. flavefaciens* need to be consider. Because when Mg was absent of $NH_3$-free cellulose media no degradation occurs, despite the normal concentration of $Ca^{+2}$. May the variations in the response to increased $Ca^{+2}$ concentrations was the reflection of Mg content in those media, and not the direct effect of $Ca^{+2}$ concentrations. Also a possible interaction between the two cations need to be evaluate. These are aspects that deserve more attention and research.

The determination of mineral requirement for bacteria and their different functions, may appear simple; however, the differences among species and strains and the lack of information about their basic physiological functions in rumen bacteria make this area of research somewhat complex. Without doubt, more research needs to be undertaken, considering the variety of responses obtained. By using a higher number of concentration levels, increasing the number of replicates, using larger size of culture, (particularly for cellulose degradation, so multiple samples can be taken from the same vessel, to decrease the error for been inoculating so many individual tubes) it should be possible to achieve more precision in the data. This should allow better

estimates of requirements and reflect the biology of the bacteria. In this study, the statistic methods used to determine requirements have not always been satisfactory to reach a solution; possibly due to high variation and also because the particular response of the bacteria to the $Ca^{+2}$ or Mg concentrations, which no necessarily describes a linear or quadratic function.

In light of the results obtained in this study, there are many questions that need to be addressed in future research. The role of $Ca^{+2}$ and Mg in the rumen bacterial cell wall (ion channels, enzymes, receptors, lipid composition, etc.). Identify what cellulolytic enzymes are affected (amount, activity) by $Ca^{+2}$ or other cations such as Mg. Identify why $Ca^{+2}$ is an absolute requirement for *F. succinogenes* in cellulose degradation. Is $Ca^{+2}$ having a signalling role for cell division in *F. succinogenes,* as it has in *E. coli*?. Why Mg is required in absolute form for growth and apparently, also, for cellulose degradation for *R. flavefaciens?*.

## CONCLUSIONS

From the present study it is concluded that:

1. The different rumen cellulolytic species studied responded differently to $Ca^{+2}$ and Mg supplementation in the growth medium.

2. No $Ca^{+2}$ requirement could be demonstrated for growth for *Ruminoccocus flavefaciens*.

3. Although relative normal growth was achieved with very low $Ca^{+2}$ concentrations, the $Ca^{+2}$ requirement to maximize growth was higher than expected. The biological importance of the concentration required for maximum response is questionable in the majority of the cases. Relatively low concentrations can permit normal growth, as the small amount of $Ca^{+2}$ contained by the reagent grade minerals used to prepare the media.

4. *Fibrobacter succinogenes* A3c showed some evidence of a special $Ca^{+2}$ requirement. Pleomorphism was observed at lower $Ca^{+2}$ concentrations, suggesting that $Ca^{+2}$ acts as a signal for cell reproduction.

5. Only *Fibrobacter succinogenes,* both strains, has an absolute $Ca^{+2}$ requirement for cellulose degradation.

6. $Ca^{+2}$ requirement for cellulose degradation for *Ruminococcus flavefaciens* and *R. albus* was demonstrated, athough is not an absolute requirement.

7. Only *Ruminococcus flavefaciens* has an absolute Mg requirement for growth.

8. For growth all the species and strains studied responded to increasing Mg concentrations, having an specific requirement.

9. *Ruminococcus flavefaciens* also exhibits a Mg requirement for cellulose degradation.

# APPENDIX

## APPENDIX A

## Stock solutions, and different anaerobic media.

### 1.- Stock solutions:

### 12 % Sodium Carbonate:

Thirty-six g of anhydrous $Na_2CO_3$ are dissolved in deionized $H_2O$ and brought to a final volume of 300 ml. The solution is transferred to a 500 ml round-bottom flask and bubbled with $N_2$ for at least 30 minutes. Tube in 10 ml aliquots under a stream of $N_2$. Sterilize the tubes at 120° C° for 20 min.

### 3% Cysteine·HCl:

Add 300 ml deionized $H_2O$ to 500 ml round-bottom flask. Heat to dispel gasses while gassing with $N_2$. When fairly well reduced (about 20-30 minutes), add 9.0 g cysteine·HCl. Continue gassing with $N_2$ for 15 minutes. Tube in 5.0 and 10.0 ml aliquots under $N_2$. Autoclave in tubes at 120° C° for 20 minutes.

### 0.1% Resazurin solution:

Add 0.1 g of resazurin to 100 ml volumetric flask and make to volume with deionized water.

### 0.1% Hemin solution:

100 mg of hemin is dissolved in 0.02% NaOH and then diluted to a final volume of 100 ml with deionized water.

**Vitamin mixture:** (1 liter) (Scott and Dehority, 1965)

200 mg Pyridoxine HCl

200 mg Riboflavin

200 mg Thiamine HCl

200 mg Nicotinamide

200 mg Ca-D-Pantothenate

10 mg Paraaminobenzoic acid (PABA)

5 mg Folic acid

5 mg Biotin

0.5 mg $B_{12}$ Vitamin,

make to 1 liter volume with deionized water.

**Volatile fatty acid mix:** (31 ml) (Caldwell and Bryant, 1966)

17 ml Acetic acid (54.84 % v/v)

6 ml Propionic acid (18.75%)

4 ml Butyric acid (12.9%)

1 ml Isobutyric acid (3.23%)

1ml n-Valeric acid (3.23%)

1 ml Isovaleric acid (3.23%)

1 ml 2-methyl Butyric acid (3.23%)

**Mineral solution I:** (Bryant and Burkey, 1953)

0.3 % $K_2HPO_4$

**Mineral solution II:** (Bryant and Burkey, 1953)

0.3 % $KH_2PO_4$

0.6 % $(NH_4)_2SO_4$

0.6 % NaCl

0.06 % $MgSO_4$

0.06 % $CaCl_2 \cdot 2H_2O$

**Mineral solution A:** (Scott and Dehority, 1965)

4.5 g/l $KH_2PO_4$

**Mineral solution B:** (Scott and Dehority, 1965)

4.5 g/l NaCl

4.5 g/l $(NH_4)_2SO4$

0.33 g/l $CaCl_2 \cdot 2H_2O$

0.25 g/l $MgSO_4$

0.10 g/l $MnSO_4 \cdot H_2O$

0.10 g/l $FeSO_4 \cdot 7H_2O$

0.10 g/l $ZnSO_4 \cdot 7H_2O$

0.01 g/l $CoCl_2 \cdot 6H_2O$

**Mineral solution B, $NH_3$-free:** (modified from Scott and Dehority, 1965)

4.5 g/l NaCl

0.33 g/l $CaCl_2 \cdot 2H_2O$

0.25 g/l $MgSO_4$

0.10 g/l $MnSO_4 \cdot H_2O$

0.10 g/l $FeSO_4 \cdot 7H_2O$

0.10 g/l $ZnSO_4 \cdot 7H_2O$

0.01 g/l $CoCl_2 \cdot 6H_2O$

**Mineral solution divalent-cation-free:** (modified from Scott and Dehority, 1965)

4.5 g/l NaCl

4.5 g/l $(NH_4)_2SO_4$

3.0 g/l $KH_2PO_4$

**Mineral solution B, selective cation-free:** (modified from Scott and Dehority, 1965)

To achieve selective cation-free media, the cation to be eliminated was not included in Mineral solution B. Thus Mineral solution B, $Ca^{+2}$-free, was prepared by eliminating $CaCl_2$; Mineral solution B, Mg-free was prepared by eliminating $MgSO_4$; Mineral solution B, $Ca^{+2}$-Mg-free was prepared by eliminating both $CaCl_2$ and $MgSO_4$.

**Phosphate buffer:**

10 ml 7.5% $NaH_2PO_4$

10 ml 7.5% $K_2HPO_4$

280 ml of deionized $H_2O$

Tube 9 ml aliquots without gassing, stopper with rubber stoppers and autoclave at 120° C° for 20 minutes.

**2.- Media:**

**Rumen fluid-glucose-cellobiose-agar slants medium (RGCA):** (600 ml)

90 ml Mineral solution I

90 ml Mineral solution II

0.6 ml of 0.1% Resazurin solution

1.2 g glucose

1.2 g cellobiose

9.0 g agar

150 ml distilled water

240 ml rumen fluid (Strained through a double layer of cheesecloth and centrifuged 10 minutes at 1000 x g).

Gas with $CO_2$, bring almost to a boil over Bunsen burner, seal anaerobically, and autoclave for 5 minutes at 100° C°. Place flask in 45° C° water bath, open anaerobically under $CO_2$ and add 20 ml of 12% $Na_2CO_3$ and 10 ml of 3%

cysteine HCl, continue gassing, check and adjust pH to 6.7-6.9 if needed with NaOH or HCl. Tube 7.0 ml aliquots anaerobically into 16 x 150 mm culture tubes from slants. Autoclave at 120° C° for 20 minutes in racks.

**Complete liquid medium (cellobiose or cellulose substrate) with varing levels of $Ca^{+2}$ or Mg:**

To prepare 300 ml:

60 ml mineral solution A

60 ml mineral solution B ***** / ******

0.3 ml 0.1% rezasurin

0.3 ml 0.1% hemin

3 ml vitamin mix

1.5 g cellobiose (or 75 ml 3% cellulose ball-milled, Sigmacell® Type 20)

0.5 g trypticase

1.35 ml VFA mix

155 ml (or 85 ml) of deionized water

Adjust to pH 6.7- 6.9 with NaOH/HCl (1N)

Bubble with $O_2$-free $CO_2$, to dispel gasses; add 10 ml 12% Sodium carbonate and continue gassing with $CO_2$ for 20 to 30 minutes. Add 10 ml 3% cysteine and keep gassing with $CO_2$. Add $CaCl_2$ or $MgSO_4$, according to the experimental medium and concentrations desired, continue gassing until reduced. Check and adjust to pH 6.7-6.9 with NaOH or HCl. Tube 7 ml under $CO_2$. Sterilize at 120° C° by 20 minutes.

***** To prepare media with different $Ca^{+2}$ concentrations, $Ca^{+2}$-free Mineral solution B was used, and to achieve the different $Ca^{+2}$ concentrations different amounts of $CaCl_2$ were added to the medium.

** To prepare media with different Mg concentrations, Mg-free Mineral solution B was used, and to achieve the different Mg concentrations different amounts of $MgSO_4$ were added to the medium.

Due to the special requirement for *R. albus* 8, Phenylacetic acid (PAA) and Phenypropionic acid (PPA) were added to the medium (Stack et al, 1983), to achieve 0.25 uM as final concentration (Reveneau, 2001).

**Divalent-Cation-free cellobiose liquid media:**

To prepare 300 ml:

50 ml mineral solution divalent cation free

0.3 ml 0.1% rezasurin

0.3 ml 0.1% hemin

3 ml vitamin mix

1.5 g cellobiose

0.5 g trypticase

1.35 ml VFA mix

225 ml of deionized water

Adjust to pH 6.7- 6.9 with NaOH/HCl (1N)

Bubble with $O_2$-free $CO_2$, dispel gasses add 10 ml 12% Sodium carbonate and continue gassing with $CO_2$ for 20 to 30 minutes. Add 10 ml 3% cysteine and continue gassing with $CO_2$. Check and adjust to pH 6.7-6.9 with NaOH or HCl. Tube 7 ml under $CO_2$. Sterilize at 120° C° by 20 minutes.

**$NH_3$-free media cellulose liquid media**

To prepare this media, $NH_4SO_4$, hemin and trypticase were eliminated, and cysteine was reduced to half of normal concentration.

To prepare 300 ml:

60 ml mineral solution A

60 ml mineral solution B without $(NH_4)_2SO_4$

0.3 ml 0.1% rezasurin

3 ml vitamin mix

75 ml 3% cellulose ball-milled

1.35 ml VFA mix

90 ml of deionized water

Adjust to pH 6.7- 6.9 with NaOH/HCl (1N)

Bubble with $O_2$-free $CO_2$, to dispel gasses. Add 10 ml 12% Sodium carbonate and continue gassing with $CO_2$ for 20 to 30 minutes. Add 5 ml 3% cysteine and keep gassing with $CO_2$. Check and adjust to pH 6.7-6.9 with NaOH or HCl. Tube 7 ml under $CO_2$. Sterilize at 120° C° by 20 minutes.

For $NH_3$-free with different $Ca^{+2}$, different amounts of 1M $CaCl_2$ were added to obtain the experimental concentrations.

For $NH_3$-free $Ca^{+2}$-free / Mg-free / $Ca^{+2}$-Mg-free, specific mineral solution B $NH_3$-free were prepared, deleting the respective cation / cations.

## APPENDIX B

**Effect of consecutive transfer on $Ca^{+2}$-free, Mg-free, $Ca^{+2}$-Mg-free and Normal media on growth (Max OD) by the predominant rumen cellulolytic bacteria.**

| | Liquid Growth Medium (cellobiose)[1] | | | |
|---|---|---|---|---|
| | **$Ca^{2+}$-free** | **Mg-free** | **$Ca^{2+}$- Mg-free** | **Normal media** |
| ***F. succinogenes* S85** | | | | |
| 1st transfer | 1.04 (25h)[2] | 0.70 (25h) | n.g. | 1.35 (20h) |
| 2nd transfer | 1.00 (60h) | 0.80 (36h) | | |
| ***F. succinogenes* A3c** | | | | |
| 1st transfer | n.g.[3] | 0.70 (20h) | n.g. | 1.4 (15h) |
| 2nd transfer | | 0.66 (16h) | | |
| 3rd transfer | | 0.66 (16h) | | |
| ***R. albus* 7** | | | | |
| 1st transfer | 1.19 (14h) | 0.52 (18h) | 0.60 (25h) | 1.1(12h) |
| 2nd transfer | 1.06 (11h) | 0.53 (17h) | 0.56 (40h) | |
| 3rd transfer | 1.1(12h) | 0.45 (22h) | | |
| ***R. albus* 8** | | | | |
| 1st transfer | 0.23 (142h) | 0.43 (170h) | 0.14 (167h) | 0.56 (129h) |
| ***R. flavefaciens* B34b** | | | | |
| 1st transfer | 0.90 (20h) | n.g. | n.g. | 0.88(17h) |
| 2nd transfer | 0.86 (17h) | | | |
| 3rd transfer | 0.87 (19h) | | | |
| ***R. flavefaciens* C94** | | | | |
| 1st transfer | 0.92 (27h) | n.g. | n.g. | 0.74 (28h) |
| 2nd transfer | 0.95 (29h) | | | |
| 3rd transfer | 0.45 (24h) | | | |

[1] Calcium concentration in $Ca^{+2}$-free medium: 0.046 and 0.014 mM as total and ionized, respectively.
Magnesium concentration in Mg-free medium: 0.3 ug/ml total Mg.
Normal medium: 0.36 mM $Ca^{+2}$ and 0.41 mM Mg
[2] Maximum Od and hours to reach max OD [3] n.g. no growth.

## APPENDIX C

### Quadratic functions used to estimate the maximum point of the curve.

**1. $Ca^{+2}$ Growth experiments.-**

**- Maximum growth (A)** (OD units)

FS-S85 $A=1.2404 + 0.5424x - 0.05805x^2$ ($P=0.0205$; $r^2=0.27$).

**- Rate of growth (B)** (OD units/hour)

FS-A3c $B= 0.1468 + 0.2745x - 0.3216x^2$ ($P=0.0001$; $r^2=0.72$)

RA-7 $B= 0.2607 - 0.1375x + 0.1905x^2$ ($P=0.2131$; $r^2=0.12$)

**2. Magnesium Growth experiments.-**
(Equations calculated with Magnesium concentration in mg/ml; and results are reported in tables and figures as mM)

**-Maximum growth (A)** (OD units)

FS-S85 $A= 0.9851 + 65.1899x - 2385.65x^2$ ($P=0.0001$; $r^2=0.72$ )

RA-7 $A= 0.9296 + 58.1543x - 2287.38x^2$ ($P=0.0073$; $r^2=0.37$)

RA-8 $A= 0.4861 + 17.3054x - 704.8414x^2$ ($P=0.0203$; $r^2=0.31$)

RF-B34b $A= 0.4566 + 71.1056x - 2715.37x^2$ ($P=0.0001$; $r^2= 0.78$)

**-Rate of growth (B)** (OD units/hour)

RF-B34b $B= 0.0478 + 7.48237x - 2777.69x^2$ ($P=0.0258$; $r^2=0.35$)

**-Lag time (C)** (hours)

RF-B34b C= 14.4296 - 572.90X + 16220$x^2$ (P=0.0021; $r^2$=0.52)

RF-C94 C= 22.4134 – 2165.48x + 81667$x^2$ (P=0.0004; $r^2$=0.60)

**3. $Ca^{+2}$ Cellulose degradation experiments**
(Equations calculated as g, in tables and figures are expressed as mg)

**-Maximum degradation (A)** (g)

RA-7 A= 0.03452 + 0.01497x – 0.02661$x^2$ (P=0.1440; $r^2$=0.12)

**-Lag time (C)** (hours)

RA-7 C= 14.19354 – 35.8740x + 53.5384$x^2$ (P<0.0001; $r^2$ =0.54)

RF-C94 C= 17.5969 – 2.80475x - 14.0903$x^2$ (P=0.2659; $r^2$=0.06)

**4. $Ca^{+2}$ Cellulose degradation $NH_3$-free experiments**
(Equations calculated as g, in tables and figures are expressed as mg)

**-Maximum degradation (A)** (g)

FS-A3c A= 0.00869 + 0.01549x – 0.01644$x^2$ (P=0.0328; $r^2$=0.35)

**-Rate of degradation (B)** (g/hour)

FS-A3c B=0.00022248 + 0.00209x – 0.00258$x^2$ (P=0.0106; $r^2$=0.43)

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