CHARACTERIZATION β-1,3-1,4-GLUCANASE FROM *Bacillus subtilis* GN156 FOR SILAGE FERMENTATION

INTRODUCTION

Since dairy products and the demand for premium meat quality have recently increased, livestock farming in Thailand has therefore grown rapidly. However, the lack of grass roughage in dry period is a big problem. Thus, both good quality and large amount of grass commonly used as roughage are needed. Therefore, grass storage in a form of silage has been proposed to benefit both preservation and nutritional improvement.

Silage is a fermented feed produced and stored under anaerobic condition. During ensiling, water-soluble carbohydrates (WSC) in the crop is consumed by lactic acid bacteria to produce lactic acid and some acetic acid that causes the pH decrease of ensiled material. Consequently, spoilage microorganisms in silage are also inhibited (Henderson, 1993). The major problem of grass ensiling in Thailand is the lack of a sufficient amount of suitable WSC. A shortage of sugar will cause poor fermentation due to lactic acid reduction, increase in pH, ammonia and also other fermentation products (Stetälä, 1989). Generally, tropical grass contains mainly cellulose, hemicellulose and other carbohydrates which cannot be metabolized by lactic acid bacteria. To improve fermentability, it is necessary to breakdown carbohydrates into soluble and consumable form. Addition of enzymes to forage is becoming more common for the purpose of enhancing the WSC in the ensiling process (Stefanie *et al.*, 2000). Applications of hydrolytic enzymes from fungi have been previously studied. Jaakkola (1990) found that addition of an enzyme mixture of cellulase, hemicellulase and glucosidase from different fungal sources does not affected cell wall degradation. However, when higher cellulose concentration was added, the degadation activity increased, while commercial hydrolytic enzymes from *Acremonium* sp. applied to various varities of grass in Thailand did not improve silage

quality (Ohmomo *et al.*, 1995). It seems that the proper enzyme mixture has the potential to improve degradation activity.

Bacillus subtilis GN156 was screened from silage and deposited in the Department of Biotechnology, Department of Agro-Industry, Kasetsart University. It exhibits the effective grass degradation and enhances silage quality (Buwjoom, 1998).

In this study, purification and characterization of hydrolytic enzymes from *B. subtilis* GN156 were studied. In addition, synergism of hydrolytic enzymes obtained in grass degradation was also studied.

OBJECTIVES

The objectives of this study are:

1. Study the enzyme system of *B. subtilis* GN156, find out the most effective enzyme involving in grass degradation and characterization of its properties.

2. Purify and characterize of effective hydrolytic enzymes involved in grass degradation.

3. Study the synergistic actions of the combination systems to increase hydrolysis efficiency.

LITERATURE REVIEW

1. Silage

1.1 Ensiling

Ensiling is a forage preservation method based on a spontaneous lactic acid fermentation under anaerobic condition. The epiphytic lactic acid bacteria ferment the water-soluble carbohydrates (WSC) in the crop to lactic acid, and to a lesser extent to acetic acid. Due to the production of these acids, the pH of the ensiled material decreases and spoilage microorganisms are inhibited (Woolford, 1985; Stokes, 1992; Henderson, 1993; Sheperd *et al.*, 1995; Sheperd and Kung, 1996b; Stafanie e*t al*, 2000). The successful outcome of the ensiling process depended on the silage microflora, which can be divided into 2 groups. The desirable microorganisms are the lactic acid bacteria and the undesirable ones are the organisms that can cause anaerobic spoilage (e.g. clostridia and enterobacteria) or aerobic spoilage (e.g. yeasts, bacilli, listeria and molds). Many of these spoilage organisms do not only decrease the nutritional value of the silage, but also have a detrimental effect on animal health and/or milk quality (e.g. listeria, clostridia, molds and bacilli) (Stafanie *et al*., 2000)

The ensiling process can be divided into 4 stages (Weinberg and Muck, 1996., Merry *et al*., 1997. *cited by* Stafanie *et al.*, 2000)

1.1.1 Aerobic phase: This phase normally takes only a few hours in which the atmospheric oxygen present between the plant particles is reduced, due to the respiration of the plant material and aerobic and facultative aerobic microorganisms such as yeasts and enterobacteria. Furthermore, plant enzymes such as proteases and carbohydrases are active during this phase, provided the pH is still within the normal pH range for fresh forage juice of pH 6.0-6.5.

1.1.2 Fermentation phase: This phase starts when the silage becomes anaerobic, and it continues for several days to several weeks, depending on the properties of the ensiled forage crop and the ensiling conditions. The fermentation proceeds successfully by the development of lactic acid bacteria, which becomes the predominant population during this phase. It causes the pH to decrease to 3.8 - 5.0 due to the production of lactic and other acids.

1.1.3 Stable phase: As long as air is prevented from entering the silo, relatively little microbial activity occurs. Most microorganisms slowly decrease in numbers. Some acid tolerant microorganisms survive as an inactive state, others such as clostridia and bacilli survive as spore-forming. Only some acid tolerant proteases and carbohydrases and some specialized microorganisms, such as *Lactobacillus plantarum* and *Lactobacillus buchneri* etc are still active at a low level.

1.1.4 Feed-out phase or aerobic spoilage phase: This phase starts as soon as the silage gets exposed to air. The process of spoilage can be divided into 2 stages. The onset of deterioration is due to the degradation of preserving organic acids by yeasts and occasionally acetic acid bacteria. This will cause a rise in pH, and thus the second spoilage stage is started, which is associated with increasing temperature, and activity of spoilage microorganisms such as bacilli. The last stage also includes the activity of many other (facultative) aerobic microorganisms such as moulds an enterobacteria. Aerobic spoilage occurs in almost all silages that are opened and exposed to air. However the rate of spoilage is highly dependent on the numbers and activity of the spoilage organisms in the silage. Spoilage losses of 1.5 - 4.5 % dry matter loss/day can be observed in affected areas. These losses are in the same range as losses that can occur in airtight silos during several months of storage (Honing and Woolford, 1984 *cited by* Woolford, 1985)

To avoid failures, it is important to control and optimize each phase of the ensiling process. The proper management of the silo, filling techniques and the right size of grass will help to minimize the amount of oxygen present between the plant particles in the silo (Shinoda *et al*., 2001). Good harvesting techniques combined with

good silo filling techniques will thus minimize the loss of water-soluble carbohydrates, which an available for lactic acid bacteria in fermentation phases. To optimize and stabilize the fermentation process, silage additives are usually used such as molasses and enzymes.

1.2 Control of fermentation

The quality of silage depends on fermentation efficiency of the organisms in terms of acid production and dry matter (DM) content of the forage. At intermediate levels of DM, clostridial inhibition is derived from the combined influence of acids and moisture availability. Generally crops are ensiled directly after cutting at a DM level of 200 g/kg resulting lactic acid fermentation with the pH drop to 4.2 to stabilize the process. At DM content of less than 150 g/kg, as would be the case of silage crops harvested in wet weather, the excessive moisture can counteract the preservative effect of the acid fermentation. Under this condition clostridia may not be inhibited at a pH as low as 4.0. At high DM level to 300 g/kg, those clostridia are inhibited by lack of moisture whilst lactic acid bacteria can tolerate DM environments as high as 700 g/kg. (Woolford, 1985) The presence of suitable, osmotolerant lactic acid bacteria could become a limiting factor in the ensiling process by these high dry matter silages. But these bacteria represent only a small percentage of the indigenous microflora on forage crops, especially forage with DM content above 50 % is difficult to ensile. (Staudacher *et al*., 1999 *cited by* Stefanie *et al*., 2000) Therefore silage fermentation from moderate DM control forages used as raw materials should be one way to optimize lactic acid production and inhibit clostidial activity.

The major problem of the ensiling fermentation process is low numbers of lactic acid bacteria which causes poorly silage fermentation. This can be due to lack of a sufficient amount of suitable water-soluble carbohydrates in forage. To overcome the insufficient fermentable substrates, many additives are used in silage-making such as direct addition of sugar (e.g. molasses) or addition of enzymes to release extra sugar from the crop. (Stefanie *et al.*, 2000)

1.3 Additive improving silage fermentation

In order to improve the ensiling process, there are many kinds of additives used. Silage additives may be chemical or biological substances, and can be identified within a few categories by the modes of action (Table 1).

Table 1 Categories of silage additive

Additive category	Selection of active ingredients	Remark
Fermentation stimulants	Lactic acid bacteria Sugars (molasses) Enzymes	To encourage rapid proliferation of lactic acid bacteria or stabilizes dominance of these organisms
Fermentation inhibitors	Formic acid Lactic acid Mineral acids Sodium chloride Sulfite salts	To inhibit the microflora in general
Nutrients	Urea Ammonia Minerals	Can improve aerobic stability
Absorbents	Dried sugar beet pulp straw	To reduce moisture content
Specific antimicrobial agents	Nitrite salt Antibiotics	To discourage clostridia directly

Source: adapted from McDonald *et al*.(1991) *cited by* Stefanie (2000), Woolford (1985).

Tropical grass biomass increases with maturity, but decreases in nutritive value. To solve this problem these grasses are frequently ensiled at an early growing stage. However, young plants have a high moisture content, high buffering capacity and a low level of soluble carbohydrates. According to Woolford (1984) *cited by* Neiva *et al*. (2001), these factors have a negative influence on the fermentation process. To increase water soluble carbohydrates in silage-making, there are 2 kinds of additive improving silage fermentation which are usually used.

1.3.1 Molasses

Molasses is the carbohydrate source used most frequently as the fermentation stimulant and employed in silage-making for crops low in soluble carbohydrates such as grass and legumes (Henderson, 1993). Since molasses has a high viscosity, it is diluted with water before use. This disadvantage results in a high moisture content in silage and clostridial spoilage can thereby be induced. (Woolford, 1984 *cited by* Neiva *et al*., 2001) Therefore, to obtain the maximum benefit it must be used in relatively high concentration (about 40 - 50 g/kg) with an optimal proportion of the added carbohydrate and DM content of the crop. On the other hand, if the treated crop has a very low DM content, the addition of carbohydrate may be lost in the effluent during the first few days of ensilage (Mhlbach, 2000)

However, many studies propose the addition of molasses to low DM content crop such as grass for increasing WSC. Boin (1975) found that adding 3 % sugarcane molasses (w/w fresh basis) to Napier grass (12.9 % DM, 6.6 % WSC) could produced silage of good fermentation quality (*cited by* Mhlbach, 2000)

Guinea grass with 4 weeks (18.6 % DM) and 8 weeks (26.5 % DM) of growth was ensiled with and without 4 % molasses in 400 g laboratory silos, resulting in pH value of 4.4 - 5.4 and 4.0 - 4.7 and ammonia-nitrogen value of 23.5 - 35.3 and 15 - 39, respectively. The molasses treated silages did exhibit better quality than the untreated ones according to the lower pH and ammonia-nitrogen value. (Esperance *et al*., 1985 *cited by* Mhlbach, 2000)

However, Neiva *et al*. (2001) have not observed significant differences in ammonia-nitrogen and pH value between the Napier grass silages treated with 4 levels (0, 5, 10, 15 %) of dehydrated sugarcane. Whilst, the DM levels increased and crude protein levels decreased linearly with the addition of dehydrated sugarcane. Similar results were obtained by Almeida *et al.* (1986) and Tosi *et al.* (1989) as well. The conclusion may therefore be drawn that the wilted Napier grass made well-preserved silage without sugarcane or bagasses.

1.3.2 Enzymes

The addition of enzymes to forage is becoming more common for the purpose of enhancing the breakdown of forage cell walls during the ensiling process. When water-soluble carbohydrates are limiting, enzyme additives should improve fermentation characteristics by releasing soluble sugars to support the growth of lactic acid bacteria during the fermentation process, resulting in silage with a lower final pH and higher lactic acid content. Enzyme additives may also degrade portions of the plant cell wall during storage, thereby reducing fiber content and potentially improving animal performance. (Sheperd and Kung, 1996a)

Summarized in Table 2 are the effects of lactic acid bacteria and cell wall degrading enzymes on the fermentation of grass-legume silage. The treated silage with the combination of lactic acid bacteria plus cell wall degrading enzymes have a higher lactic acid content than untreated control silage , whilst pH and cell wall components are decreased (Harrison *et al*., 1994)

Similar improvements in fermentation quality or reductions in structural carbohydrate have been reported with forages after ensilage with enzymes. (Sheperd *et al.*, 1995; Stokes, 1992; Chen *et. al.*, 1994)

Moreover, Chen *et al.* (1994) demonstrated that treatment of haycrop silage with a commercially available enzyme-inoculant mixture improved silage fermentation and composition. When dairy cows consumed treated silage

which had high quality, there were higher rate of fermentation in the rumen and improved milk production. In contrast, treatment of corn silage with enzyme-inoculant mixture did not improve silage preservation or composition, ruminal degradation of silage and cow performance.

Table 2 Effect of lactic acid bacteria and cell wall degrading enzymes on composition of grass-legume silage

Treatment	Untreated	Lactic acid Bacteria	Cell wall degrading enzymes	Lactic acid bacteria plus cell wall degrading enzymes
pH	4.45	4.04	4.17	4.02
Lactic acid (% of DM)	4.25	5.95	5.26	6.22
NDF reduction[1] (%)	0	0	1.8	3.5
ADF reduction[1] (%)	0	NS[2]	NS	4.0

Source : Harrison *et al.* (1994)

[1]Percentage of reduction from preensiled fiber composition

[2] $p > 0.10$

Although many studies have been reported in either fermentation or animal production when silage was treated with additives, results of some experiments conflicted. Stokes (1992) found that silage mixed with an inoculant and an enzyme system did not improve the silage fermentation compared to the control (Table 3). This might have been due to the enzyme having no action in the silage environment.

Table 3 Silage fermentation characteristics

	Silage treatment			
	Control	Enzyme Treatment	Inoculant treatment	Mixed enzyme and inoculant treatment
DM (% fresh weight)	30.70	28.11	32.22	29.40
PH	4.25	4.04	4.06	4.22
Lactic acid (% of DM)	9.71	7.42	10.92	8.69

Source : Stokes (1992)

Sheperd and Kung (1996b) found that a commercial enzyme could be used to decrease the fiber content of corn silage but had minimal value relative to improving the silage fermentation.

1.4 Grass silage

Grass silage, which has undergone a good fermentation, would be defined by pH of < 4.5, a predominance of lactic acid versus acids, ammonia N content of < 1 % of DM and < 0.5 % butyric acid in DM (Harrison *et al.*, 1994)

Grass silage has been considered as an important source of nutrients for dairy cattle, for the prolonged and intensive feeding of high-producing cows (Ingham, 1949). Nowadays grass silage is a part of the grassland program because it is good for the cow, good for the milk, and good for the budget. But it does not mean that feeding animal with grass silage can always cause good animal health and productivity. If dairy cows are fed low quality silage, it is impossible to guarantee their good health and productivity. The quality of grass silage is significant for dairy farms (Chen *et al.*, 1994.; Harrison *et al.*, 1994).

The substrates for the fermentation occurring in the silage, are very critical factors affecting silage quality. A shortage of sugar will cause reduction in the lactic acid content in the silage, increase in pH, ammonia and also other fermentation products typical for poor fermentation (Stetälä, 1989). The major problem of the grass ensiling process in Thailand is a lack of sufficient amount of suitable water soluble carbohydrates. One way to overcome this problem is enzyme addition to release sugars from structural carbohydrates of the crop (Stefanie *et al*., 2000). Therefore, understanding of plant cell structure is necessary for the use of enzymes to degrade structural carbohydrates with efficiency.

2. Grass components

Carbohydrate is a major component of forage crops which can be divided into 2 groups as non-structural carbohydrate and structural carbohydrate. The first one can be dissolved in water at room temperature and is called water soluble carbohydrate (WSC). Fructose, glucose, sucrose and fructosans are the principal sugars in the group of water soluble carbohydrates and are easy to use by lactic acid bacteria during ensiling, (Woolford, 1985) but they are of low content in grass.

Structural carbohydrate, the second group of carbohydrate in forage crops, is composed of complex components including cellulose, hemicellulose, and pectins. All carbohydrates present in grass are shown in Table 4. The most resistance part to degradation is lignin which is not a carbohydrate but a polyphenolic substance. While lignin, the most resistant part in degradation is not a carbohydrate but is a phenolic substance, which supports and protects the cellulose in the plant cell.

Table 4 Carbohydrate content of grasses, % of dry matter

Components	Tropical grasses	Temperate grasses
Sugar	5	10
Fructosans	0	1-25
Starch	1-5	0
Pectin	1-2	1-2
Cellulose	30-40	20-40
Hemicellulose	30-40	15-25

Source : Van Soest, 1982 *cited by* Stetälä (1989)

2.1 Cellulose

Cellulose is the principle cell-wall component of plants and hence the most abundant organic compound, and the most abundant carbohydrate, on earth. (Whistler and BeMiller, 1997; Hon and Shiraishi, 1991) It is a high molecular weight, linear, insoluble homopolymer of repeating β-D-glucopyranose units joined by 1,4-glycosidic linkages as shown in Figure 1.

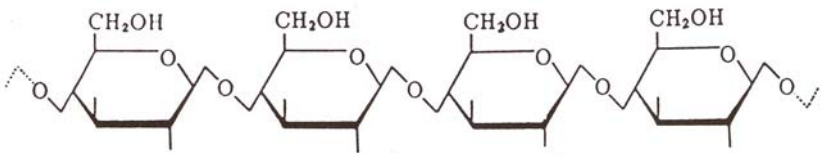


Figure 1 Structure of cellulose
Source: Lee, 1992

2.2 Hemicellulose

The designation *hemi*, meaning half, was included as part of the name by early investigators because of their belief that the hemicelluloses were on their way to becomimg cellulose. However, hemicellulose is defined as a separate family of

unrelated polysaccharide structures (Whistler and BeMiller, 1997). They vary in amount and in structure, depending on the plant type and the location of the cells within the plant. Many hemicelluloses are fairly long polysaccharide chains with frequent branches consisting of one to several sugar units, often of different sugar types (Table 5).

Table 5 Type and structure of hemicellulose

Type of Hemicellulose	Structure	
	Backbone	Branch chain
Xylan	β-1,4 linked xylose	α-linked-4-0-methylglucuronic acid on C2 of some xylose residues. α-linked arabinose or acetyl ester on C2 or C3 of some xylose residues
Mannan and galactomannan	β-1,4 linked mannose	galactose linked α-1,6 to mannose galactose linked β-1,6 to mannose
Glucuronomannan	β-1,4 linked mannose and β-1,4 linked glucuronic acid	and arabinose linked β-1,3 to mannose
Xyloglucan	β-1,4-linked glucose	xylose or fuc α-1,2-Gal β-1,2 or ara-1,2 linked α-1,6 to glucose backbone
Callose	β-1,3-glucan	
β-1,3 and β-1,4- glucan	Mixed-link glucan of 1,3 and 1,4 linkages	
Arabinogalactan II	β-1,3 and β-1,6-linked galactose	arabinose linked β-1,3 to galactose

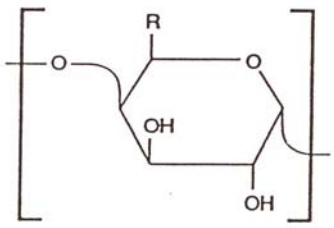
Source : Brett and Waldron (1996)

The most common hemicellulose in annual plants, especially in farm crops, consist of a core composed of a chain or chains of (1,4) linked β-D-xylopyranose units. Some are essentially linear molecules with only a few short side chains. Others are highly branched, bushlike structures with short side chains on a central core structure. The short side chains are from one to a few units long and are usually composed of L-arabinofuranose, D-galactopyranose, D-glucuronopyranose, and/or 4-O-methyl-D-glucuronopyranose units. Occasionally present are L-rhamnopyranose, L-arabinopyranose, L-galactopyranose, and/or L-fucopyranose unit and/or various methylated sugars.

For the mature grass, arabinoxylans and β-1,3-1,4-glucans are the major components (Brett and Waldron, 1996). Especially, β-1,3-1,4-glucans which are restricted in the monocots of the family Poaceae (cereals and grasses).

2.3 Pectin

Pectin is a linear chain of (1,4)-linked α-D-galactopyranosyluronic acid units.Pectinis, therefore, a α-D-galacturonan i.e., a poly (α-D-galactopyranosyluronic acid) or a α-D-galacturonoglycan. (Figure 2) Some pectins contain covalently attached, highly branched arabinogalactan chains and/or short side chains composed of D-xylosyl units on the rhamnogalacturonoglycan backbone. Neutral sugars, rhamnose, arabinose and galactose, may also present in the branch chains (Table 6).



$R = -CO_2CH_3, -CO_2^-, or -CO_2H$

Figure 2 Representative segment of anonamidated α-D-galacturonan (i.e., pectin) molecule (Whistler and BeMiller, 1997).

Table 6 Type and structure of pectin

Type of Hemicellulose	Structure	
	Backbone	Branch chain
Rhamnogalacturonan	α-1,4 linked galacturonic acid and α–1,2-linked rhamnose	arabinose and galactose linked to rhamnose.
Arabinan	α-1,5-linked arabinose	α-1,2 or α-1,3-linked arabinose
Galactan	β-1,4-linkec galactan or 1,6-linked galactan	
Arabinogalactan I	β-1,4-linked galactan	α-1,5-linked arabinose on C3 of galactose
Homogalacturonan	α-1,4-linked galacturonic acid	
Rhamnogalacturonan II	A complex structure includes galacturonic acid , rhamnose , arabinose and galactose in the ratio of 10 : 7 : 5 : 5	

Source : Brett and Waldron (1996)

Although, the amount of pectin in grass is low when compared with the β-1,3-1,4-glucan content, many reports of plant cell hydrolysis have suggested that pectinase appeared to be the most important enzyme. Because pectin is a complex structural polysaccharide that occurs mainly in the middle lamella of plant cell walls, functioning as a cementing agent in the cell wall. Pectin hydrolysis is needed to allow other enzymes attack additional plant components (Moloney *et al.,* 1984; Considine *et al.,* 1988; Spagnuolo *et al.;* 1997).

2.4 Lignin

Lignin is a phenolic polymer that is laid down after cell elongation has ceased The precursors of lignin are coumaryl , coniferyl and sinapyl alcohols (Figure 3) which give rise to the ρ–coumaryl , guiacyl and sinapyl propane subunits, respectively. These precursors are linked by a wide variety of bonds and formed an irregular pattern. Lignin is therefore the most complex polymer among naturally occurring high-molecular-weight materials. (Figure 4) (Hon and Shiraishi, 1991., Brette and Waldron, 1996.) and its chemical structure is not yet completely known.

The presence of many complex carbon-to-carbon linkages between the units makes it difficult to degrade the polymer to low-molecular-weight fragments. Furthermore, it has not yet been possible to isolate all components of lignin completely from plant tissues without engendering structural change (Hon and Shiraishi, 1991).

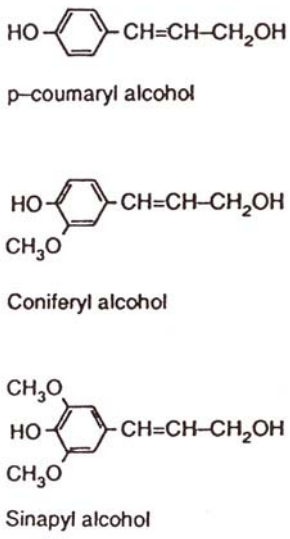


Figure 3 The aromatic alcohols that are precursors in the synthesis of lignin
Source: Brett and Waldron, 1996

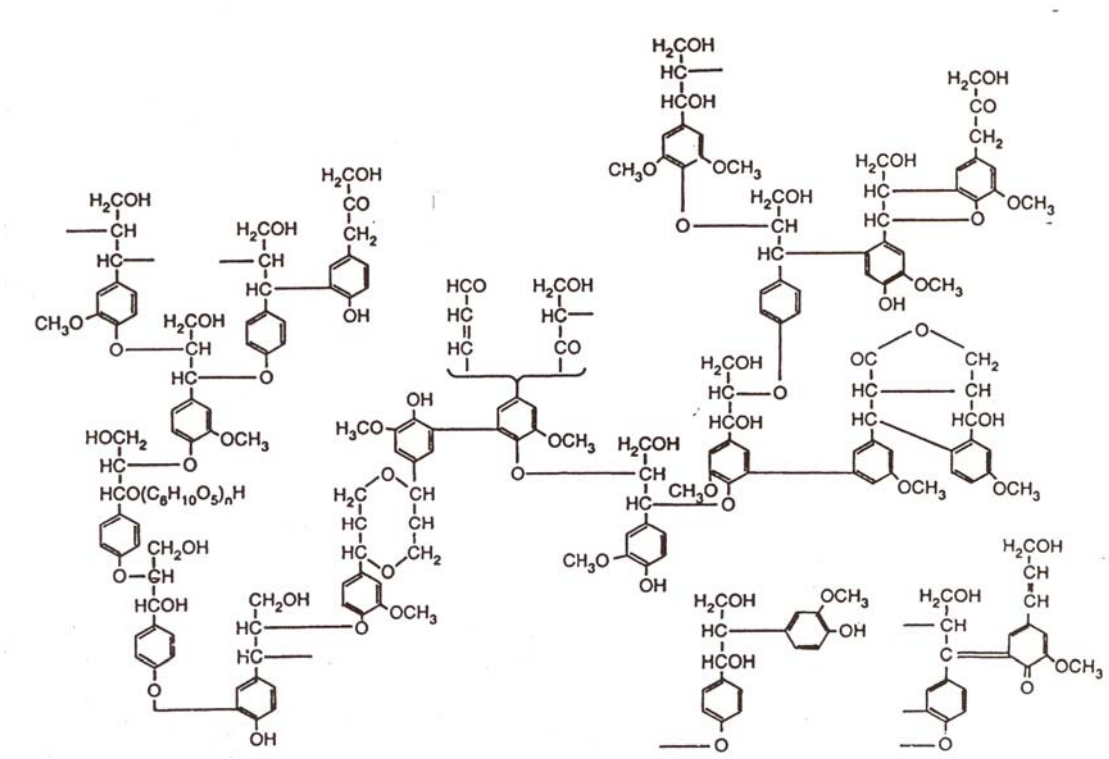


Figure 4 Partial structure of spruce lignin

Source: Freudenberg and Neish, 1968 c*ited by* Brett and Waldron, 1996

2.5 Architecture of grass cell wall

Plant tissue is a natural composite material and a chemical complex of cellulose, lignin, hemicellulose and extractives. Cellulose provides strength and flexibility, while lignin supports and protects the cellulose from biological and chemical attack. Hemicellulose bonds lignin to cellulose. These three components in the plant cell are the constructing materials in the plant cells structure.

Since the surface of the glucan consists mainly of hydrogen atoms, the molecule becomes hydrophobic. The β–1,4-linkage forces the alternate chain units to be rotated 180°C around the main axis. The extended molecule thus forms a flat ribbon, which is further stiffened by intramolecular hydrogen bonds, especially between the hydroxyl group at C-3 and the ring oxygen atom of the neighbouring unit. Another intramolecular H-bond between the hydroxyls on C-2 and C-6 may exist

(Figure 5) The glucan molecule exhibits a C-1 end with a reducing aldehyde hydrate group and a C-4 end with a non-reducing alcoholic hydroxyl groups (Figure 6) (Franz and Blaschek, 1990).

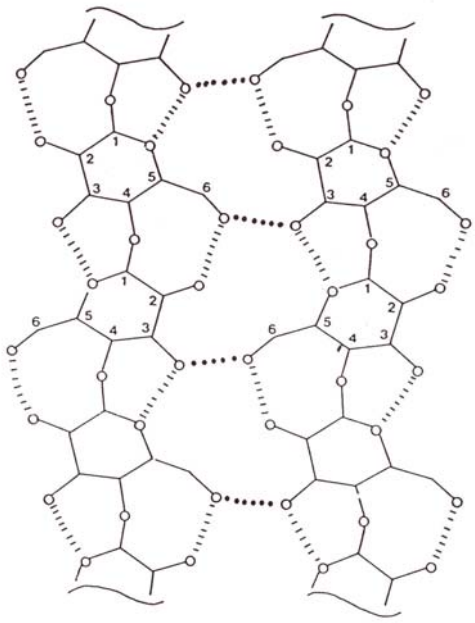


Figure 5 Intramolecular (III) and Intermolecular (…) hydrogen bonds of two adjoining glucan chains in cellulose

Source: Franz and Blaschek , 1990

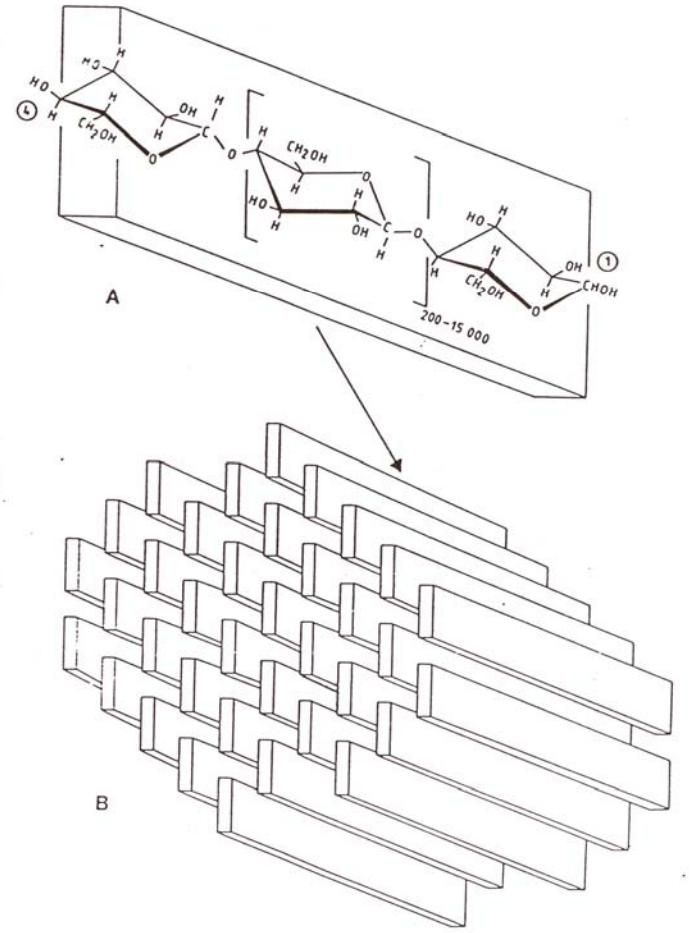


Figure 6 Plant cell structure

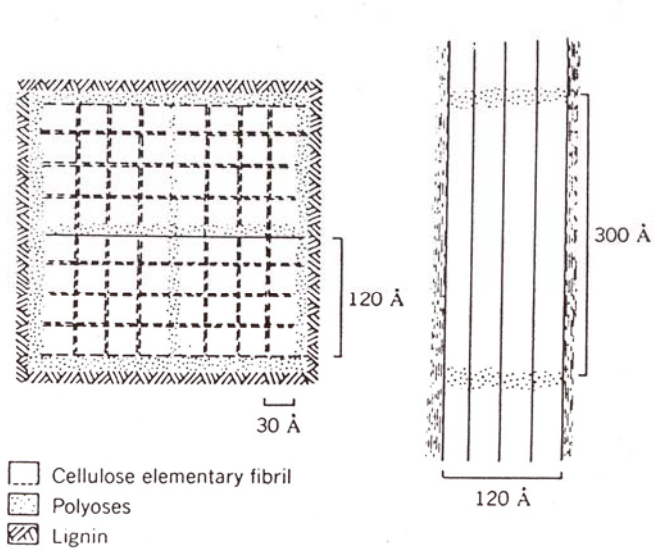
(A) β–1,4-glucan chain forming a flat ribbon with a reducing C-1 and a non-reducing C-4 end.

(B) A cellulose microfibril of about 3.5 mm. Diameter composed of about 36 glucan chains , which are help together by hydrogen bonds.

Source: Franz and Blaschek, 1990

The linear polymers, glucan chains, are linked together to form elementary fibrils (or photofibrils). The linear polymers in an elementary fibril are oriented in a parallel alightment and are bounded by hydrogen bonds to form a crystalline region, which is surrounded by a disordered layer of cellulose molecules, an amorphous region or paracrystalline region (Ranby, 1969. c*ited by* Lee, 1992).

A model for the ultrastructural organization of the cell wall components which was postulated by Fengel (1971) (*Cited by* Lee , 1992) is shown in Figure 10. It is shown that there are two different size of fibrils, 120 A° and 250 A° in diameter. The 120 A° fibril consists of 16 elementary fibrils and the 250 A° consists of four 120 fibrils. The 250 A° are also called microfibrils. Hemicelluloses are arranged around the fibrils of form the microfibrils, which are again surrounded by a hemicellulose layer and lignin. (Figure 7) (Lee, 1992)



(a) (b)

Figure 7 A model for the ultrastructural organization of the cell wall component

(a) Cross section

(b) Longitudinal Section

Source: Fengel, 1971 Cited by Lee, 1992

Pectin is found to bind to cellulose and other components like a network (Figure 8). So, pectin appears to control the pore size in the wall, and hence the movement of the macromolecules through the wall. Therefore, it may control the access of enzymes to their substrates, and thus make an indirect but important contribution to the control of wall mechanical properties (Brett and Waldron, 1996).

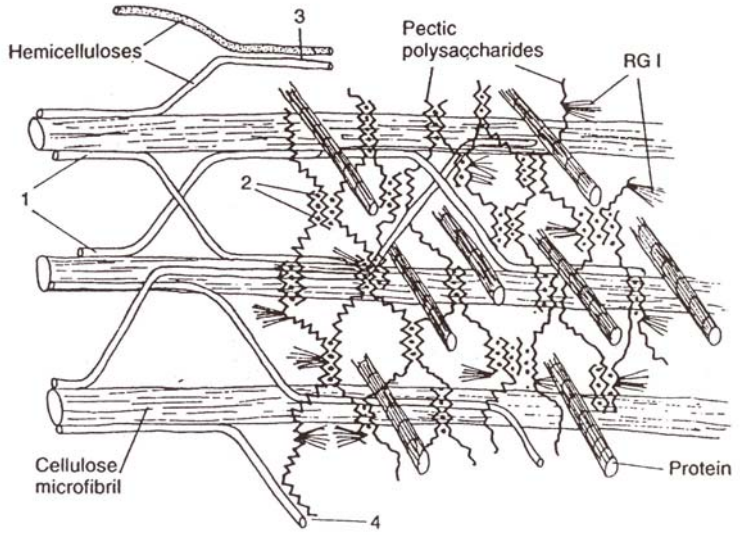


Figure 8 Possible interactions between several classes of cell wall polymer and the likely relative orientation

Source: Brett and Waldron, 1996

Structural carbohydrates including lignin are plant cell components which are arranged in plant cell wall. Within the cell wall, 3 distinct concentric morphological layers can be distinguished. There are middle lamella, primary wall and secondary wall (Figure 9). The secondary wall forms during growth and maturation of the cell and contains 3 layers , an outer layer, S_1 , a middle layer, S_2 and an inner layers, S_3. The microfibrils of the outer layers are wound in flat helices. The direction of winding alternates to form overlapping spirals. In the middle layer, S_2, the cellulose fibrils are tightly packed in a steep parallel helix. The innermost S3 layer consists of helical microfibrils as shown in Figure 10 (Tsao *et al*., 1987; Hon and Shirashi, 1991).

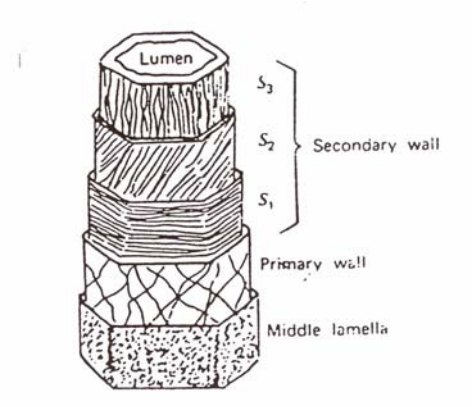


Figure 9 Architecture of a cell wall

Source: Tsao *et al*., 1987

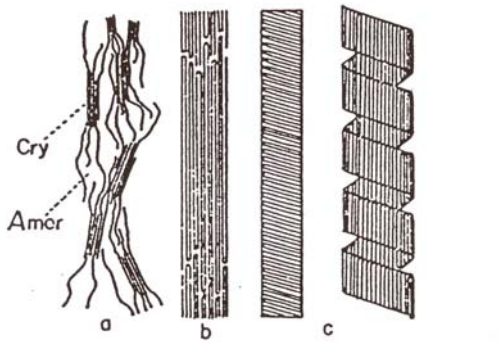


Figure 10 Supermolecular models of native cellulose.

(a) Frings micelles in a microfibril. Cry and Amor represent crystalline and amorphous regions respectively.

(b) Folded chains along the fiber axis.

(c) Folded chains form a flat ribbon on a helix of the ribbon.

Source: Nisizawa , 1973

In the primary wall, the microfibrillar structure appears as a loose and random network. The microfibrils are oriented around the fiber at approximately right angles to the axial fiber direction. The middle lamella, which is heavily lignified and contains lignin and hemicellulose, is the earliest-formed layer, found at the center of the double wall formed by two adjacent cells. It is derived from the cell plate, which and is often stretched during cell growth, the middle lamella is frequently extremely thin. It is often thickest at the cell corners (Figure 11).

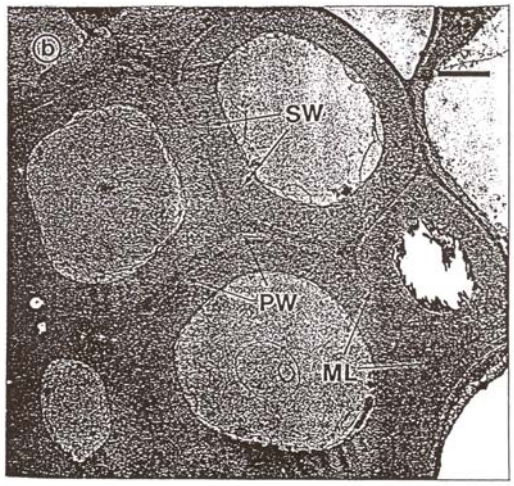


Figure 11 The major layers in the cell wall. ML, middle lamella, PW, primary wall, SW, secondary wall

Source: Brett and Waldron, 1996

The overall structure of the cell wall must be extremely complicated, given the great complexity and variety of the components. The composition of the primary and secondary walls varies in different species and different cell typs. Especially, grasses have primary walls with little pectin and with arabinoxylans and β–1,3 , β-1,4 glucans as the major hemicelluloses. But secondary walls are less known. (Brett and Waldron, 1996).

3. Cell wall degrading enzymes

The use of cell wall degrading enzymes as silage additives has been considered from two points of view, first, as a means of increasing the content of water soluble carbohydrate as substrate for lactic acid bacteria, and second, as a method of improving the digestibility of the organic matter of the crop (Mc Donald *et al*., 1991 c*ited by* Henderson, 1993).

In order to hydrolyze the different components of grass, the concerted action of various enzymes is needed. Generally, they are cellulases, hemicellulases and pectinases (Table 7).

Table 7 Enzymes used for grass silage making

Enzyme	Reference
Cellulase	Vaisto *et. al*.(1978) Henderson *et. al*.(1982)
Cellulase, hemicellulase, cellobiase and Pectinase	McHan (1986)
Cellulase and amylase / amylogucosidase	Agosin *et. al*.(1982) Robinson *et. al*.(1985)
Cellulase, glucaose oxidase	Heikonen *et. al*.(1984)
Polymethygalacturonase, polygalacturonase, Pectinaseterase, acid protease and hemicellulase	Udalova *et. al*.(1976)
Tekadiasterase, cellulase	Tsutsumi and Abe (1977)

Source : Stetälä (1989)

3.1 Cellulase

The term cellulase refers to the group of enzymes that contributes to the degradation of cellulose to its monomer glucose. There are 3 major types of cellulolytic activity which involved in the enzymatic degradation of cellulose. (Enari, 1983; Franz and Blaschek, 1990)

A complete set of cellulolytic enzymes is minimally composed of the following enzymes:

3.1.1 Endo-β-1,4-glucanases which split the native macromolecular chains, producing shorter units with a lower degree of crystallinity and a series of new chain ends (EC 3.2.1.91)

3.1.2 Exo-β-1,4-glucanases (cellobiohydrolases) which act from either reducing and non-reducing end of the shorter chains by continuously producing cellobiose in a synergistic way with the endoglucanase (EC 3.2.1.4). Cellobiohydrolases hydrolyses cellodrextrins but not cellobiose.

3.1.3 β-1,4-glucosidases hydrolyse cellobiose and cello-oligosaccharides to glucose (EC 3.2.1.21). The enzyme does not attack cellulose or higher cellodextrins.

In addition to these enzymes, a minor activity, glucan glucohydrolase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.71) removes glucose units from the non-reducing end of the chain. (Enari, 1983)

According to the model for enzymatic hydrolysis of cellulose (Figure 12), endoglucanase acts on amorphous region in the cellulose fibres. It opens up new chain-ends for attack by cellobiohydrolase, which then removes cellobiose units from the non-reducing ends of the chains. The synergy between the two enzymes thus results from the fact that new substrate (free chain-ends) for cellobiohydrolase is formed by the action of endoglucanase. β-glucosidase further enhances the total hydrolysis by removing cellobiose, the end-product inhibitor of cellohydrolase and endoglucanase. In this scheme endoglucanase is believed to be the rate-limiting activity. (Enari, 1983)

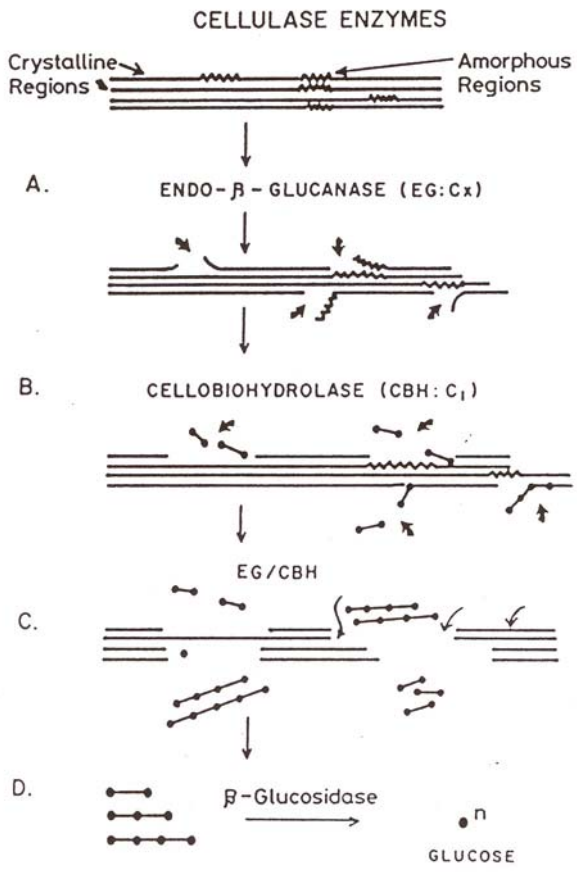


Figure 12 Schematic presentation of sequential stages in enzymatic hydrolysis of cellulose Reproduced with permission of the Technical Association of the Pulp and Paper Industry.

Source: Montenecourt and Eveleigh, 1979 *cited by* Enari, 1983

The degradation of cellulose by bacteria differs from the fungal cellulolytic system. Bacterial extracellular endoglucanase breaks down cellulose to short chain oligosaccharides. These are then hydrolysed to glucose by a periplasmic β-glucosidase (Figure 13). In some bacteria the uptake of cellobiose is mediated by a phosphorylase system.

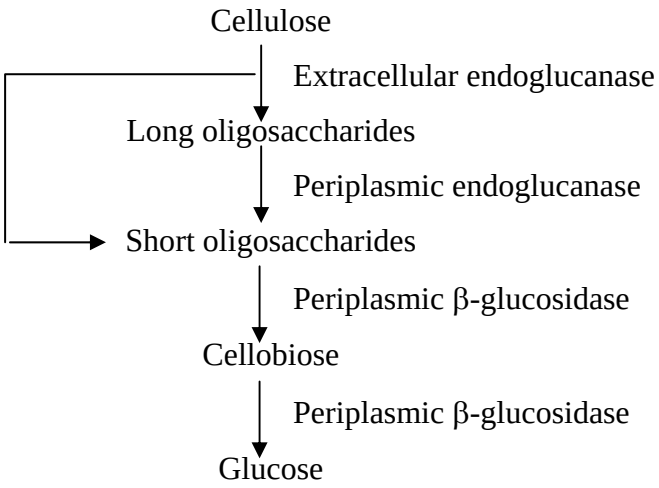


Figure 13 Mechanism of bacterial cellulolytic Reproduced with permission of the Society for General Microbiology.

Source: Rhamasamy and Verachtert, 1980 *cited by* Enari, 1983

3.2 Hemicellulase

Hemicelluloses are composed of both linear and branced heteropolymers of D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose and D-glucuronic acid. These individual sugars may be acetylated or methylated. Most hemicelluloses are based on a β-1,4-linkages of their backbone sugars (Eriksson, *et al.*, 1990). The classification of these hemicelluloses fractions depends on the types of sugar moieties present. There are actually several types of hemicelluloses. However, with regard to grass components, β-1,3-1,4-glucans and arabinoxylans are the main types (Henderson, 1993).

Hemicellulases are referred to the enzymes which break down hemicelluloses. They are defined and classified according to the substrates on which they act. They are collectively grouped as glycan hylrolases (EC 3.2.1). (Bastawde, 1992)

3.3 Pectic enzyme

Pectic substrances are the major components of the middle lamella, a thin layer of adhesive extracellular material found between the primary cell wall of adjacent young plant cell. In addition, they constituente important parts of the primary plant cell wall (Alkorta *et.al.*, 1998).

The enzymes that hydrolyze pectic substrances are broadly known as pectinase, which are classified base on their attack on the galacturonan backbone of the pectic substrance molecule (Alkorta *et.al.*, 1998).

Because of the universal occurrence of pectic polysaccharides, the enzyme systems capable of degrading these structures are varied in their mechanism of action. Pectin enzymes have been classified to a report of the Enzyme Commission on nomenclature as shown is Table 8 (Fogarty and Kelly, 1983)

Table 8 Classification of pectinolytic enzymes

A. Esterase
Pectinesterase , PMGE , EC 3.1.1.11 , de-esterifies pectin to pectic acid by removal of methoxyl residues.
(S.n.)[a] : pectin pectyl-hydrolase.
(R.n)[b] : Polymethylgalacturonate esterase.
B. Depolymerases
1. Acting on pectin
1.1 Polymethylgalacturonase (PMG)
(a) Endo-PMG hydrolyses pectin in a random fashion.
(S.n.) : Poly(methoxylgalactosiduronate) glycanohydrolase.
(R.n) : Endopolymethylgalacturonase.

Table 8 (Continued)

(b) Exo-PMG hydrolyses pectin in a sequential fashion.

(S.n.) : Poly(methoxylgalactosiduronate) exohydrolase.

(R.n) : Exopolymethylgalacturonase.

1.1 Polymethylgalacturonate lyase (PMGL)

(a) Endo-PMGL , EC 4.2.2.10 , causes random cleavage in pectin by a transelimination process.

(S.n.) : Poly(methoxylgalactosiduronate) endolyase.

(R.n) : Endopolymethylgalacturonate lyase (endopectin lyase).

(b) Exo-PMGL cause sequential cleavage in pectin by a transelimination process.

(S.n.) : Poly(methoxylgalactosiduronate) exolyase.

(R.n) : Exopolymethylgalacturonate lyase (exopectin lyase).

2 Acting on pectic acid (polygalacturonic acid)

2.1 Polygalacturonase (PG)

(a) Endo-PG, EC 3.2.1.15 , hydrolyses pectic acid in a random fashion.

(S.n.) : Poly(1,4-α-D-galactosiduronate) glycanohydrolase.

(R.n) : Endopolygalacturonase.

Exo-PG-1, EC 3.2.1.67 , hydrolyses pectic acid releasing D-galacturonate , i.e. hydrolyses successive bonds.

(S.n.) : Poly(1,4-α-D-galactosiduronate) galacturonohydrolase.

(R.n) : Exopolygalacturonase.

(c) Exo-PG-2 , EC 3.2.1.82 , hydrolyses pectic acid from non-reducing end releasing digalacturonate , i.e. hydrolyses alternate bonds.

(S.n.) : Poly(1,4-α-D-galactosiduronate) digalacturonohydrolase.

(R.n) : Exopolydigalacturonase.

Table 8 (continued)

2.2 Polygalacturonate lyase (PGL)

(a) Endo-PGL , EC 4.2.2.2 , causes random cleavage in pectic acid by a transelimination process.

(S.n.) : Poly(1,4-α-D-galactosiduronate) endolyase.

(R.n) : Endopolygalacturonate lyase (endopectate lyase).

(b) Exo-PGL, EC 4.2.2.9 , cause sequential cleavage in pectic acid by a transelimination process.

(S.n.) : Poly(1,4-α-D-galactosiduronate) exolyase.

(R.n) : Exopolygalacturonate lyase (exopectate lyase).

3. Acting on oligo-D-galactosiduronates

3.1 Oligogalacturonase (OG)

OG hydrolyses oligo-D-galactosiduronate.

(S.n.) : Oligo-D-galactosiduronate hydrolase.

(R.n.) : Oligogalacturonase.

3.2 Oligogalacturonate lyase (OGL)

OGL, EC 4.2.2.6, causes cleavage of oligo-D-galactosiduronate by a transelimination process.

(S.n.) : Oligo-D-galactosiduronate lase.

(R.n.) : Oligogalacturonate lyase.

Source : Fogarty and Kelly (1983)

[a] (S.n.) : systematic name.

[b] (R.n.) : recommended name.

The mode of actions of the most frequent depolymerase on the pectin molecule are summarized in Figure 14.

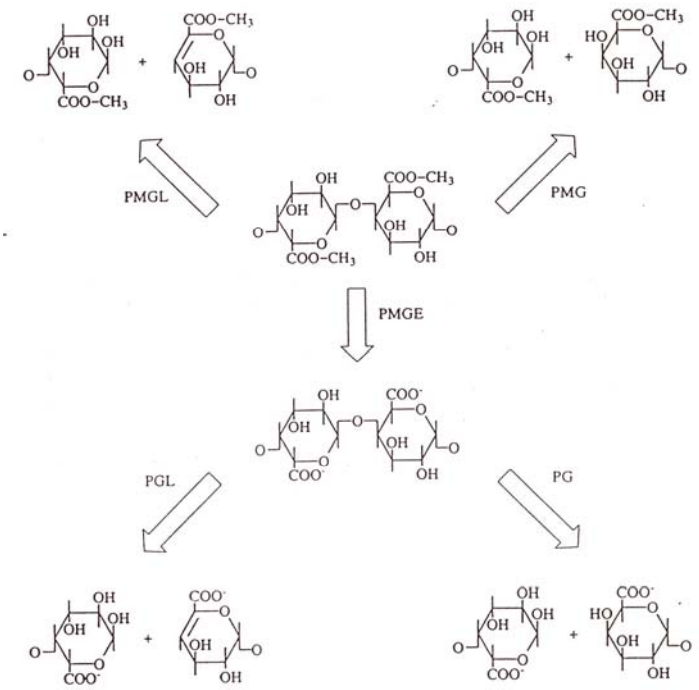


Figure 14 Enzymatic mode of action of the most frequent depolymerases on the pectin molecule, PMGL: polymethylgalacturonate lyase (pectin lyase), PMG: polymethylgalacturonase (pectin hydrolase), PMGE: polymethylgalacturonate esterase (pectinesterase), PGL: polygalacturonase (pectate lyase), PG: polygalacturonase (pectate hydrolase.

Source: Serra *et.al.*, 1992 *cited by* Alkorta *et.al.*, 1998

According to the complex of plant cell wall, the different components hydrolysis is the action of various enzymes that is not only synergistic activities of free noncellulosomal enzymes but those synergistic action by enzymes incorporation into the cellulosome complex has been reported as well (Bayer, *et al.*, 2004).

Cellulosomes are multi-enzyme complexes, many of the known cellulosomes include different types and mixed compositions of hemicellulase (e.g. lichenase, xylanases, mannanases, arabinofuranusidases and pectin lyases) in addition of cellulases (Bayer, *et al.*, 2004).

4. *Bacillus subtilis* GN156

To solve the problem of insufficient amount of WSC in the grass ensiling process, the addition of enzymes to release structural carbohydrates is the aim of this study. Since, the fermentation conditions, like extreme pH and temperature change during the ensiling process, effective enzymes, which are stable at wide pH and temperature range, are needed.

Bacillus subtilis GN 156 was screened from corn silage. It showed effective hydrolysis of grass. Grass silage, treated with lactic acid bacteria and hydrolytic enzymes from *B. subtilis* GN 156, seemed to have a higher quality than the control based on its high lactic acid content and low pH (Buwjoom,1998). In this study, the hydrolytic enzymes from *B. subtilis* GN 156 were purified and the synergistic effect of these enzymes on grass was proven by comparison of sugars which were released during grass hydrolysis.

5. β-1,3-1,4-glucanase (Lichense or 1,3-1,4-β-D-glucan-4-glucano hydrolase; EC 3.2.1.73)

The enzymatic depolymerization of β-1,3-1,4-glucans is catalysed by endogenous glycosyl hydrolases with at least three different specificities: 1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4), 1,3-β-D-glucan 3-glucanohydrolase (EC 3.2.1.39) and 1,3-1,4-β-D-glucan 4-glucanohydrolase (EC 3.2.1.73). β-glucosidase (EC 3.2.1.21) is also present, as well as β-glucan exohydrolase (EC 3.2.1.91).

Endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (EC 3.2.1.91) and β-1,4-glucosidase (EC 3.2.1.21) are cellulases, and refer to the enzymes that contribute to the degradation of cellulose to its monomer glucose. (Enari, 1983, Franz and Blaschek, 1990, Kim, 1995). In contrast, β-1,3-glucanase (EC 3.2.1.39) and endo-β-1,3-1,4-glucanase (EC 3.2.1.73) are considered hemicellulases because of

their ability to break down hemicellulose. These enzymes are therefore classified according to the substrates which they act on. (Bastawde, 1992)

Among the endo-glycosidase, the most active is the β-1,3-1,4-glucanase. It hydrolyses 1,4-β-D-glycosidic linkages in β-D-glucans containing 1,3 and 1,4 bonds, but does not hydrolyses β-D-glucans containing only 1,3 or only 1,4 bonds (Dixon and Webb, 1979).

Structurally, β-1,3-1,4-glucans are linear glucans of up to 1,200 β-D-glycosyl residues linked through β-1,3 and β-1,4 glycosidic bonds, with variation in the proportion of both types of linkages (25 - 30% β-1,3) and in the length of the mixed-linked segments (Planas, 2000).

As shown in Figure 15, the main final hydrolysis products from barley β-glucans are the trisaccharide-1 (3-*O*-β-cellobiosyl-D-glucopyranose) and the tetrasaccharide-2 (3-*O*-β-cellotriosyl-D-glucopyranose) (Akiyama *et al.*,1996; Planas, 2000).

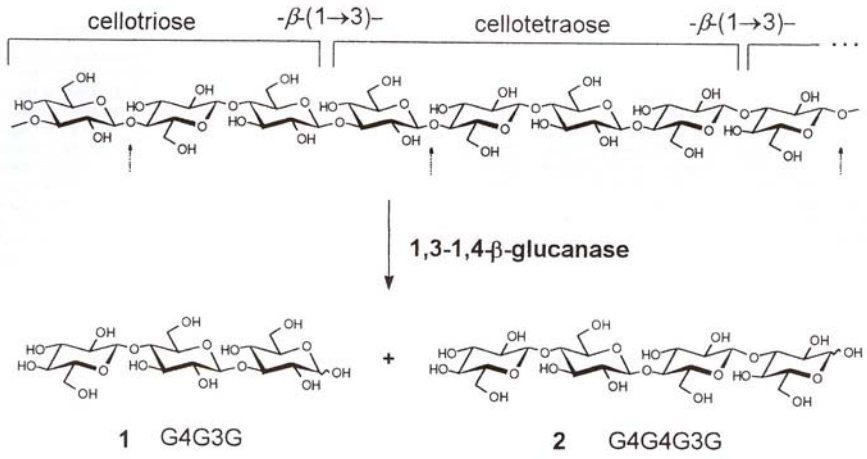


Figure 15 Enzymatic depolymerisation of barley β-glucan by β-1,3-1,4-glucanases. Source: Planas, 2000

5.1 β-1,3-1,4-glucanase producing organisms

β-1,3-1,4-glucanase are produced by plants, bacteria, actinomycetes and fungi, which have been reported as listed in Table 9.

Table 9 β-1,3-1,4-glucanase producing microorganisms

Organism	Reference
Bacillus amyloliquefaciens	Borris and Zemek(1980, 1981),
	Borris *et al.* (1980);
Bacillus amyloliquefaciens	Hofemeister *et al.* (1986);
	Hoj *et al.* (1992);
	Meldgaard and Svendsen (1994)
Bacillus brevis	Louw, Reid (1993)
Bacillus circulans	Borris and Zemek(1980, 1981),
	Borris *et al.* (1980)
Bacillus circulans WL-12	Bueno *et al.* (1990)
Bacillus laterosporus	Borris and Zemek(1980, 1981),
	Borris *et al.* (1980)
Bacillus licheniformis	Malet *et al.* (1993),
	Juncosa *et al.* (1994),
	Hahn *et al.* (1995),
	Pons, Planas and Querol (1995),
	Malet and Planas (1997),
	Pons, Querol and Planas (1997)
Bacillus macerans	Borris and Zemek(1980,1981),
	Borris *et al.* (1980),
	Borris, Buettner and Maentsaelae (1990),
	Meldgard and Svendsen (1994),
	Bueno *et al.* (1990),
	Hahn *et al.* (1995)

Table 8 (Continued)

Organism	Reference
Bacillus IMET B376	Borris (1981)
Bacillus pumilus	Borris and Zemek(1980,1981), Borris *et al.* (1980)
Bacillus polymyxa	Borris and Zemek(1980,1981); Borris *et al.* (1980); Gosalbes *et al.* (1991)
Bacillus subtilis HL-25	Yuuki, Tezuka and Yabuuchi (1989)
Bacillus subtilis NCIB 8565	Hinchliffe (1984)
Bacteroides succinogenes	Erfle *et al.* (1988)
Clostridium thermocellum	Schimming, Schwarz and Staudenbauer (1991)
Orpinomyces sp. PC-2	Chen, Li and Ljungdahl (1997)
Ruminococcus flavefaciens	Flint, McPhersan and Bisset(1989)
Streptomyces bovis JB1	Ekinci, McCrae and Flint (1997)
Streptomyces narbonensis 2a	Elinov *et al*. (1982)
Barley	Woodward and Fincher (1982), Olesen and Thomsen (1989); Slakeski *et al.* (1990); Doan and Fincher (1992); Chen, Fincher and Hoj (1993) Keitel, Thomsen and Heinemann (1993)
Wheat	Lai, Hoj and Fincher (1993)
Rice	Akiyama, Kaku and Shibuya (1996)

Source: adapted from Planas (2000)

Although, β-1,3-1,4-glucanases are formed by many organisms, the important enzymes, obtained for application in the brewing and animal feedstuff industries, are usually from bacteria, especially hyperproducing selected strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Planas, 2000).

5.2 Mode of action of β-1,3-1,4-glucanase

Generally, the basic concept of the action pattern of β-1,3-1,4-glucanase is understood and the main final hydrolysis products from barley β-glucan are the trisaccharide and the tetrasaccharide. This concept has been confirmed by many reports.

Malet *et al.* (1993) studied hydrolysis of barley β-glucan by *B. licheniformis* β-1,3-1,4-glucanase. The final oligosaccharide products were trisacchride and tetrasaccharide.

In contrast, Akiyama *et al.* (1996) examined the action pattern of the purified rice β-1,3-1,4-glucanase by using barley β-glucan as substrate. They found that the major products were two oligosaccharides with mobility intermediate between cellobiose and cellotetraose, but no disaccharide was detected.

Besides, barley β-glucan which usually is used as substrate for determination of enzyme action, lichenan and oat spelt β-glucan were used to examine the action of β-1,3-1,4-glucanase of *Bacteriodes succinogenes* secreted from *E.* coli. It was found that the major products with lichenin as substrate were trisaccharide (82%) and pentasaccharide (9.5%). While the hydrolysis of oat β-glucan yielded trisaccharide (63.5%) and tetrasaccharide (29.6%) as the major products. However, chromatographic patterns of the products from the cloned enzyme appear to be similar to those reported for the β-1,3-1,4-glucanase isolated from *B. subtilis* (Elfle *et al.*, 1988)

From the previous studies, it is concluded that the major products from the action of β-1,3-1,4-glucanase may be different. They depend on both type of substrate and the sources of the enzyme used.

5.3 β-1,3-1,4-glucanase properties

The physical and chemical property of β-1,3-1,4-glucanase are summarized in Table 10

Table 10 β-1,3-1,4-glucanase properties (adapted from Planas, 2000)

Organisms	MW (kDa)	pI	pH_{opt}	$Temp_{opt}$ (°C)	Reference
Bacillus subtilis HL-25	24	8.55			Yuuki *et a l.*(1989)
E-1	24	8.75			
E-2					
Bacillus subtilis 103	30	5.4			Firantene *et al.*(1981)
Bacillus IMET B376	26		6-8		Borriss (1981)
Clostidium thermocellum	38		6.6-10	80	Schimming *et.al.*(1991)
Fibrobacter succinogenes	37		6.0	50	Elfle *et al.*(1988)
Orpinomyces sp.	26		6	45	Chen *et al.*(1997)
Ruminococcus flavofaciens	90				Flint *et al.*(1993)
Streptococcus bovis JB1	25		6.5	50	Ekini *et al.*(1997)
Talaromyces emersonii	40.7		4.8	80	Tuohy *et al.**
Barley EI	28	8.5			Woodward and
EII	33	>10			Fincher (1992)
Rice	34	4.9	5.5	50	Akiyama *et al.*(1996)
Wheat	30	8.2			Lai *et. al.*(1993)

*Personal communication by Planas (2000)

Borriss (1981) purified β-1,3-1,4-glucanase, with the molecular weight of 26 kDa, from the culture fluid of *Bacillus* IMET B376. The enzyme has a broad pH optimum between 6 and 8 and was particularly thermostable in precence of Ca^{++}. The K_m value for lichenan and barley-glucan was determined to be 1.43 and 1.15 mg/ml, respectively.

β-1,3-1,4-glucanase from *B. subtilis* strain 103 was purified to 142-fold. It was found that the molecular weight of the enzyme was 30 kDa, with an isoelectric point of pH 5.4. (Firantene, *et al.*, 1981)

Two β-1,3-1,4-glucanases, named E1 and E2 , were purified from the culture supernatant of *B. subtilis* HL-25 by Yuuki *et al.* (1989). Both purified enzymes have an identical molecular weight of 24 kDa but their isoelectric points were pH 8.55 and 8.75, respectively.

Louw *et al.* (1993) have cloned a gene coding for β-1,3-1,4-glucanase from *B. brevis* into *Escherichia coli*. The enzyme produced from the recombinant *E. coli*, has an optimum temperature and pH for enzyme activity around 65-70°C and 8-10, respectively. The molecular weight was estimated to be about 29 kDa on SDS-PAGE.

The gene bga A encoding an akaline β-1,3-1,4-glucanase from an alkalophilic *Bacillus* sp. strain N137 was cloned and expressed in *E. coli* by Tabernero *et al.* (1994). Its activity is stable between pH 6 and 12 and shows optimal activity between 60 and 70°C.

6. <u>Synergistic effect of enzymes</u>

The complexity of the plant cells content of celluloses, hemicelluloses and pectins, the concerted action of different enzymes is necessary for efficient degradation.

Molony *et al.* (1984) found that a combination of cellulolytic activity from *Talaromyces emersonii* UCG 208 culture filtrates and a commercial pectinase activity converted beet pulp efficiently to soluble sugars.

In 1988, Considine *et al.* studied the extracts of solid state cultures of *Talaromyces emersonii* UCG 208, *Trichoderma reesei* QM 9414, *Trichoderma reesei* MCG 77 and *Pennicillium capsulatum* growing on beet pulp. They found that the extracts of these strains contained adequate amounts of cellulase and hemicellulase activities, while the extract of *P. capsulatum* had also pectinase activity. When the degree of pulp hydrolysis was studied, the results showed that the extract of *P. capsulatum* was higher in activity than the activity of *T. reesei* MCG 77. They concluded that hydrolysis of the pectin afforded the greatest access of the cellulases and hemicellulases to their respective substrates.

Spagnuolo *et al.* (1997) used cellulase, hemicellulase and pectinase, separately, in binary or ternary combinations to hydrolyze dried beet-pulp. They found that a low rate of degradation was brought about by the presence of a barrier composed of pectic substances. Pectinolytic enzymes were therefore necessarily to remove this protection to allow the attack on the other components. Pectinases appear to be the most important enzymes, by facilitating the subsequent degradation of cellulose and hemicellulose by the corresponding enzymes.