CHAPTER 2
LITERATURE REVIEW

2.1 Non-starch polycaccharide

2.1.1 Definition and classification of non-starch polysaccharide

Polysaccharides are polymers of monosaccharides joined through glycosidic linkage, and are defined and classified in terms of the following structural consideration:

- identity of the monosacharides present
- monosaccharide ring form (6-membered pireates or 5-membered furanose)
- positions of the glycosidic linkage
- configurations (α or β) of the glycosidic linkages
- sequence of monosaccharide residues in the chain
- presence of absence of non-carbohydrate substituents

(Choct, 1997)

2.1.2 β-Glucan

β-glucan is one of the non-starch polysaccharides in cereals, and is derived from the polymerization of β-D-glucose monomers. Analyzing the chemical linkages in the β-glucan found the two types of linkages established were β-(1,3) and (1,4) (Figure 2.1) (Onwurah, 2001).

The β-glucan in rice accounts for only about 0.4–0.9% of the water-soluble endosperm starches while β-D-glucan in barley is about 3.2–4.6% (Table 2.1) (Demirbas, 2005).

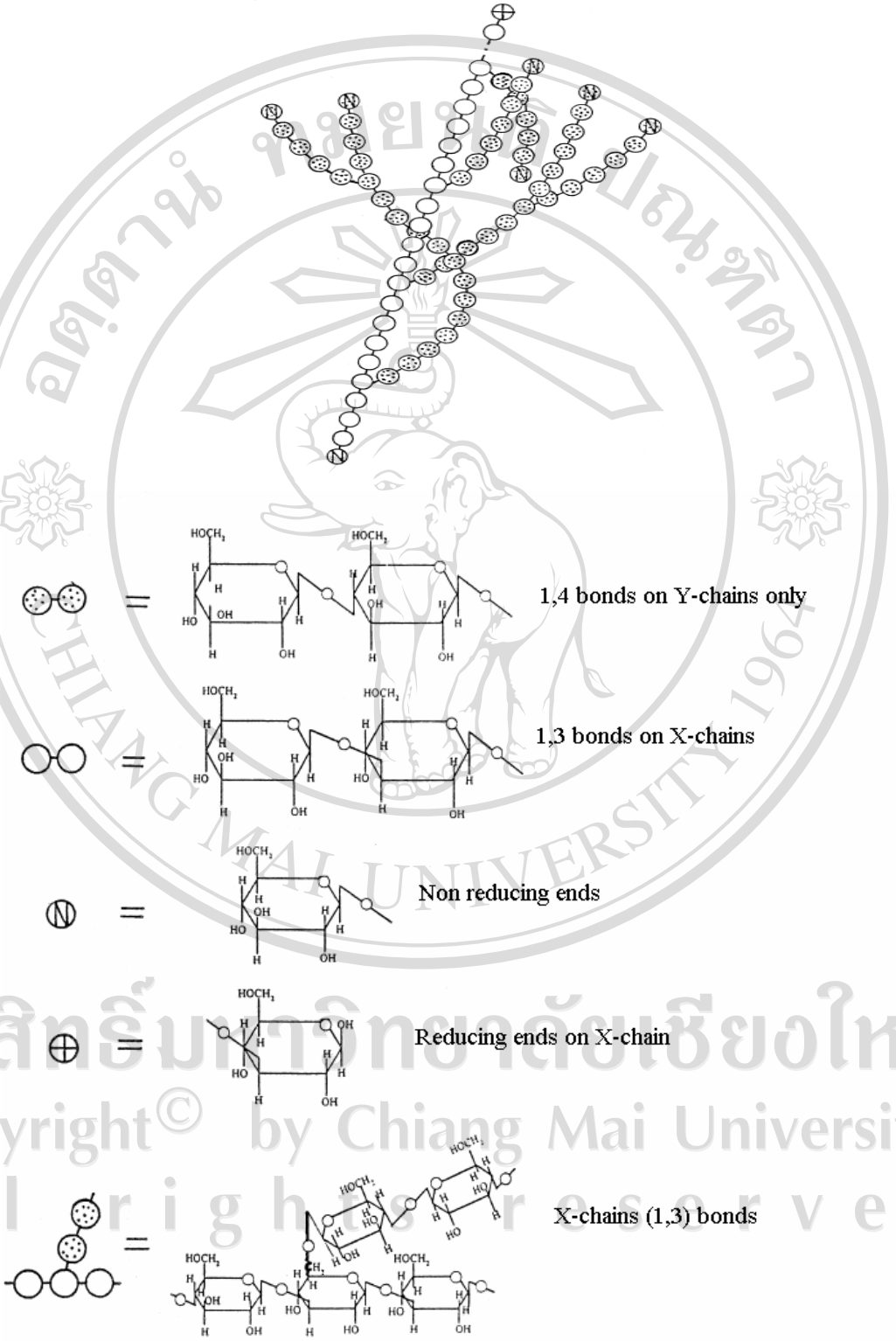

Figure 2.1 Chemical structure of β-glucan (Onwurah, 2001)

Table 2.1 Average β-glucan content of cereal grain (Demirbas, 2005)

	β-glucan content of grain (%)	
Grain	The lowest	The highest
Barley	3.2	4.6
Beans	2.4	3.5
Canary seed	1.1	2.3
Corn/Maize	0.5	1.3
Flax	0.3	0.7
Lentils	0.4	1.1
Millet	0.5	1.0
Oat	3.9	5.7
Peas	0.3	0.7
Rice	0.4	0.9
Rye	0.7	1.5
Spelt	0.6	1.2
Spring wheat	0.6	1.1
Winter wheat	0.5	1.0

2.1.3 Grain structure

Whether the grain used is wheat, barley or maize, the most important nutrient is starch, which accounts for approximately 60-70% of grain weight (Annison, 1990). Starch is located in the thin-walled endosperm which, with the embryo, constitutes the contents of the grain. The whole endosperm is enclosed by a thin layer of cells with very thick cell walls, known as the aleurone layer, which itself contains the digestive enzymes required for release of nutrients from the endosperm for germination to proceed (Figure 2.2). Enclosing the aleurone layer is the pericarp which consists of several layers of cells and is designed to protect the grain. Thus, in order for a non-ruminant to access the starch contents of a grain, it must be able to penetrate the tough, fibrous pericarp and the aleurone layer. The final barrier is the endosperm cell wall itself which is far more easily ruptured by feed processing and the mechanical grinding of the teeth/gizzard than the walls of the aleurone. The problem component in cereal endosperm cell walls is not the thin cellulose component however, but the soluble NSPs which encrust it. The bulk of the NSP encrusting the endosperm cell wall in wheat, rye and triticale endosperm is composed of arabinoxylan (Henry, 1985). This is a linear polymer, of variable length, which is constructed of D-xylose linked β, 1-4 with arabinose substituted along the backbone. If the arabinose were absent, the xylan polymer would be able to interact with other xylan molecules and

precipitate. The presence of the arabinose renders this polymer soluble, however, and in so doing allows long polymers to entangle in solution which presents the non-ruminant with a viscous problem. Mixed-link β-glucans, which are variable length linear molecules of glucose linked β,l-4 with intermittent β,1-3 links, comprise the bulk of the NSPs in oat and barley endosperm cell walls (Henry, 1985). Mixed link β-glucans are also soluble by virtue of the fact that the β, 1-3 link introduces a link in the structure of the polymer which prevents large scale aggregation and therefore prevents precipitation (McCleary and Glennie-Holmes, 1985). Again, these molecules present the non-ruminant with a viscous problem in the intestine.

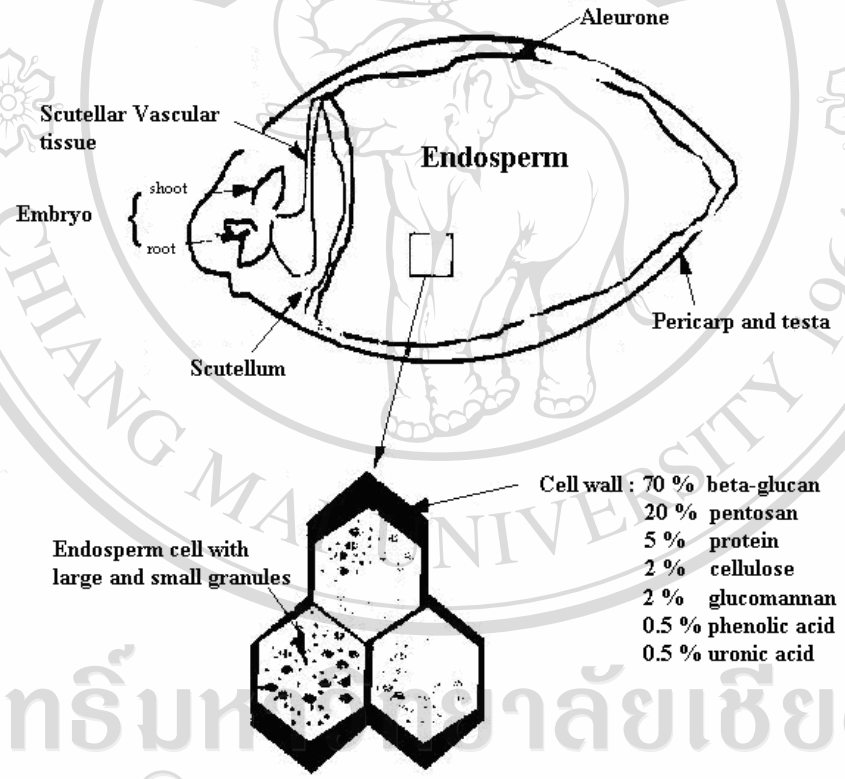

Figure 2.2 Structure of the barley grain (Annison, 1990)

2.1.4 Mechanism of action

The term non-starch polysaccharide (NSP) represents a diversity of compounds possessing different physicochemical properties; thus their nutritional effects in animal are also diverse and, in some cases, extreme. Digestion of non-starch

polysaccharide fractions tends to be more variable due to lack of digestive enzymes and their tendency to create a viscous environment within the intestinal lumen (Choct *et al*., 1996). However, generally conceded that the detrimental effect of NSP in viscous grains is associated with the viscous nature of these polysaccharides, their physiological and morphological effects on the digestive tract, and the interaction with the microflora of the gut. The mechanisms include altered intestinal transit time and modification of the intestinal mucosa, as well as changes in hormonal regulation due to varied rate of nutrient absorption.

The wheat arabinoxylans caused a general inhibition of nutrient digestion affecting starch, fat and protein, which indicated that they acted in a similar manner as the anti-nutritive NSP of rye and barley. The arabinoxylans of rye have been reported to depress fat, protein and dry matter (DM) retention (Ward and Marquardt, 1987; Fengler and Marquardt, 1988). The depressed ileal digestibilities of the starch and protein have been observed in chickens fed barley diets which had high levels of extractable viscous β-glucans (Hesselman and Aman, 1986). Classen *et al*., (1988) demonstrated that supplement of barley diets with β-glucanase reduced the variability from 11.9 to 3.3% for weight gain and from 5.2 to 2.7% for feed conversion ratio (FCR) in broilers.

2.1.5 Viscous nature of soluble NSP

Increase in bulk and viscosity of the intestinal contents decrease the rate of diffusion of substrates and digestive enzymes and hinder their effective interaction at the mucosal surface (Edwards *et al*., 1988). Studies in vitro with guar gum showed that soluble, indigestible polysaccharides interact with the glycocalyx of the intestinal brush border, producing a thickening of the rate-limiting unstirred water layer, which results in decreased nutrient absorption (Johnson and Gee, 1981). These results lend support to the hypothesis that viscosity is involved in the anti-nutritive effect of NSP in diets. Choct and Annison (1992) compared the effects of a high molecular weight NSP isolate (Intact-NSP) (854 g arabinoxylans and 42 g glucan kg^{-1} DM; molecular weight = 758,000) and a partially depolymerized NSP isolate (Depol-NSP; 194,000) (Annison *et al*., 1992), and pentose on broiler performance and nutrient digestibility Table 2.2. When included at a level if 30 g kg^{-1} diet, the partially depolymerized NSP

increased digesta viscosity significantly compared with pentose sugars added at the same level, but it was lower than that in bird fed intact NSP. Bird performance was not significantly affected by the depolymerized NSP, indicating that birds can tolerate small increase in digesta viscosity without a detrimental effect on performance.

Table 2.2 Effect of higher molecular weight NSP, partially depolymerized NSP and pentoses on weight gain(WG), feed conversion ratio (FCR), feed intake (FI) and apparent metabolizable (AME) in broilers (n=8) (Choct and Annison, 1992)

Diet	Level added $(g\ kg^{-1})^2$	Digesta viscosity	WG $(g\ wk^{-1})^3$	FCR (g : g)	FI $(g\ wk^{-1})$	AME $(MJ\ kg^{1}\ DM)$
Control	0	1.2^a	430^a	1.589^a	681	16.13^a
Intact-NSP	30	3.0^c	325^b	1.960^b	622	14.53^b
Pentoses	30	1.2^a	394^{ac}	1.695^{ac}	658	16.23^a
Depol-NSP[1]	30	2.2^b	404^{ac}	1.649^a	661	15.74^a

Watery and sticky dropping alone may not be a sufficient indicator of the anti-nutritive effect of the arabinoxylans in poultry, as excreta from birds fed the diet containing the depolymerized arabinoxylans were watery and sticky, and those from birds given the diet containing pentose sugars also appeared more moist compared with controls. In the rat, dietary components that are not completely digested or absorbed in the small intestine give rise to an increased amount of osmotically active materials in the gut content. If these materials are utilised by the hindgut bacteria, production of low molecular weight metabolites that are not readily absorbed may occur. This can lead to further accumulation of osmotically active substances which attract a large amount of water. This may well relate to the inefficient utilization of five-carbon sugars by chickens (Wagh and Waibel, 1966). The significant improvement in performance of birds fed barley or rye supplement with β-glucanase or arabinoxylanase is not due to a complete hydrolysis of the polysaccharides and a subsequent absorption of the released sugars (White *et al*., 1981), but is due to the depolymerization of the polysaccharides into smaller polymers which do not greatly elevate the viscosity of digesta (De Silva *et al*., 1983)

2.1.6 Modification of secretary response of the gut

Viscous polysaccharides cause physiological and morphological changes to the digestive system of rats, pigs and humans (Brown *et al*., 1979; Cassidy *et al*., 1981; Morgan *et al*.,1985). The endogenous secretion of water, proteins, electrolytes and lipids can be increased markedly by NSP supplementation of the diet (Low, 1989). The metabolic cost of such processes can be considerable. Prolonged consumption of diets containing viscous polysaccharides is associated with significant adaptive changes in the digestive system in rats (Ikegami *et al*., 1990). The changes in the gastrointestinal tract are characterized by enlargement of digestive organs and increased secretion of digestive juices, accompanied by decreases in nutrient digestion. The depressed apparent ileal protein digestibility caused by the wheat arabinoxylans, therefore, may be due to an inhibition of protein breakdown and/or a reduction in amino acid absorption. It may also result from an increase in the secretion of endogenous proteins, which can be derived from gut secretion and losses of intestinal cells. In the following study (Angkanaporn *et al*., 1994), the effect of soluble wheat arabinoxylans on endogenous protein (sum of amino acids) secretions, in comparison to that of pure cellulose and an inert nutrient diluent (polythene powder), was investigated. Endogenous and exogenous amino acids were distinguished using the homoarginine marker technique (Hagemeister and Erbersdobler, 1985). The apparent protein digestibility was depressed to a similar extent by addition of wheat arabinoxylans at level of 15 and 30 g kg^{-1}, but the true protein digestibility was significant inhibited only at the higher level of inclusion. This indicated that the anti-nutritive effect of wheat arabynoxylans on the apparent protein digestibility is mainly due to increased endogenous secretion of amino acids at low levels of inclusion, while at high level a direct inhibition of protein breakdown and/or absorption occurs.

The biological effects of NSP differ considerably due to their diverse physicochemical properties. Southon *et al*. (1985) reported that a diet containing 75 g non-cellulosic polysaccharides kg^{-1} and 24 g cellulose kg^{-1} fed to rats induced higher rates of protein synthesis in the jejunum and ileum, and more rapid mucosal cell division than rats given a semi-purified diet containing cellulose as the only source of NSP. The lack of action of cellulose on the endogenous amino acid secretions was

further demonstrated in chickens by Parsons (1984), who found that when a nitrogen-free diet containing 400 g raw potato starch kg^{-1} and 100 g pectin kg^{-1} was fed to laying hens, the total endogenous amino acid secretion was increased by 40%; whereas the diet containing 500 g cellulose kg^{-1} elicited little effect. These observations indicate that the action of NSP in modification of physiological functions of the gastrointestinal tract is not a mere mechanical stimulation of the mucosa. It is possible that the soluble NSPs interact with the gut well in a way that modifies the endocrine regulation.

2.1.7 Encapsulated nutrients

Although the insoluble NSPs have mainly been regarded as a nutrient diluent in the diet, they can also affect digesta transit time and gut motility. Another face of the role of insoluble NSP in poultry diets that is worthy of reiteration is the possibility of them acting as a physical barrier to digestive enzymes, such as amylase and proteases, thus reducing their efficient digestion in the upper part of the gut. This is probably particularly important in non-viscous grains, such as sorghum. There is evidence that enzymes with affinity for insoluble NSP can also elicit a positive response in growth performance of broilers (Cowan, 1995; Choct, 1996). This indicates that breakdown of cell wall matrix, especially the insoluble components, may facilitate easier access of digestive enzymes to their substances within the short feed transit time in birds.

2.2 β-Glucan-degrading enzymes

The anti-nutritional properties of β-glucan (1,3: 1,4-β-D-glucans, mixed-linkage β-glucan) have been known for many years. In animal feeds the major source of β-glucan is barley grain. Although levels are also high in oats, these are rarely fed to chickens and pigs. The anti-nutritional properties of β-glucan are attributed to their viscosity-inducing properties, which significantly affect the rate of movement of barley-based diets through the digestive tract of chickens, and reduce the rate of nutrient absorption. This problem is effectively dissolved by the judicious use of β-glucan-degrading enzymes.

A wide range of enzymes are active on β-glucan and these include the endo-acting fungal cellulases [endo-1,4-β-D-glucanase (EC 3.2.1.4)] and the bacterial β-glucanase [lichenase; endo-1,3 : 1,4-β-D-glucanase (EC 3.2.1.73)]. Both groups of enzymes cleave within the main chain of mixed-linkage β-glucan, although their point of action is different (fig. 2.3). Fungal cellulase also hydrolyse cellulose (1,4-β-D-glucan) in an endo-hydrolytic pattern, but the bacterial 1,3 : 1,4-β-glucanases have no action on cellulose. This point of difference is important in designing specific substrates for the assay of these groups of enzymes. A range of substrate and assay prodedures is available for the measurement of bacterial β-glucanase and cellulase enzymes, some of which are listed in Table 2.3

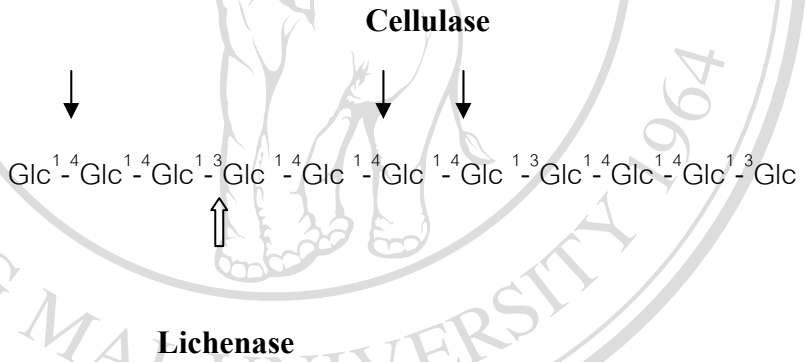

Figure 2.3 β-Glucan structure and enzymes cleave within the main chain of mixed-linkage β-glucan (Bedford and Partridge, 2001)

Table 2.3 Substrate for the assay of cellulase and β-glucanase (Bedford and Partridge, 2001)

Substrate	Nature	Assay prodedure
Cellulase		
Barley β-glucan	Soluble	Reducing-sugar or viscometric
Lichenan	Soluble	Reducing-sugar or gel plate
CMC-7M	Soluble	Reducing-sugar or viscometric
CMC-4M	Soluble/gel	Reducing-sugar
Azo-CMC	Soluble	Chromogenic substrate
Azo-barley glucan	Soluble	Chromogenic substrate
Cellazyme C tablets	Gel particles	Chromogenic substrate
Cellazyme T tablets (tamarind xyloglucan)	Gel particles	Chromogenic substrate
Beta-glucazyme tablets	Gel particles	Chromogenic substrate
1,3 : 1,4-β-glucanase		
Barley β-glucan	Soluble	Reducing-sugar or viscometric
Azo-barley glucan	Soluble	Chromegenic substrate
Beta-Glucazyme tablets	Gel particles	Chromogenic substrate

2.3 Solid-state fermentation

SSF is a process that occurs in the absence or near absence of any fluid in the space between particles. In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material. In contrast, in submerged fermentation (SmF) the nutrients and microorganisms are both submerged in water (Grigelmo and Martin, 1999).

In addition, in the solid process the proportion of each phase depends on the type of substrate being used. However, the main difference between solid and liquid fermentation is related to the fact that in the latter, the mixture between components of the process (microorganisms, nutrients and metabolites) is homogenous and uniformly distributed throughout the fermenter. This perfect mixture prevents the formation of distinct layers around the microbial cell that limit nutrient and metabolite diffusion and gas exchange, thus affecting microbial growth. In this respect, a solid medium can be considered to be more heterogenous in terms of the microbial population and solute concentration (Griffin, 1981). The higher the heterogeneity of

the mixture in solid fermentation, the less accurate are the results. To reduce heterogeneity, a mixer system, and forced aeration to remove CO_2, dissipate heat and distribute humidity is recommended (Gervais and Molin, 2003).

Among the characteristics of SSF, the low *a*w of the solid medium influences physiological aspects of the microorganism such as vegetative growth, sporulation and spore germination, as well as metabolite and enzyme production and enzyme activity. Fungal spores produced by a SSF culture are more stable, more resistant to dehydration and have a higher germination rate after freezing than spores obtained by SmF (Holker and Lenz, 2005). This has been attributed to the higher hydrophobicity, more rigid cell wall, and smaller volume of conidiophores obtained with SSF cultures (Pascual *et al*., 2000; Munoz *et al*., 1995).

Another important factor in SSF is aeration. Aeration has several functions: oxygenation, CO_2 removal, heat dissipation (regulating the temperature of the medium), distribution of water vapor (regulating humidity), and distribution of volatile compounds produced during metabolism. The aeration rate depends on the porosity of the medium; Oxygen Partial Pressure (*p*O2) and Carbon dioxide Partial Pressure (*p*CO2) should be optimized for each type of medium, microorganism and process (Chahal, 1987).

Temperature is related directly to water activity (*a*w) and aeration. One limitation of SSF is ability to remove excess heat generated by metabolism by microorganism due to the low thermal conductivity of the solid medium. In practice, SSF requires aeration more for heat dissipation than as a source of oxygen (Viesturs *et al*., 1981). Increased bioreactor temperatures cause denaturation of products, especially thermolabile substances (Santos *et al*., 2004).

Despite the problems of fermentation in solid medium whose main points have been discussed, this process has numerous advantages over liquid fermentation (Holker *et al*., 2004). First, the amount of specific enzymes produced by SSF is greater than obtained by submerged fermentation (SmF) (Aguilar *et al*., 2004). For example, the production of polygalacturonase and pectinlyase by *A. niger* was 5 and 1.3 times higher in solid-state culture than in submerged culture, respectively (Tagarano and Pilosof, 1999). Solis-Pereyra *et al*. (1993) compared the production of endo-polygalacturonase and exo-polygalacturonase by *A. niger* between SSF and

SmF. Production of both enzymes was greater in SSF cultures. Further the time required for synthesis was shorter in SSF. Enzyme synthesis was stimulated when the substrate contained higher sugar concentrations, in SSF, while in SmF, production decreased reflecting catabolite repression in SmF, but not SSF.

A. niger grew more efficiently and produced more invertase in SSF culture than in submerged culture when sucrose levels were high. These studies suggested that higher sugar levels prevented denaturation of invertase in SSF (Viniegra-Gonz´alez *et al*., 2003). In contrast with invertase, production of pectinase in SmF culture was greater; however, when sucrose was added, pectinase production was greater with SSF, in agreement with other investigators who showed that enzyme production is more sensitive to catabolite repression in SmF (Raimbault, 1998).

Other advantages of SSF compared to the traditional submerged process can be cited: higher cell mass production within a short period of time; similarity to the natural habitat of the filamentous fungi that permits use of wild-type microorganisms which often show a better performance than genetically modified strains and may enhance biosafety; downstream steps are facilitated due to the higher concentration of the product. On the other hand, disadvantages should be mentioned: problems with scale up production; difficulties in control of pH, heating, nutrient supply and humidity; impurity of the product because fiber degradation on microbial activity can generate complex and colored compounds such as melanoidins that are difficult to separate and may block columns and degrade resins during downstream processes. However, the greatest obstacle to the industrial use of SSF is the lack of knowledge about various aspects of the process and the lack of adequate fermenters (Robinson *et al*., 2001). Effect of cultural conditions on β-glucanase production is exhibited as follows.

2.3.1 Effect of Carbon-source

Carbon is the central component of the biological macromolecules, its incorporated into biosynthetic pathways may be derived form organic or inorganic sources, microorganisms which obtain their carbon in this way are described as heterotrops, and include all the fungi and protozoan as well as most types of bacteria (Hogg, 2005).

In general β-glucanase, cellulase, xylanase and pentosanase producing microorganisms produce these enzymes in the presence of the specific substrates. These are classified as inducible enzymes. Apart from inducing substrates, microorganisms required nutritional carbon and nitrogen sources for facilitation enzyme productions.

Fungi have been considered to be the organisms most adapted to SSF because their hyphae can grow on particle surfaces and penetrate into the interparticle spaces and thereby colonizing solid substrates (Santos *et al*., 2004). However, several studies also have reported. In SSF, the solid material can serve as a physical support and as a source of carbon and nutrients to sustain microbial growth or only as an inert physical support to which nutrients and the carbon source are added. This solid material generally is a natural compound consisting of agricultural and agroindustrial by-products and residues, urban residues, or a synthetic material (Pandey, 2003). Table 2.4 summarizes the literature available on by-products that can be used in enzyme production to apply on animal nutrition.

2.3.2 Effect of nitrogen source

Nitrogen is a major element in proteins, nucleic acids, and several other constituents in the cell. Nitrogen can be found in nature in both organic and inorganic forms. However, the bulk of available nitrogen in nature is in inorganic form, either as ammonia (NH_3), nitrate (NO_3^-) or N_2. Thus, the addition of urea or agroindustry by-product might be improve enzyme production (Hogg, 2005).

Hundred tonnes of such potential wastes (Ajinomoto Thailand) Co., Ltd., these wastes are ami-ami solution (waste water) from monosodium glutamate industry and still contain high level of organic nitrogen source for microbes. Ruanglek *et al*., 2006 showed that protein of ami-ami solution contains 9.27% of protein. However, these agro-industrial wastes might contain such minerals as chloride, calcium and others that could inhibit growth of microbes.

2.3.3 Effect of mineral

Except carbon and nitrogen other macro nutrient such as P, S, K, Mg, Ca, Na and Fe was essential nutrient for microbial growth. Phosphorus occurs in nature in the

form of organic and inorganic phosphates and is required by the cell primarily for synthesis of nucleic acids and phospholipids. Sulfur is required because of its structural role in the aminoacids cysteine and methionine and because it is present in a number of vitamins, such as thiamine, biotin and lipoic acid, as well as in coenzyme A. Potassium is required by all organisms. A variety of enzymes, including some of those involved in protein synthesis, specifically require potassium. Magnesium functions to stabilize ribosomes, cell membranes and nucleic acids and is also required for the activity of many enzymes. Calcium helps stabilizing the bacterial cell wall and plays a key role in the heat stability of endospores. Sodium is required by some but not all organisms and its need often reflects the habitat of the organisms. Iron plays a major role in cellular respiration, being a key component of the cytochromes and iron-sulfur proteins involved in electron transport (Hogg, 2005).

2.3.4 Effect of inoculum size

Importance of inoculum size on microbial fermentation process is widely accepted. The inoculum levels of *B. licheniformis* A99 at or above 15% were found to be suitable for enzyme production. An inoculum size of less than 10% was inadequate to allow good growth of the culture and consequently, enzyme production (Archana and Satyanarayana, 1997). The best concentration inoculum for *A. terreus* and *A. niger* was $2 \times 10^7 - 2 \times 10^8$ spores/g substrate (Gawande and Kamat, 1999).

2.3.5 Effect of moisture level

The moisture content in SSF is a crucial factor that determines the success of the process. The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate. A higher than optimum moisture level causes decreased porosity, alteration in particle structure, gummy texture, lower oxygen transfer, and enhancement of the formation of aerial mycelia (Raimbault and Alazard, 1980). Likewise, a lower moisture level than optimum leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling, and a higher water tension (Fenikwova *et al*., 1960 cited by Archana and Satyanarayana, 1997).

Table 2.4 Summarizes the literature available on by-products that can be used in enzyme production to apply on animal nutrition (Gaminha *et al*., 2007)

Substrate	Microorganism	Enzyme	Author
Rice straw	*Trichoderma reesei*	Cellulase Hemicellulase	Eun *et al*., (2006)
Barley	*Talaromyces emersonii*	Endo-β-glucanase	McCarthy *et al*., (2005)
Corn silage	*Thermoascus auruntiacus*	Xylanase	Colombatto *et al*., (2004)
Corn stalk	*Fusarium oxysporum*	Endoglucanase Cellobiohydrolase β-glucosidase	Panagiotou *et al*., (2003)
Corn straw	*Penicillium decumbens*	CMCase β-glucosidase Xylanase	Yang *et al*., (2001)
Alfalfa	*Gliocladium spp.* *Orpinomyces joyonii* *Caecomyces communis*	Cellulase, Xylanase Endoglucanase β-Glucosidase	Schimidt *et al*., (2001) Hodrova *et al*., (1998)
Forage silage	Streptomyces Achromogenes	Cellulase, hemicellulase	Hill *et al*., (2001)
Wheat straw	*Neurospora crassa* Several fungi	Cellulase Cellulase, hemicellulase Ligninperoxidase	Romero *et al*., (1999) Peiji *et al*.,(1997)
Sugarcane bagasse	*Trichoderma reesei* *Phanerochaete chrysosporum* *Coriolus versicolor*	Cellulase Ligninase	Kansoh *et al*., (1999) El-Gammal *et al*., (1998)
Sugarcane bagasse, wheat straw, corn cobs, rice husks, peanut shells	*Phanerochaete chrysosporium*	Cellulase, xylanase	El-Nasser *et al*., (1997)
Soy husks	*Coriolus versicolor*	Glucanase Cellulase	Jha *et al*., (1995)

Microbial growth and metabolism nearly always occur in an aqueous phase as does diffusion of solutes and/or substrates. CO_2/O_2 exchange, on the other hand, can occur both in the liquid and in the gas phase. In SSF, the liquid phase is represented by the aqueous film that surrounds the cells while the space between particles is occupied by the gas phase. Although replacement of the liquid phase with the gas

phase increases the transfer of oxygen, this prevents the diffusion of solutes and substrates and markedly interferes with microbial growth (Gervais *et al*., 1996).

2.3.6 Effect of incubation period

A high level of xylanase activity was produced by *Bacillus* sp. AR-009 grown by using wheat bran as a solid support. The time course of enzyme production was fallowed for 108 h. Maximum production was observed after 72 h. Further incubation after this time did not show any improvement in the level of enzyme production (Gessesse and Mamo, 1999). A low level of enzyme produced by *B. licheniformis* A99 appeared in the early stages of incubation and the enzyme levels steadily reached a maximum level by 72 h. A prolonged incubation time beyond this period did not help to further increase the yield (Archana and Satyanarayana, 1997). The duration needed for incubation is generally dictated by the properties of the strain such as its growth rate and enzyme production pattern (Park and Rivera, 1982 cited by Archana and Satyanarayana, 1997); thus, the time requirement for product formation in the case of bacteria is obviously far less due to their faster doubling rate. The best incubation period for *A. terreus* and *A. niger* was 144 h (Gawande and Kamat, 1999).

2.4 β-Glucanase production

Some microorganisms can produce endob-1,3-1,4-glucanase (cited by Tang *et al*., 2003) such as *Bacillus subtilis* (Cantwell and McConnell, 1983), *Bacillus macerans* (Heng *et al*., 1997), *Bacillus licheniformis* (Lloberas *et al*., 1991), *Bacillus amyloliquefaciens* (Hofemeister *et al*., 1986), *Bacillus polymyxa* (Gosalbes *et al*., 1991), *Bacillus brevis* (Louw *et al*., 1993), *Fibbrobacter succinogenes* (Teather and Erfle, 1990), *Streptococcus bovis* (Ekinci *et al*., 1997), *Clostridium thermocellum* (Schimming *et al*., 1991) and anaerobic fungus *Orpinomyces sp.* (Chen *et al*., 1997).

Tang *et al*., 2003 studied response surface methodology (RSM) method to optimize the medium composition for increasing the β-1,3-1,4-glucanase production by *B. subtilis* ZJF-1A5. Polysaccharides, such as barley flour, dextrin and soluble starch, were better carbon sources (Figure 2.4) than monosaccharides and disaccharides, for β-glucanase production. All inorganic nitrogen sources chosen in the experiments were not favorable for cell growth and enzyme production, yeast

extract was the best nitrogen source, followed by soybean flour (Figure 2.5). The composition of fermentation medium optimized with response surface methodology was (g/l): barley flour, 63.5; corn flour, 44.8; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1. β-Glucanase activity was 251 U/ml at 48 h using optimized medium, 1.4 times higher than that in original medium.

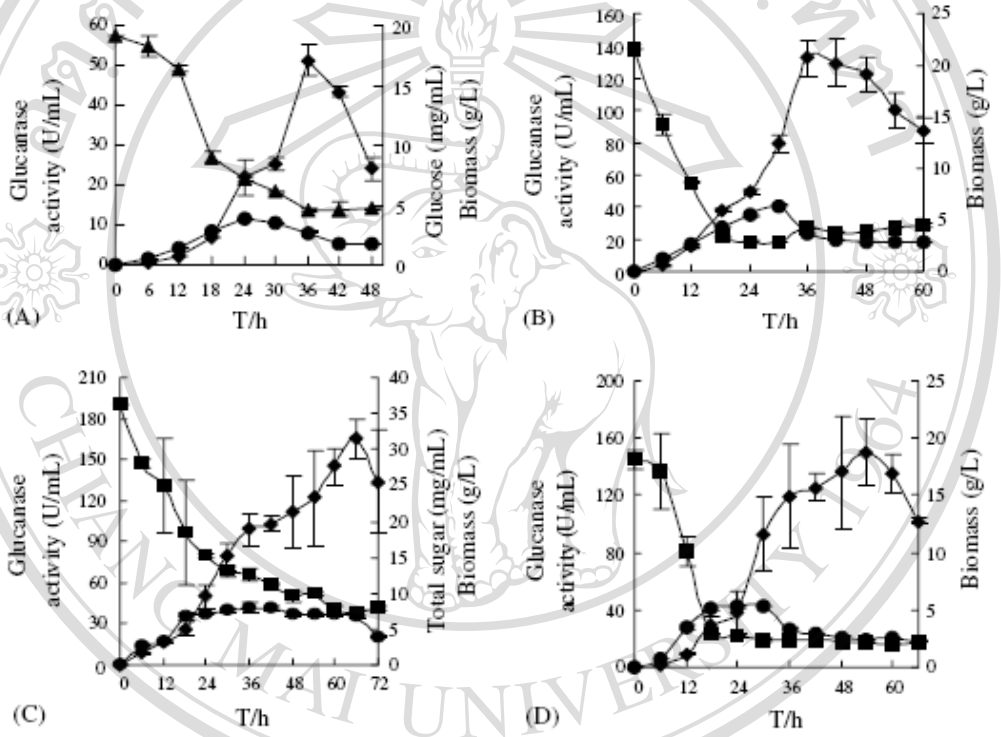

Figure 2.4 The plots of β-glucanase production by *B. subtilis* ZJF-1A5 with different carbon sources. (A) 2% glucose; (B) 2% dextrin; (C) 4% dextrin; (D) barley flour hydrolysate. β-glucanase activity (◆); glucose(▲) ; total sugar (■); biomass (●)

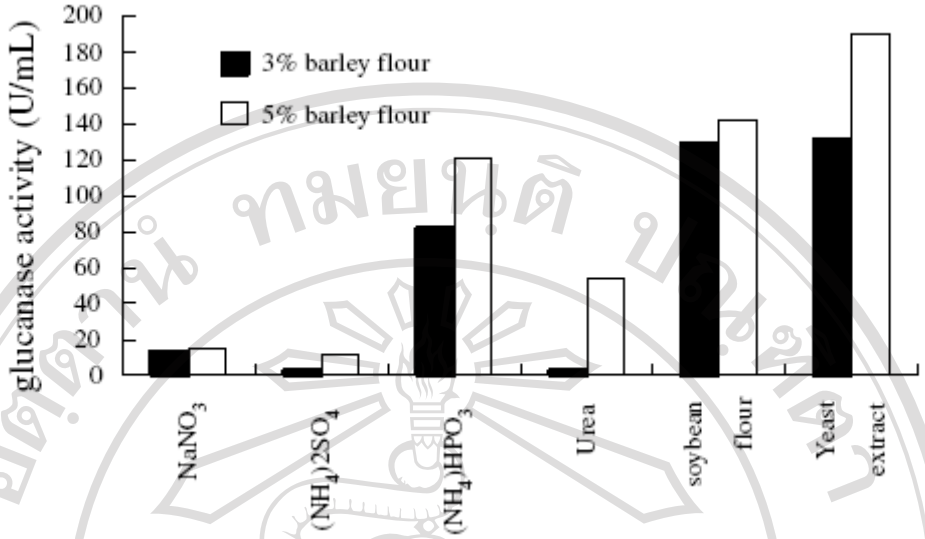

Figure 2.5 Effects of nitrogen sources on the β-glucanase production by *B. subtilis* ZJF-1A5

El-Helow and El-Ahawany (1999) studied to maximize lichenase production by *B. subtilis* strains harboring catabolite repression resistance mutations such as *crsA*, *crsE*, and *rvtA*.10. Among four isogenic catabolite repression-resistant *Bacillus subtilis* strain crsA47 were chosen. Optimized medium (tryptone, 10; yeast extract, 10; pectin, 2; and NaCl, 2 g l^{-1}) yielded a crude filtrate with lichenase activity of 114 U ml^{-1} and a specific activity of 20 U mg^{-1} dry protein within 24 h of cultivation. However, the formulate a medium suitable for lichenase formation by *B. subtilis* from renewable agro-industrial by-products. Powdered dry peels of lemon, orange, mango, and onion in addition to brewery spent grains, palm seeds, wheat straw, rice husk, and corn cob were individually used to replace pectin in the optimized medium at a concentration of 10 g l^{-1}. According to the results presented in Figure 2.6, lemon peel was chosen because its culture filtrate attained maximum grades of lichenase activity (100.9 U ml^{-1}) and specific activity (18 U mg^{-1} protein). Morover, nitrogen sources was approached by examining the fermentation of lemon peel (10 g l^{-1}) combined with an equal concentration of dry powdered wheat bran or meals of cotton seed, linen seed, sunflower seed, black seed, or soybean. Highest lichenase activity (120.1 U ml^{-1}) was recorded by the sunflower meal culture (Figure 2.7). However, as a result of minimum protein accumulation, maximum specific activities were introduced by

wheat bran and cotton seed meal cultures. Based on its availability in large quantities as a by-product with limited usage in Egypt, the latter was chosen for optimization experiments.

An optimum lemon peel-cotton seed meal ratio was investigated with a constant substrate mixture, formulations with nine different levels of lemon peel, supplemented with their complementary weights of cotton seed meal, were prepared. The substrate ratio of lemon peel/cotton seed meal = 4/16 (Figure 2.8) was selected because it showed the highest specific activity grade (46.4 U mg^{-1} protein). Nine different concentrations (ranging from 10–50 g l^{-1}) of the selected substrate composite were then tried. As shown in Figure 2.9, maximum lichenase production (123 U ml^{-1}) with a specific activity grade of 38 U mg^{-1} protein was given by the culture containing 45 g l^{-1} substrate mixture.

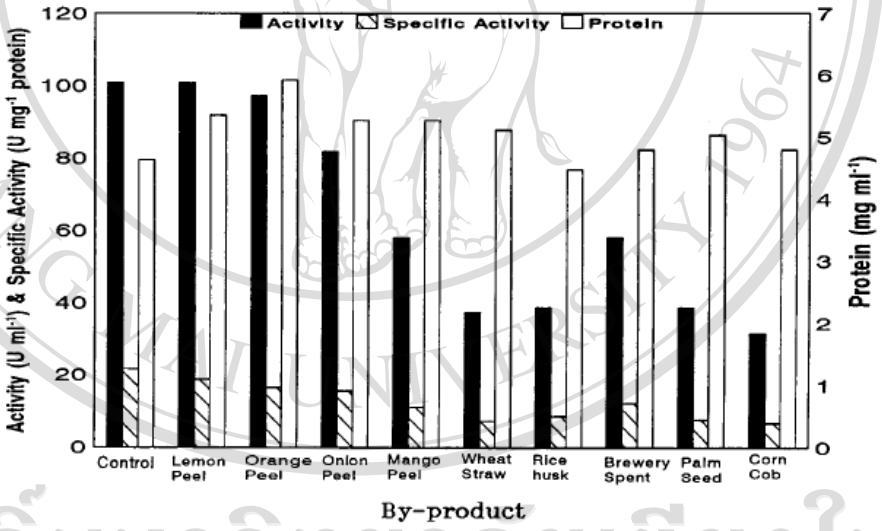

Figure 2.6 Screening for lichenase induction in *B. subtilis* crsA47 by agro-industrial by-products

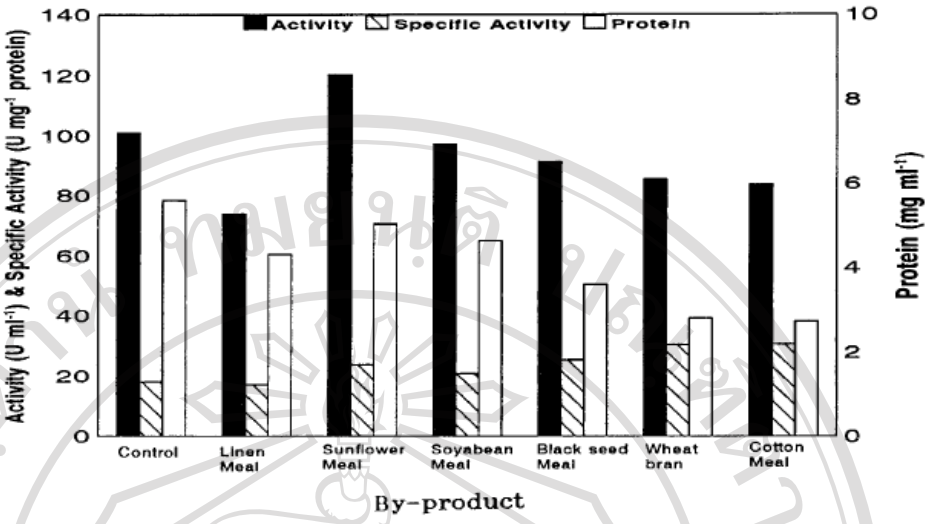

Figure 2.7 Screening for fermentable agro-industrial by-products served mainly as nitrogen sources for lichenase production by *B. subtilis* crsA47

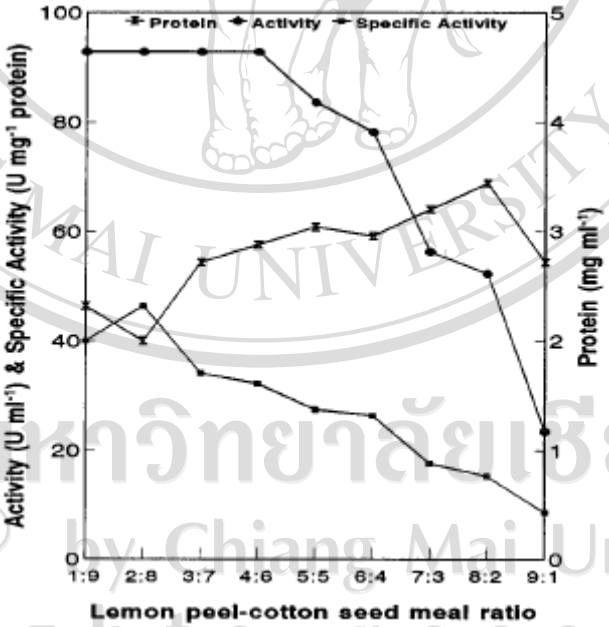

Figure 2.8 Effect of lemon peel-cotton seed meal ratio on lichenase formation and protein secretion by *B. subtilis* crsA47

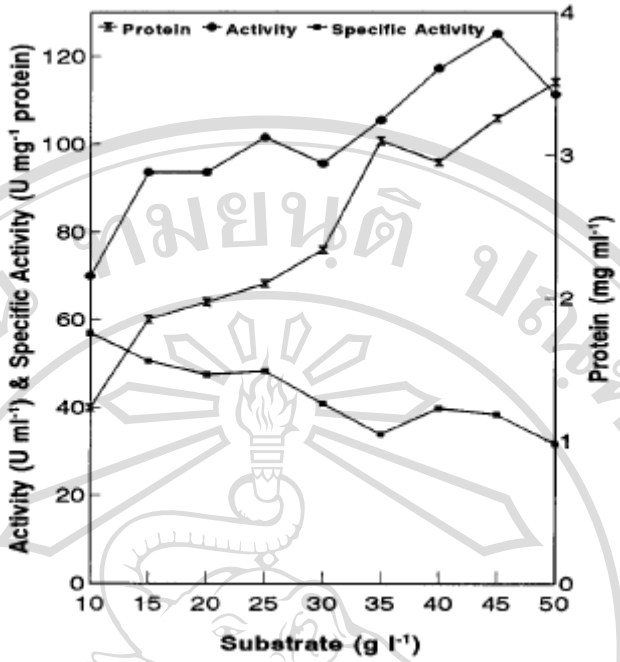

Figure 2.9 Effect of substrate concentration on lichenase formation and protein secretion by *B. subtilis* crsA47

Jecu (2000) studied the endoglucanase production from lignocellulosic materials under solid state fermentation (SSF) by *Aspergillus niger* 38. The effects of fermentation conditions, such as moisture content, initial pH, temperature, and composition of mixed substrate (wheat straw and wheat bran) on endoglucanase production by *Aspergillus niger* 38 were studied. With a moisture content of 74% a pH range of 4.5–5.5 on mixed substrate containing wheat straw : wheat bran of 9:1, 14.8 international units (IU) endoglucanase activity : ml were obtained in 96 h.

2.5 Evaluation of safety of enzymes supplement in feed

Several selected strains of fungi and bacteria are being successfully used in biotechnological processes for producing various useful substances for animal. Such strain are usually stable and not connected to toxicological or hygienic concerns. Hence, before application with animals selected microorganisms have to be tested to toxins mostly found in animal feed and causing problems for animals. Feed enzymes are subjected to toxicological evaluation according to EU (European union communicate) regulation. The main issues are evaluation of toxicity to the target

animal and to the consumer via residues in human feed. Because of the protein nature of the enzymes, toxicity of the enzymes, as such, is not expected. Enzymes are, like other native proteins, naturally occurring constituents of feed and food.

All feed untreated in a way which leads to denaturation of protein (e.g. heat, acid treatment) also contains enzymes of various origins. For instance, phytase activities are contained in cereal seeds at various levels. Yet, other compounds originating from the producing organism may be toxic, thus we must calculate the degree of purity required for the preparation of the desired product (Simon, 1996)

Table 2.5 Detection of toxin in animal feed and grain by Elisa kit (The American Association of Feed Microscopists, 1978; Cheeke and Shull, 1985; Miller, 1994; อินทิรา, 2540 and Miller, 2001 cited by พันทิพา, 2544)

Toxin	**Microorganisms**
Aflatoxin	*Aspergillus flavus, A. parasiticus, A. ruber, A. oryzae, A. niger, A. ostiamus, A. ochraceus, Penicillium puberulum, P. variable, P. citrinum, P. frequentans, Rhizopus sp.*
Fumonisin	*Fusarium moniliforme, F. anthophilum, F. dlamini, F. napiforme, Alternaria spp.*
Ochratoxin	*A. ochraceus, P. viridicatum*
T-2 toxin	*Fusarium sp., F. roseum, F. tricinctum, F. sporotriciodes, F. poae*
Vomitoxin (Deoxynivalenol, DON)	*Fusarium sp., F. roseum, F. graminearum*
Zearalenone (ZEN, F-2 toxin)	*Fusarium sp., F. roseum, F. graminearum*
Rubratoxin	*P. rubrum*
Citrinin	*Penicillium spp.*
Diacetoxyscirpenol (DAS)	*F. tricinctum, F. sporotrichiodes, F. poae, F. lateritium, F. equiseti, F. semitectum*
Nivalenol (NIV)	*F. graminearum, F. nivale, F. lateritium, F. equiseti, F. semitectum*
Moniliformin	*Fusarium sp.*
Fusarochormanone	*Fusarium sp.*
Tricothecenes	*Fusarium sp.*
Ergot	*Claviceps*

The toxin in feed majoring concern on the level of aflatoxin and ochratoxin which may be produced by some kind of microorganism. Table 2.5 show aflatoxin and ochratoxin produced by the metabolism of certain fungi *Aspergillus* and *Penicillium*. Thus, the screening of β-glucanse producing strain may be analyse only aflatoxin and ochratoxin which can produce by microorganisms used in the study.

2.5.1 Aflatoxin

Aflatoxin is a toxic and carcinogenic substance produced by certain strains of the molds *Aspergillus flavus* and *A. parastiticus*. Aflatoxin B1 is the most frequently encountered and the most virulent. The commodities most affected by aflatoxin are corn, peanuts, cottonseed, milo, and the majority of tree nuts.

The effects of aflatoxin in animals of ingesting excessive amounts of the toxin range form chronic ill-health and performance problems to death. Aflatoxin causes liver damage or cancer, decrease in milk and egg production and immune suppression. In addition, it interferes with reproductive efficiency. Swine are highly susceptible to aflatoxins. Extreme effects can lead to death, but the greatest impact comes from reduced reproductive capability, suppressed immune function, reduced productivity capability and various pathological effects on organs and tissues (Binder *et al*., 2007)

In animals, the effects of ingesting excessive amounts of the toxin range from chornic ill-health and performance problems to death (Hermandez, 2003). Aflatoxin has been shown to cause liver damage or cancer, decreased milk and egg production, immune suppression and interference with reproductive efficiency (Food Safety Research Office, 2003)

The Food and Drug Administration (FDA) has set maximum allowable levels of aflatoxin in food and feed. Therefore, accurate determination of the presence of the toxin is of major importance for those monitoring the quality of food and feed in which aflatoxin may occur. Testing these commodities for the toxin requires careful sampling, chemical extraction, sanitation and quantitative analysis.

Although there has been advisory and regulatory level for aflatoxin issued by the Food and Drug administration, many agree that levels about 20 parts per billion (ppb) for commodities destined for human or animal consumption may cause health problems and economic loss.

2.5.2 Ochratoxin

Ochratoxin, commonly produced by the molds *A. ochraceus* and *Penicillium viridicatum*, can be found in corn, barley, wheat and milo. Ochratoxin may be present in conjunction with aflatoxin. One of the most naturally occurring carcinogens. In fact, ochratoxin is a suspected carcinogen (Neogen Corporation, no date).

The toxic effects of ochratoxin A have been studied extensively in a variety of experimental animals. All the animals studied so far have been susceptible to orally administered ochratoxin A, but to various degrees. At high levels of ochratoxin A, changes were found in the kidneys and also in other organs and tissues. However, only renal lesions were observed at exposure levels identical to those occurring environmentally.

Feed levels as low as 200 μg/kg produced renal changes in the course of 3 months in rats and pigs. Field cases of ochratoxin A-induced nephropathy are regularly encountered in pigs and poultry. Ochratoxin A is teratogenic in the mouse, rat, and hamster. Ochratoxin B, rarely found as a natural contaminant, is much less toxic; the other ochratoxins have never been encountered in natural products. The nephrotoxic potential of ochratoxin A is well documented from all experimental studies, with a feed level of 200 μg/kg causing nephropathy in pigs and rats. Lower levels have not been tested. Field cases of ochratoxin A-induced nephropathy in farm animals have long been recognized.

Ochratoxin affects kidneys in animals exposed to naturally occurring levels of this mycotoxin. Turkeys and other poultry exhibit lower productivity levels during field outbreaks of ochratoxicosis. Symptoms include retarded growth and decreased feed conversion (Leatherland Food International, no date). It has been known to affect egg production in laying hens (Ministry of Health, Singapore, no date).

Although there has been no advisory or regulatory level for ochratoxin issued by the Food and Drug Administration, many agree that levels between 10-20 parts per billion (ppb) for commodities designed for human or animal consumption may cause health problems and economic loss. Some foreign markets have set regulation limits ranging from 5 to 50 ppb.

The best protection against mycotoxins is monitoring for their presence in feeds and foods. That means testing all along the pathway from initial harvest of grains to the finished product.

2.6 Why use enzymes in animal feed?

The main purpose for the use of enzyme is to improve the nutritive value of feedstuffs. All animals use enzyme in the digestion of food, those produced by animal itself or by the microbes in the digestive tract. However the digestive process is not 100% efficient; for example swine can not digest 15-25% of the food they eat (Sheppy, 2000). Therefore, it is necessary to add enzymes in animal feeds is to increase the efficiency of digestion.

In animal production systems, the main cost and profitability. Which is depends on the relative cost and nutritional value of the available feeds. Often, the limiting factor when formulating rations is the animal's ability to digest different constituent parts of the feed ingredients, particularly fiber. This inefficiency in the utilization of nutrients can result in a cost to the farmer, the feed company and the environment. There are four main reasons for using enzymes in animal feed (Bedford and Partridge, 2001):

1. To break down anti-nutritional factors that are present in many feed ingredients. These substances can not be digested by the animal's endogenous enzymes, moreover it can interfere with normal digestion, causing poor performance.

2. To increase the availability of starches, proteins, and minerals that are enclosed with in fiber-rich cell walls and not accessible to the animal's own digestive enzymes, or bound in a chemical form that the animal is unable to digest (e.g. phosphorus as phytic acid).

3. To break down specific chemical bonds in raw materials that are not usually broken down by the animal's own enzymes, thus releasing more nutrients.

4. To supplement the enzymes produced by young animals where endogenous enzyme production may be inadequate, because of the immaturity of their own digestive system.

2.7 The current enzyme used in animal feeds

Broadly, there are four groups of enzymes used in farm animal (Bedford and Partridge, 2001).

2.7.1 Fiber-degrading enzymes

One of the main limitations to digestion is the monogastrics animal (pigs and poultry) do not produce the enzymes to digest fiber. In diets containing ingredients such as wheat, barley, rye or triticale, a large proportion of this fiber is soluble and insoluble arabinoxylan and β-glucan (White *et al*., 1983). The soluble fiber can increase the viscosity of the contents of the small intestine, impeding the digestion of nutrients and thereby reducing the growth of the animal.

The fiber content of wheat and barley can vary considerably according to variety, growing location, climatic conditions, etc. This in turn means that there can be considerable variability in the nutritional value of these ingredients and hence diets containing them. In breaking down the fiber, enzymes (e.g. xylanase targeting arabinoxylans, β-glucanase targeting β-glucans) can reduce this variability in nutritional value, giving rise to improvements in the performance of the feed and the consistency of the response (Bedford and Classen, 1992).

2.7.2 Protein-degrading enzymes

Various raw materials contribute to the protein content in the diet and ultimately the amino acids the fuel lean meat deposition. There is considerable variability in the quality and availability of protein from the different raw materials typically found in monogastric diets. Within the primary vegetable protein source such as soybean meal, certain anti-nutritional factors (ANFs), such as lectins and trypsin inhibitors, can lead to damage to the absorptive surface of the gut, impairing nutrient digestion (Liener, 1994). In addition, the underdeveloped digestive system of young animals may not be able to make optimal use of the large storage proteins found in the soybean meal (glycinin and β-conglycinin) (Pusztai *et al*., 1990).

The addition of a protease can help to neutralize the negative effects of the proteinaceous ANFs in addition to breaking down the large storage protein molecules into smaller, absorbable fractions (Pusztai *et al*., 1990).

2.7.3 Starch-degrading enzymes

To many nutritionists maize is viewed as the "gold standard" of raw materials. Most nutritionists do not consider maize digestion as being poor. In fact, most would argue that is better than 95% digested. However recent evidence presented by Noy and Sklan (1994) suggests that, at the ileal level, starch digestibility rarely exceeds 85% in broilers between 4 and 21 days of age. The addition of an amylase to animal feed can help to expose the starch more rapidly to digestion in the small intestine, and in doing so lead to improved growth rates from enhanced nutrient uptake.

At weaning, piglets often suffer a growth check because of changes in their nutrition, environment and immune status. The addition of an amylase, usually in conjunction with order enzymes, to augment the animal's endogenous enzyme production has been shown to improve nutrient digestibility and absorption and hence growth rate for a range of diet (Close, 1995).

2.7.4 Phytic acid-degrading enzymes

Phosphorus is required for bone mineralization, immunity, fertility and growth and is an essential mineral for all animals. Swine and poultry digest only about 30-40% of the phosphorus found in feedstuffs of vegetable origin, with the remainder being tied up in a form inaccessible to the animal-phytic acid (Ravindran *et al*., 1995). In many instances, additional phosphorus must be added to the diet to meet the animal's requirement. More than half of the phosphorus consumed from such feedstuffs is excreted in the feces, which can result in major environmental pollution. By adding a phytase to the diet, the phytic acid is broken down, liberating more of the phosphorus for use by the animal.

The two main benefits of phytase supplementation are, firstly, the reduction in feed costs from the reduced additional supplementation of phosphorus to the diet and, secondly, environmental from reduced excretion of waste products and the threat of pollution.

Until recently, the majority of feed enzyme research has been performed on cereal based diets, for example, barley and wheat, particularly for poultry diets. The type of feed enzymes currently available are outlined in Table 2.6.

Table 2.6 Feed enzymes in use today. (Ogden, 1995 cited by Rotter *et al*., 1989; Cowan, 1992)

Enzyme	Action	Target substrate	Type of feed	Expected benefits
β-glucanase	β-glucans to oligosaccharides and glucose	Barley, oats and rye based diets	Poultry and pig diets	Reduction of sticky droppings, improved feed utilization
Amylase	Degrade cereal starch to dextrins and sugars	High starch cereal diet	Early pig/calf diets	Increase availability of cereal
Cellulase	Cellulose to low molecular weight products and glucose	High fiber diets	Poor-grade forages	Improved energy availability
Pentosanases (Xylanases)	Arabinoxylans to low molecular weight products and sugars	Rye, barley and wheat	Pig and poultry diets	Improved litter quality, improved feed utilization
α-Galactosidase	Degrades oligosaccharides and ANFs	Soybean and other legumes	Pig diets	Improved energy availability, reduced scours in piglets
Phytases	Increases availability of phosphorus from phytic acid	Many different diets	Pig and poultry diets	Reduces need for inorganic phosphorus
Proteases	Protein to peptides and amino acids	Wheat by-products, legume proteins	Milk replacer using soybean or soybean protein	Higher protein digestibility, lower nitrogen excretion
Lipases	Fats to fatty acids	Animal and vegetable fats	Pet diets/broiler diets	Improved digestibility of fat and enhanced energy retention as a result

2.8 Presentation of Exogenous Enzymes to the Animal

The most common method of feeding enzymes is with dried feed. The enzymes are usually being added to the feed during blending, often prior to processing treatments such as pelleting. However, most of the enzymes currently of interest to the animal feed industry, such as β-glucanase, amylase, proteases and phytase, suffer temperature-related reduction in activity due to heat treatments. Therefore, an enzyme must be able to remain active after exposure to the high temperature and pressure of a pellet mill.

An alternative to the use of exogenous enzymes in dry diets is the pretreatment of the diet or its components prior to secondary processing. Adding enzymes to complete compound feed may be appropriate when the enzyme is targeted at cereals, which make up the bulk of the diet.

There are many factors evolving in the animal feed industry which suggest that the use of exogenous enzymes will become more important in the future (Johnson *et al*., 1993). These include:

(i) An increasing shift in the use of 'alternative' feedstuffs in formulating diets

(ii) The use of enzymes known to be effective against particular dietary components

(iii) The production of novel by-product (for example, linseed meal derived after linseed oil production) that have a depressing effect on growth

(iv) The increased availability of free amino acids which may reduce the requirement for high quality protein supplements

(v) Novel feed systems, such as liquid feed systems and those that take advantage of pretreatment, which support the use of in-feed enzymes

(vi) The introduction of pollution control, for example of nitrogen and phosphorus (enzymes that reduce excretion of these substances would be advantageous)

(vii) The possibility of indirect physiological actions on problems of post-weaning diarrhoea in pigs

(viii) The possible reduction in animal performance due to the restricted use of growth-promoting antibiotic

2.9 Action of feed enzymes in the animal

The application of exogenous enzymes to dry diets presupposes that the enzyme will be active in the digestive tract of animal, and it must therefore fulfil a number of criteria. The enzyme must be active under the physiological conditions prevailing in the animal's digestive tract; it must be able to resist proteolysis by the animal's endogenous proteases and supplement rather than antagonize the animal's digestive enzymes. Species differences in the anatomy and physiology of the digestive tract are likely to affect exogenous enzyme activity in this respect. For example, between pigs and poultry these differences include the following (Pattridge *et al*., 1993)

1. Anatomical: in poultry, feed passes into the crop, where any added enzymes can act for several hours at a pH of approximately 6.0 before passing into the acid environment of the gizzard, whereas in the pig, feed passes directly into the acid environment of the stomach immediately after ingestion.

2. Digestive capacity: poultry have a shorter small intestine and thus reduced possibilities for enzyme inactivation by the microflora, a shorter mean retention time in the small intestine (1-2 h in poultry versus 4-5 h in the pig) and a lower water content in the upper part of the gastrointestinal tract.

3. Bacterial activity: the importance of the microflora in the gut of poultry is much less than in the pig.

4. Fiber fermentation: lower in poultry than in pigs, due to their widely different hind gut capacities.

Survivability of enzymes in the digestive tract varies widely. Thacker and Baas (1996) demonstrated that 84% of pentosanase and 26% of β-glucanase activity was recovered in the duodenal digesta of pigs 4 h after feeding diets supplemented with these enzymes. Approximately 75% of exogenous protease has been detected in the ileal digesta of young pigs fed protease-supplemented diets.

The rusult of enzyme use is an increase in the rate of nutrient digestibility. This is important since it moves the site of digestion and absorption of starch and protein to a more anterior site wherein animal has a greater competitive edge over its resident microflora. This is more the case as the bird ages and its intestinal tract

matures and becomes more heavily populated. Fig2.10. illustrates the case in discussion (Bedford, 2000).

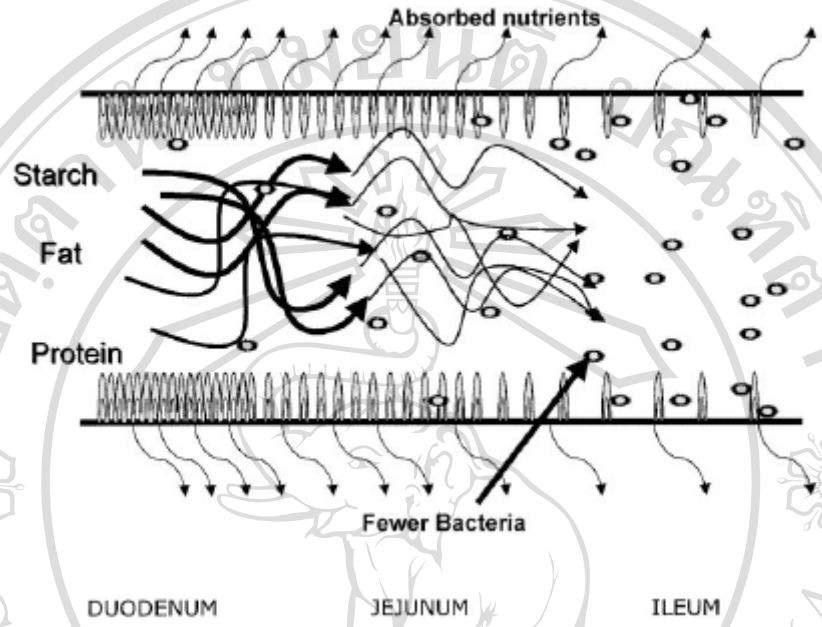

Figure 2.10 Relationship between the rate of digestion of a diet and microbial population density. A rapidly digestible ration supports fewer microbes. (Bedford, 2000)

As feed passes through the stomach/gizzard, it is largely sterilized by the extremes of pH and activity of pepsin. In addition, as it enters the duodenum it is exposed to a rapid and significant pH shift towards neutral which further stresses any bacterial survivors of gastric transit. Large influxes of digestive enzymes, bile acids, lecithin and lysozyme further test the surviving bacteria such that the duodenum is largely devoid of bacteria. In the upper regions of the gut, digestive efficiency is maximal due to the high concentrations of pancreatic enzymes and efficient and highly active absorptive enterocytes (Uni *et al*., 1999). As feed passes though the small intestine, there is a progressive decline in digestive enzyme and bile acid concentration as these are either catabolised and/or absorbed (Campell *et al*., 1983). As a result, the environment of the small intestine becomes increasingly hospitable to bacterial colonisation.

If the diet being fed is highly digestible then the majority of nutrients are digested and absorbed prior to the establishment of an environment favourable to bacterial growth. As a result, the populations of the lower small intestine are kept to a minimun essentially through substrate limitation. With a poorly digested diet, however, nutrients evade digestion and absorption by the bird and as a result enter the mid-lower small intestine where the bacterial population are able to make good use of such substrate, and flourish as has been shown when comparing rye (poorly digested) with corn (well digested) based diets (Wagner and Thomas, 1987). In stimulation of bacterial growth, there are inevitably species which are able to colonize the anterior reaches of the intestine by production if enzymes which actively degrade the very antimicrobials the bird produce, such as bile acids (Christl *et al*., 1997). Through deconjugation and dehydroxylation, these compounds lose their antibacterial effect and as a result the sensitive bacteria are able to thrive. Elimination of these active compounds also results in impaired fat digestion since bile acids are essential for efficient micelle formation (Campbell *et al*., 1983)

Evidently, the consequences of reduced diet digestibility overgrowth are manifold, not least since the presence of a greater population will demand a greater energy and protein requirement from the diet which is ultimately taken at the expense of the host.

The consequences of reduced diet digestibility, therefore, need to be assessed from two viewpoints if the benefits of cereal targeted enzymes are to be correctly assessed. The first is direct effects of a poorly digested diet on the nutrient assimilation rate of the host and the second is the ramifications that such an increase in substrate delivery will have on microfloral populations inhabiting both the small intestine and the caeca. The former will of course limit the growth rate of the animal and the latter may result in a less efficient utilisation of digested and/or utilised nutrients through competition for substrates and interactions with the health status of the animal.

Enzymes have clearly been demonstrated to increase the digestibility of poorly digested cereals to a much greater extent than well digested cereals (Classen *et al*., 1995). There are two consequences of such an effect of enzyme addition as far as the feed compounder is concerned:

1. Variation between the best and worst samples of a given grain is reduced.
2. In practice, the average nutrient content of the cereal is greater in the presence of enzyme than in the absence. As a result, addition of an enzyme allows feed formulation nutrient matrix values to be elevated.

2.10 Supplementing β-glucanase to animals

In highly developed animal, nutrients like proteins, fat, and carbohydrates can only be absorbed in nutritionally significant quantities in the form of free amino acids, fatty acids, and monosaccharides, or oligopeptides-saccharides. Such digestive processes will not occur without the relevant enzymetic reactions. The endogenous enzymes produced by animals may be insufficient in special circumstances. Supplementation of diets with exogenous enzymes would therefore help the animal cope in these situations.

The change in environment at weaning often remarkably challenges an animal's ability to secrete endogenous enzyme. Before piglets can satisfactorily cope with the post-weaning diet, therefore, they may need a period of adaptation to increase the physical size of the gastrointestinal tract; its capacity to secrete digestive enzyme, HCl, bicarbonates, and other chemicals; and its absorptive capacity. Linderman *et al*., (1986) reported that the total amount of enzymes secreted and the amount per gram of pancreas increased linearly in the pig after birth. Weaning at 28 d of age caused a sudden drop in the activities of amylase, protease, and lipase the following week. This resulted in severe digestion problems, including reduced nutrient absorption and diarrhea. Adding the appropiate enzymes to the diet, in combination with an optimal feed composition, should overcome such problems.

Enzyme manufacturers provide plenty of data showing responses to β-glucanase enzymes. Summaries of some typical studies are given in Table 2.7

2.10.1 Effects to digestibility

Several studies have investigated the effects of enzyme supplements on nutrient digestibility. Li *et al*., (1996b) determined the effect of β-glucanase supplementation to hulless barley-soybean meal (HB+SBM). Twelve piglets, average BW 7.3 kg, were fitted with a simple T-cannula at the distal ileum, approximately 5

cm from the ileo-cecal sphincter. After a 7-d recuperation period, six pigs were allotted to each dietary treatment according to a two-period crossover design. β-glucanase supplementation to the hulless barley + Soybean meal diet increased ($P < .05$ or $P < .01$) the ileal digestibilities of gross energy (GE), crude protein (CP), β-glucans, and the majority of the amino acids and the fecal digestibilities of GE, CP and all amino acids (Table 2.8).

Gdala *et al*., (1997) studied 8-12 week old piglets fitted with a T-shaped cannula at a terminal ileum. The supplementation of barley and wheat based diet with enzyme mixture, increased the digestibility of xylose, arabinose, mannose and dry matter.

Bergh *et al*., (1999) reported ileal digestibility of nutrients was generally improved among chickens given enzyme supplemented diets when compared to animals fed diets without enzyme, the greatest increase in ileal fat digestibility (19% increase) and ileal starch digestibility (9% increase) being observed among animals given the enzyme supplemented waxy diet in comparison to the unsupplemented waxy diet.

Moreover, the ileal digestibility of total β-glucan was also improved by enzyme supplementation and it was concluded that the β-glucanase used in this study may have reduced β-glucan-enhanced intestinal viscosity or nutrient encapsulation due to the presence of intact cell walls and thus, facilitated nutrient uptake (Bergh *et al*., 1999; Jensen *et al*., (1998).

Yin *et al*., (2001) conducted to investigate the effect of β-glucanase on nutrient and energy digestibilities at ileal and total tract levels on young pigs based hulless barley. The results (Table 2.9) showed that the supplementation of β-glucanase significantly ($P<0.05$) improved apparent ileal digestibility of dry matter (DM), gross energy (GE), crude protein (CP), amino acids (AA), neutral detergent fibre (NDF) and total non-starch polysaccharide (NSP). Reduction in hindgut fermentation was observed probably due to the improved ileal absorption of nutrients.

Table 2.7 Enzyme efficacy: digestibility and production performance

Enzymes	Diet	Animal	β-glucan (%)	Response (%)	Reference
β-glucanase	Barley based	Broiler	3.9	Improve FI, DG and FCR	Almirall and Estive-Garcia (1995)
β-glucanase	Barley based	Piglet	5.8	Increase ileal digestibility of GE, CP, β-glucan and amino acid Increase fecal digestibility of GE, CP and amino acid	Li *et al*., (1996)
β-glucanase	Barley based	Piglet	5.08	Increase digestibility of DM, CP and energy	Li *et al*., (1996)
β-glucanase	Barley based	Broiler	NA	Improve FCR (4.3%) and live weight (4.5%) Reduce sticdy droping	Bustany (1996)
β-glucanase, xylanase α-amylase, protease and αgalactosidase	Barley and Wheat based	Piglet	NA	Improve ileal digestibility of DM, arabinose, xylose and mannose.	Gdala *et al*., (1997)
β-glucanase, xylanase	Barley based	Poultry	3.62-4.40	Improve AME of barley	Villamide *et al*., (1997)
β-glucanase	Barley based	Broiler	3.0-4.0	Improve FI, body weight, FCR. Increase total HDL-cholesterol Increase ileal digestibility of fat, starch and β-glucan Reduce intestinal viscosity	Bergh *et al*., (1999)
β-glucanase	Barley based	Piglet	2.6-4.8	Increase digestibility of β-glucan and reduce digesta viscosity in the upper of GI tract	Jensen *et al*., (1998)
Roxazyme® G	Barley based	Broiler	NA	Improve body weight and FCR	King and Moughan (1998)
Roxazyme® G	Wheat and Rye base	Laying hen	NA	Improve AME and FE Increase egg production	Pan *et al*., (1998)
β-glucanase	Barley based	broiler	4.0-7.3	Improve BWG, FI, FCR and starch digestibility	Ankrah *et al*., (1999)
β-glucanase	Barley based	piglet	4.02-6.15	Improve ileal digestibility of DM, GE, CP, AA, NDF and NSP	Yin *et al*., (2001)
β-glucanase, xylanase	Rye based	Broiler	7.6	Improve DG, FI, FCR . Increase AME Increase villus size, villus height to crypt depth ratio Increase concentration of bile acids in small intestine	Mathlouthi *et al*., (2002)

NA = Not available, FI = Feed intake, DG = Daily gain, FCR = Feed conversion ratio, FE = Feed efficiency, BWG = Body weight, GE = Gross energy, AME = Apparent metabilizable energy, CP = Crude protein, DM = dry matter, AA = Amino acids, NDF = Neutral detergent fiber, NSP= Non-starch polysaccharide

Table 2.8 Effects of β-glucanase supplementation on ileal and fecal digestibilities (%) of dry matter (DM), organic matter (OM), crude protein (CP), gross energy (GE), β-glucans, and amino acids in the hulless barley + soybean diet (Li *et al*., 1996)

	Ileal digestibility		**Fecal digestibility**	
Item	Control	β-glucanase	Control	β-glucanase
DM	60.1c	66.7b	84.6c	86.4b
OM	63.0c	69.6b	87.0c	88.9b
CP	65.2C	73.5B	80.5C	85.9B
GE	64.9c	71.1b	84.6C	87.2B
β-glucans	80.1C	92.1B	99.4	99.5
Aminoacids				
Indispensable				
Arginine	79.4C	84.3B	88.8C	91.6B
Histidine	76.9c	81.9b	87.9C	91.0B
Isoleucine	71.0C	77.6B	81.9C	86.2B
Leucine	71.6C	78.3B	84.2c	87.6b
Lysine	69.0c	73.1	80.9C	85.5B
Phenylalanine	74.1C	80.1B	86.0C	89.0B
Threonine	57.9C	67.7B	79.8c	84.7b
Valine	69.9C	76.7B	82.8C	86.9B
Dispensable				
Alanine	61.8C	70.3B	78.7c	83.4b
Aspartic acid	70.4	73.9	85.3c	88.0b
Glutamic acid	79.5	83.4	91.4C	93.5B
Glycine	57.7	64.9	81.5C	85.4B
Serine	68.9C	75.2B	86.2c	88.8b
Tyrosine	66.7c	77.1b	81.5C	86.8B

b,c Means in the same row, within ileal or fecal digestibilities, with different superscript letters differ ($P < 0.05$).

B,C Means in the same row, within ileal or fecal digestibilities, with different superscript letters differ ($P < 0.01$).

Table 2.9 Effects of adding enzyme preparations on apparent ileal and overall digestibilities (%) and hindgut fermentation (HF, %) of dry matter, gross energy, crude protein, neutral detergent fiber (NDF) and ileal total non-starch polysaccharides (NSP) (Yin *et al*., 2001)

	Control	β-glucanase
Ileal digestibility		
Dry matter	60.3b	68.7a
Gross energy	62.1b	69.8a
Crude protein	59.6b	66.7a
NDF	16.0c	29.6ab
NSP	37.2b	48.3a
Overall digestibility		
Dry matter	81.4	82.7
Gross energy	80.3	81.6
Crude protein	69.5b	73.9ab
NDF	60.9b	68.1a
HF1e		
Dry matte	21.1a	14b
Gross energy	18.2a	11.8ab
Crude protein	9.9a	7.2b
NDF	44.9	38.5
HF2f		
Dry matter	55.1a	44.7b
Gross energy	48.0a	39.1ab
Crude protein	25.4a	21.6b
NDF	53.4	54.6

a,b,c Values in the same row with different superscript letters differ ($P < 0.05$).

e Fermentation expressed as the difference between overall and ileal digestibility.

f Fermentation expressed as a proportion of undigested material reaching hindgut.

2.10.2 Effect to villus size and the concentration of bile acids in the small intestine

Mathlouthi *et al*., (2002) was studied with growing chickens (4 to 22 d of age) to evaluate the effects of feeding a rye-based diet supplemented with commercial enzyme preparation containing xylanase and β-glucanase (Quatrazyme HP, Nutri-Tomen, France) on small intestine wall morphology and bile acid composition. Nutrient digestibility, bird performance compared with unsupplemented.

The addition of xylanase and β-glucanase to the rye-based diet improved ($P \leq 0.05$) weight gain, feed intake, and feed efficiency, and decreased water intake. The

digestibility of nutrients and apparent metabolizable energy were also increased ($P \leq 0.05$). Addition of xylanase and β-glucanase increased ($P \leq 0.05$) villus size and the villus height to crypt depth ratio (Table 2.10), as well as the concentration of conjugated bile acids ($P \leq 0.05$) in the small intestine contents (Table 2.11). Exogenous enzymes improved nutrient digestibility and broiler chicken performance, probably by improving the absorption capacity of the small intestine through increased villus surface and intestinal concentration of conjugated bile acids.

Table 2.10 Histological measurement of the ileal wall in broiler chickens fed corn or rye based diets supplemented or not with enzyme (Mathlouthi *et al*., 2002)

	Dietary group		
Variable	**Corn based diet**	**Rye based diet**	**Rye based diet + E**
Villi			
Villus length, cm	686a	579b	770a
Villus width, µm	382a	294b	395a
Villus surface, mm^2	0.27a	0.16b	0.31a
Crypts			
Crypt depth, µm	163a	157a	170a
Crypt width, µm	70a	75a	74a
Crypt surface, mm^2	0.022a	0.021a	0.017a
Villus length : crypt depth ratio	4.49a	3.73b	4.62a

a,b Means in the same row with different letters are significantly different ($P<0.05$).
E= xylanase and β-glucanase addition at the level of 20 mg/kg of diet.

Table 2.11 Bile acid concentrations (mg/g of freeze-dried content) in the small intestinal contents of broiler chickens fed corn or rye based diets supplementor not with enzyme (Mathlouthi *et al*., 2002)

Bile acids	Dietary group		
	Corn based diet	**Rye based diet**	**Rye based diet + E**
Cholic (total)	2.83a	2.46b	2.83a
conjugated	2.34a	1.02a	2.43b
unconjugated	0.49a	1.44b	0.40c
Chenodeoxycholic (total)	10.36a	3.56b	4.19c
conjugated	8.42a	1.81b	3.21c
unconjugated	1.94a	1.75b	0.98c
Deoxycholic (total)	0.36a	0.30a	0.31a
conjugated	0.28a	0.14b	0.31c
unconjugated	0.08a	0.16a	0
Lithocholic (total)	0	0.75a	0.06b
conjugated	0	0.75a	0.06b
unconjugated	nd	nd	nd
Total bile acid (total)	13.56a	7.08b	7.39b
conjugated	11.04a	3.73b	6.01c
unconjugated	2.53a	3.35a	1.38b

a,b Means in the same row with different letters are significantly different ($P<0.05$).
E= xylanase and β-glucanase addition at the level of 20 mg/kg of diet

The effect of rye feeding was strong stimulation of the early deconjugation of taurochenodeoxycholic and taurocholic acids, which are the predominant bile acids in chicken bile (Elkin *et al*., 1990), but this was counteracted by enzyme supplementation.This strongly suggests the presence of some species of bacteria in the small intestine of rye-fed chickens that can deconjugate bile acids (Hylemond, 1985)

This coincides with morphological results, demonstrating that intestinal mucosa was modified in chickens receiving rye alone, but not in those receiving rye

plus enzymes. This damage to the small intestinal mucosa may be caused indirectly by the viscous characteristics of nonstarch polysaccharides (Stanogias and Pearce, 1985). Sakata (1987) demonstrated that an increase in bacterial activity in the gastrointestinal tract was associated with a change in the morphology of the gut wall. This reinforces the idea that exogenous enzymes might exert their beneficial action by influencing the intestinal microflora (Figure 2.11) (Bedford, 2000). As a result, a decrease in total bile acid concentration aggravated by early bacterial deconjugation, which made fat emulsification less effective (Coates *et al*., 1981) and the decrease in the absorptive capacity of the intestinal mucosa might together contribute to lower fat digestibility in rye-fed broiler chickens. Furthermore, the present study indicates that feeding a rye-based diet not only reduces fat digestibility, but also reduces protein digestibility. This could be explained by bacteria overgrowth in the small intestine, which increases the loss of endogenous nitrogen (Smits *et al*., 1997) by incorporating amino acids into microbial proteins (Salter and Coates, 1974) and thus reduces apparent protein digestibility (Angkanaporn *et al*., 1994). Second, the increase in digesta viscosity might reduce the diffusion rate of digestive enzymes such as proteases (Larsen *et al*., 1993).

Moreover, enzyme supplementation restored the absorptive capacity of the intestinal mucosa by increasing intestinal villi size, and it suppressed early bile acid deconjugation. This is probably due to a decrease in bacteria that hydrolyze conjugated bile acids (Smits and Annison, 1996). In fact, Bedford (2000) reported that the positive effect of adding exogenous enzymes is thought to be related to changes in microflora activity rather than to the direct effect of the enzyme on diet digestibility.

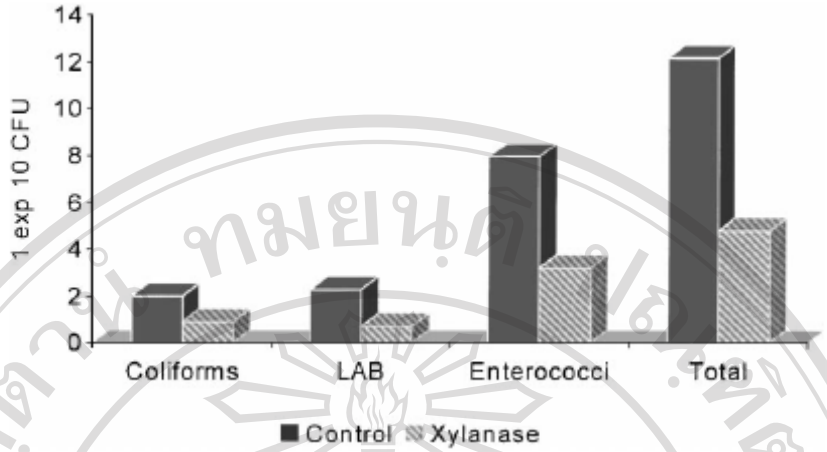

Fig 2.11 Influence of enzyme addition on total ileal microbial counts (Bedford, 2000). LAB: lactic acid bacteria.

2.10.3 Effect to growth performance

Table 1.1 summarizes the results of several studies on the performance response of broilers to enzyme supplements. Most of the studies recorded daily gain and feed conversion rates. Reported improvements were 5-45 % for daily gain and 3-15% for feed conversion rate. Recent data obtained by Ankrah *et al*, (1999); Berg *et al*.(1999) and Mathlouthi *et al*. (2002) showed that β-glucanase addition improved body weight gain, feed intake and FCR.

The inconsistent responses in growth performance described in the previous section indicate a complex interaction between enzymes and substrate, which is further complicated by the age and dietary background of the animal and by the experimental procedure. In poultry, the most commonly accepted mechanism of enzyme action is the reduction of digesta viscosity, which thereby facilitates the interaction between substrate and digestive enzymes. However, the structure and characteristics of the pig's digestive tract differ from those of poultry in many respects. Microflora in the lower part of the small intestine may play an important role in rendering NSPs available to pigs, particularly finishing pigs. The predominant bacterial genus, *Lactobacilli*, can degrade mixed-linkage β-glucans about 75% of these β-glucans are digested in the ileum. Nevertheless, even in growing–finishing pigs the digestibility of cell-wall components from various fibrous feed ingredients is low. The relative economic benefit of enzymes for piglets, however, is minor, as only

4% of the total feed used is consumed during the 21-d post-weaning period, whereas the feed consumed by grower–finisher pigs represents 62–68% of the total feed used. In commercial pig production, even small improvements in feed conversion can result in considerably increased profits. For this reason, enzyme technology should be directed not only to the diets of young pigs but also to the areas in which the greatest overall improvements can be obtained, such as in the digestion of fibrous feeds.

One of the remaining problems with using exogenous enzymes to digest cell-wall components is that they do not target specific substrates. Chesson (1987) concluded that the successful use of enzymes was reported mostly in studies in which the problem was "relatively simple and well-defined." To go beyond this will require enzyme formulations designed to target specific substrates. The challenge will be to degrade polysaccharides to a substantial extent. Most dietary-fiber polysaccharides are cell-wall components closely associated with other polysaccharides or noncarbohydrates, such as protein and lignin (Annison, 1991). Currently, it is difficult to design an effective enzyme mixture that digests cell walls, as their makeup is largely unclear and may be variable in different feedstuffs. The structural makeup and physicochemical implications of cell-wall polysaccharides may be determined by two factors: the pattern in which the polysaccharides and other components are arranged; and the bonding between molecules of cell-wall components. Christensen (1989) proposed a general model of the primary plant-cell wall, showing it to be composed of cellulose fibers to which strands of hemicelluloses are attached. Degradation of these complex and insoluble compounds probably requires multiple enzymes (Chesson 1987). Commercial-enzyme manufacturers have been responsible for the development and production of multi-enzyme systems. Many of these enzyme preparations can effectively degrade fiber polysaccharides in pig diets and therefore significantly improve the energy availability. However, further developments will be required to produce cost-effective and appropriately targeted enzyme preparations.

Another concern is the effects that pelleting or other types of processing and the conditions in the digestive tract have on the stability of enzyme preparations. Although formulators will take measures to protect enzymes such as selecting suitable microbial strains, improving production procedures, and providing a substrate carrier to which the enzymes can bind and become immobilized aggressive processing

substantially jeopardizes enzyme activity. For instance, the recovery of β-glucanase decreased from 56% to 31% after the conditioning time was changed from 15 s to 15 min at 85 °C and decreased from 16% to 11% at 95 °C (Inborr and Bedford, 1993). Furthermore, methods to monitor enzyme activity are problematic since there are as many methods as there are enzyme manufacturers, and measurements of enzyme activity in feeds are usually inaccurate and subject to high variation as a result of low enzyme concentrations and relatively poor desorption recovery. Therefore, it is important to develop a reliable and consistently used method for both quality control and rating of the products offered to feed mills and farms.

Feed-enzyme technology has developed considerably in recent years. At present, multi-enzyme preparations containing β-glucanase for the barley diets of young pigs and phytase to control pollution play important roles. Although there has been this selective use of enzymes in pigs' diets, more complex uses have yet to be elucidated. Also, the efficacy of enzyme treatment in pig husbandry appears uncertain, as well as its cost relative to benefits. More detailed research is needed in the key area of the utilization of fiber polysaccharides to improve energy availability and hence feed efficiency in finisher pigs. Natural products, such as feed enzymes, may also prove to be effective replacements for antibiotics and growth promoters, which have an adverse impact on human health and the environment.