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Review

Enzyme production by solid-state fermentation: Application to animal nutrition

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Abstract

Many microorganisms that decompose lignocellulosic material are being studied as producers of enzymes to perform enzymatic hydrolysis of the lignocellulosic material present in residues from the agroindustries. Although the cellulose and hemicellulose present in these materials have their value for feeding cattle, their bioavailability requires breakdown of the bonds with indigestible lignin. Predigestion of such materials with ligninases, xylanases and pectinases (cellulase free) may transform the lignocellulosic substrate into a feed with greater digestibility and higher quality for ruminants. This review provides an overview of variables to be considered in the utilization of fungal plant-depolymerizing enzymes produced by solid-state fermentation from agricultural production residues in Brazil.

Keywords: Animal nutrition; By-products; Enzymes; Solid-state fermentation; Thermophilic fungi

Abbreviations: ADF, acid detergent fiber; NDF, neutral detergent fiber; DM, dry matter; SSF, solid-state fermentation; SmF, submerged fermentation; a_w, water activity.

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Contents

1. Introduction

Brazilian economy is based heavily on agricultural production, with the main products being soy, corn, cotton, sugarcane, coffee, cassava, and several fruits. In addition to the commercialization of agricultural products *in natura*, the agroindustrial sector involved with the processing of fruit juices and production of alcohol and sugar has become an important export.

Industrial processing of feeds destined for animal consumption and human nutrition results in high amounts of agroindustrial residues. Most of these residues have a nutritional potential, mainly in the formulation of cattle diets. These residues are receiving greater attention in terms of quality control and have been classified as agroindustrial by-products (Galati et al., 2004; Eun et al., 2006). Many crops have co-products suitable for ruminant feeding, *e.g.*, cotton, peanuts, soy, rice and winter cereals, sugarcane, citrus fruits, cassava, corn and sorghum, fruits and vegetables.

Including enzymes as additives for ruminant diets has become of interest to researchers and animal breeders due to the positive responses observed in experiments (Colombatto et al., 2003b). In many studies, milk production response to dietary enzymes has been substantial (Lewis et al., 1999; Rode et al., 1999; Yang et al., 2000).

This paper provides an overview of the main variables to be considered when using fungal plant-depolymerizing enzymes produced by solid-state fermentation from agricultural residues for ruminant feeding.

2. Agricultural by-products and their potentials

Agricultural residues are also produced in large quantities in Brazil (Table 1). These abundant wastes are mostly left in the field, causing problem of disposal for the agroindustries. Because these residues are nutritious, a small portion is being used directly as feed or as components for industrially formulated cattle diets (Yang et al., 2001).

Table 1
Production data of different crops in a 1000 tonnes and waste production forecast in Brazil (2005/2006)

Region	Culture								
	Cotton (total production)			Wheat			Soy		
	2004/2005[a]	2005/2006[b]	Residue[c]	2004/2005[a]	2005/2006[b]	Residue[c]	2004/2005[a]	2005/2006[b]	Residue[c]
N	12.0	–	–	–	–	–	1404,8	1305.2	978.9
NE	762.5	849.7	399.4	2.5	2.5	1.2	3953.1	3713.1	2784.8
WC	2232.0	1536.2	722.0	298.0	194.2	92.2	28594.8	29058.5	21793.9
SE	310.1	174.8	82.2	191.0	159.2	75.6	4705.7	4535.3	3401.5
S	92.0	38.0	19.9	5354.4	4368.1	2074.8	12793.1	19562.9	14672.2
N/NE	774.5	849.7	399.4	2.5	2.5	1.2	5357.9	5018.3	3763.7
SC	2634.1	1749.0	822.0	5843.4	4721.5	2242.7	46093.6	53156.7	39867.5
Brazil	3408.6	2598.7	1221.4	5845.9	4724.0	2243.9	51451.5	58175.0	43631.2

Region	Culture								
	Corn (1st and 2nd crop)			Peanuts (1st and 2nd crop)			Sugarcane[d]		
	2004/2005[a]	2005/2006[b]	Residue[c]	2004/2005[a]	2005/2006[b]	Residue[c]	2004/2005[a]	2005/2006[b]	Residue[c]
N	1125.0	1108.6	554.3	–	–	–	899.6	1011.8	101.2
NE	2904.2	2541.7	1270.8	10.9	12.0	9.0	65039.1	61369.1	6136.9
WC	7911.4	8729.9	4365.0	33.3	34.0	25.3	37982.3	38807.1	3880.7
SE	10302.8	10991.2	5495.6	245.7	223.6	166.6	276582.3	305580.4	30558.1
S	12733.5	18293.3	9146.7	11.8	17.1	12.7	35181.4	30012.8	3001.3
N/NE	4029.2	3650.3	1825.2	10.9	12.0	9.0	65938.7	62380.9	6238.1
SC	30947.7	38014.4	19007.2	290.8	274.7	204.6	349755.8	374400.3	37440.0
Brazil	34976.9	41664.7	20832.4	301.7	286.7	213.6	415694.5	436781.2	43678.1

Source: CONAB—Survey: January 2006.

[a] Preliminary data: not defined datas.

[b] Estimated data: not defined datas.

[c] Estimate of waste production based on production forecast of 2005/2006.

[d] *Source*: CONAB—Survey: December 2005.

Table 2
Quantity of the main crops and the type of by-products produced in Brazil

Species	Crops	Quantity (kg DM/tonnes)	By-products produced
Gossypium spp.	Cotton	530	Seed, bran and wax
Arachis hypogaea	Peanut	740	Husk and wax
Glycine max	Soy	750	Husk and wax
Oryza sativa	Rice	557	Straw and bran
Triticum spp.	Wheat	475	Straw and bran
Hordeum vulgare	Barley	492	Straw and bran
Zea mays	Corn	550	Stem, leaf and cob
Saccharum spp.	Sugarcane	150	Tops and sugarcane bagasse

Source: Peixoto et al. (1995).

The main crops grown in Brazil and the type of by-products produced are shown in Table 2.

Agricultural and agroindustrial activities produce thousands of tonnes of by-products, *e.g.*, sugarcane bagasse, citrus bagasse, fruit peel, corn straw and corncobs. Nutritionally, the agroindustry produces two groups of residues: fibrous residues (high and low digestibility) and brans. Fibrous residues can be divided into those with high digestibility, *e.g.*, citrus pulp, corn gluten bran, soy husk and brewing residues (barley), and those with low digestibility, *e.g.*, sugarcane bagasse, cereal, corn, soy and cotton straw, cotton husk, soy husk, peanut husk and harvest remnants of forage grass seeds. Brans include rice, peanut, soy and cotton (Balsalobre, 2006, personal communication).

Sugarcane bagasse or 'bagasse' is the fibrous material resulting from the extraction of juice by pressing the sugarcane and is generally burned in the factoring to generate electric energy or for boiler heating. Bagasse is composed by around (g/100 g oven-dry basis) 32–40 cellulose, 19–24 hemicellulose, 23–32 lignin and 1.5–5.0 ash (Pandey et al., 2000a). Another important residue in Brazil, in terms of the volume and composition, is citrus bagasse. Consisting of peel, pulp and seeds, which correspond to half of the fresh fruit weight (Garzón and Hours, 1992). Brazil processes about 80 million tonnes of orange and lemons annually and produces 30–40 million tonnes of these solid residues which are pelleted after neutralization and dehydration with calcium oxide, most of it is used as a component of animal feed in European countries and Brazil (Martins et al., 2002; Barreto De Menezes et al., 1989).

3. Chemical and structural characteristics of the fibrous components of lignocellulosic residues

The cell wall of plants is a complex biological structure containing polymers and other molecules whose proportions and structural organization vary with type and age of the plant.

The growth of the primary wall starts with the elongation of the plant cell (Fig. 1). During progressive thickening of the secondary wall, deposition of cellulose exceeds that of xylan, pectin and ferulic acid, which are no longer added to the wall, and deposition

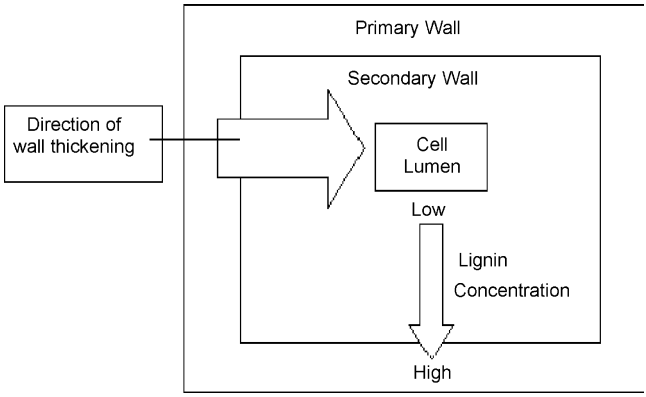

Fig. 1. Schematic representation of a plant cell and wall development (Jung and Allen, 1995 adaptation).

of lignin starts (Jung et al., 1992). This may explain why ruminal microorganisms degrade polymers from the lumen of the cell outward and why the middle lamella and primary wall regions of lignified cells are never completely digested (Jung and Allen, 1995).

Cellulose and hemicellulose represent the largest fraction of the plant cell wall and of agricultural residues such as straw from wheat, corn, rice, soy and cotton, sugarcane bagasse and orange bagasse, bonds with the lignin in the cell wall matrix need to be broken. For this reason, the direct feeding fiber has not yielded the expected favorable results considering the nutritional and energetic potential of these fibers (Colombatto et al., 2003a). Dietary fibers are components of plant walls, which are not digested by enzymes of the animal's digestive system and therefore cannot provide calories. Fibers are the predominant fraction of the plant cell wall and mainly consist of carbohydrates. The primary component fibers are cellulose, hemicellulose and lignin. Chemically, cellulose consists of linear chains of glucose. Starch, the carbohydrate source in grains and nutritionally available to animals, also consists of glucose molecules. However, the glucose molecules of cellulose are linked together by β-1,4 bonds whereas starch has α-1,4 and α-1,6 glucosidic bonds. Only microbial enzymes are able to digest the β-1,4 bond of cellulose. The digestion of hemicellulose also depends on microbial enzymes because of its complex structure, *i.e.*, mainly xylose linked by -1,4 bonds. Hemicellulose is associated intimately with lignin, which exerts a strong negative influence on fiber digestion (Kung, 2006).

According to Alves De Brito et al. (2003), evaluation of the nutritional quality of a forage plant requires the detailed analysis of the composition of its cell wall because cell walls are the main component of tropical plants. The digestibility of cell wall is influenced by both the content and physical characteristics of wall polysaccharides such as degree of crystallinity and polymerization (Fritz et al., 1990).

Hemicellulose is the fraction of the cell wall affected most by lignification. Although this fraction has higher potential digestibility than other cell wall components, its degradation is not maximized because hemicellulose concentrated in the primary wall. Degradation of the cell starts in the lumen and proceeds toward the secondary wall. By the time the

microorganism reaches the primary wall, *i.e.*, the outer portion of the cell, the particle already may be leaving the rumen. Thus, in forage plants of older physiological age or in flowering plants, the digestibility of hemicellulose often is lower than that of cellulose (Messman et al., 1991). Consequently, an increase in lignin content directly affects the degradation of neutral detergent fiber (NDF) (Caballero et al., 2001). Because lignin content increases at a higher rate than NDF content as the plant matrices, degradation of dry matter is correlated more closely with NDF digestibility than with NDF content (Dechamps, 1999). Consequently, treatment with cellulase-free ligninases may promote a "partial digestion". The breakdown of the bonds between lignin and cellulose, and particularly hemicellulose transforms the lignocellulosic substrate into a high-quality feed for ruminants (El-Nasser et al., 1997). Other enzymes such as xylanases, pectinases and endoglucanases have also been applied experimentally to release cellulose fibers (Sørensen et al., 2004; Eun et al., 2006).

Table 3 lists the chemical compositions of the cell walls of some crops and forages used for feeding ruminant animals.

Fiber-degrading bacteria and fungi usually adhere to the surface of plant cell walls failure to exploit this process may be one reason for problems with establishing inoculant microorganisms in the rumen (Gregg et al., 1998; Arcuri et al., 2006). The intimate association of microbes with plant cell walls has a sophisticated molecular structure that facilitates adherence (Fig. 2). This attachment process is called cellulosome and consists of a multienzyme complex produced by many cellulolytic rumen organisms. The cellulosomes associated with the microbial cell surface mediate cell attachment to the insoluble substrate and degrade it to soluble products that then are absorbed by the microbe (Krause et al., 2003).

4. Solid-state fermentation and its application in animal nutrition

The use of solid-state fermentation (SSF) to upgrade the nutritive value of agricultural by-products and for enzyme production has been increased due to the higher quantity of residues produced in several countries (~3.5 billion tones per year), representing a potential solution to feeding animals in developing countries (Robinson and Nigam, 2003). The advantage of using SSF to achieve both goals is the low-tech fermentation system required plus the possibility of having it carried out on farms.

4.1. General aspects of solid-state fermentation and their influence on microorganism physiology and product extraction

SSF is a process that occurs in the absence or near absence of any fluid in the space between particles (Lonsane et al., 1985). 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).

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).

The three phases, *i.e.*, solid, liquid and gas phases are present in both liquid and solid medium but not in the same proportions; they differ in diffusion kinetics and rheologic and thermodynamic (water activity (a_w), osmotic pressure) characteristics. 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

Table 3
Cell wall components (g/kg dry matter) of some important crops used as ruminant feed in Brazil

Crops	Cellulose	Hemicellulose	Lignin	Reference
Corn silage	380–400	280	70–210	Reddy and Yang (2005)
Corn				
Straw	335	249	78	Cruz (1992)
Stalk	336	237	87	Cruz (1992)
Leaf	245	273	54	Cruz (1992)
Cob	377	396	73	Cruz (1992)
Bran	338	393	49	Cruz (1992)
Coconut fiber	360–430	15–25	410–450	Reddy and Yang (2005)
Wheat straw	330–380	260–320	170–190	Reddy and Yang (2005)
	400	260	200	Tabka et al. (2006)
	270	210	98	Prates (1995)
Wheat bran	300	500	150	Couto and Sanromán (2005)
Rice bran	350	250	170	Couto and Sanromán (2005)
Rice straw	280–360	230–280	120–140	Reddy and Yang (2005)
	320–470	190–270	50–240	Karimi et al. (2006)
	352	221	43	Prates (1995)
Sorghum stalk	270	250	110	Reddy and Yang (2005)
Barley bran	230	320	214	Cruz et al. (2000)
Barley straw	310–450	270–380	140–190	Reddy and Yang (2005)
	316	254	110	Prates (1995)
Oat bran	493	250	180	Couto and Sanromán (2005)
Oat straw	300	220	85	Prates (1995)
Grape seed	71	311	435	Couto and Sanromán (2005)
Grape vine	299	353	229	Couto and Sanromán (2005)
Coniferous plants	400	300	200–300	Mandre et al. (2006)
Forages				
Andropogon gayanus	415	308	90	Reis et al. (1990)
Brachiaria decumbens	395	311	81	Reis et al. (1990)
Hyparrhenia rufa	402	275	90	Reis et al. (1990)
Brachiaria brizantha	412	308	75	Reis et al. (1995)

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).

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 satisfactory results in terms of obtaining different products by SSF using bacterial cultures

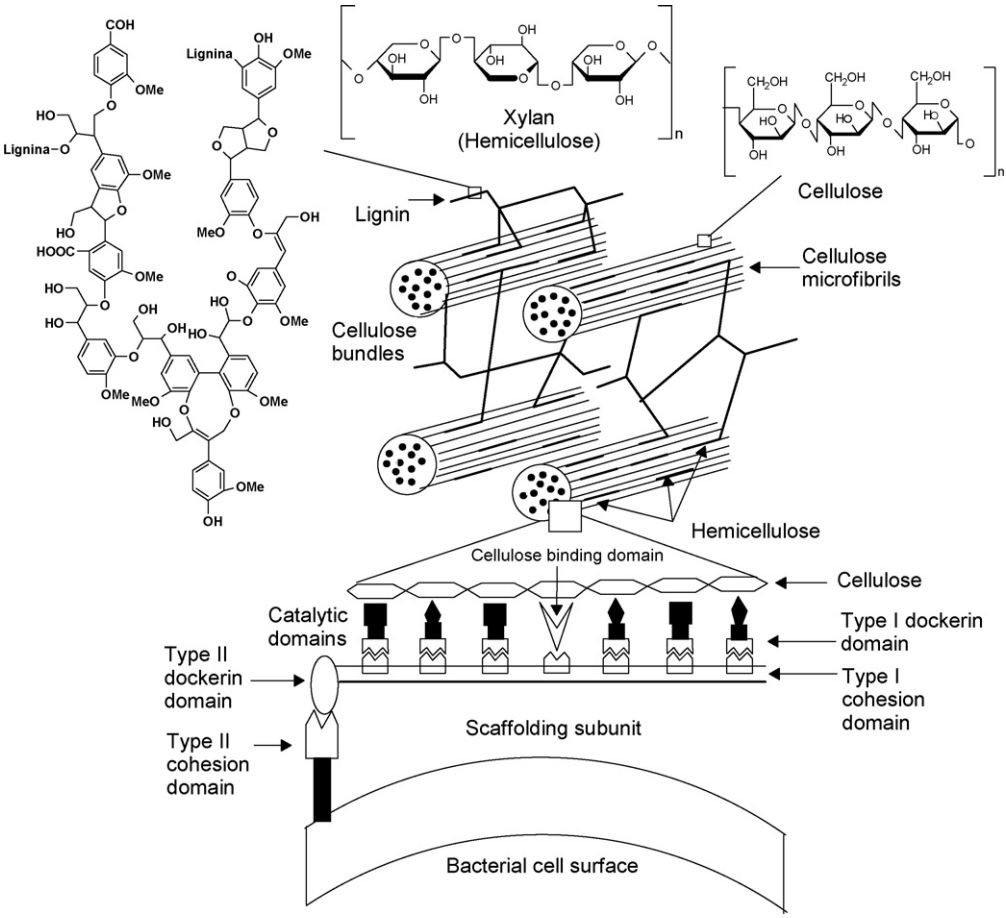

Fig. 2. Representation of fiber and its component cellulose, microfibrils, hemicellulose, and lignin that are degraded via the bacterial cellulosome complex (Krause et al., 2003 adaptation). The multiple subunits of the cellulosome are composed of numerous functional domains that interact with each other and with the cellulosic substrate.

(Prakasham et al., 2006; Sabu et al., 2006; Virupakshi et al., 2005; Kashyap et al., 2003; Kapoor et al., 2000; Cao et al., 2000).

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).

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 (Hölker 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).

Fungi grown in SSF culture at low a_w tend to accumulate polyols such as glycerol, mannitol, erythrol and arabitol in their cells. The composition of the polyol pool depends on growth conditions and represents an adaptation to the low humidity condition in SSF needed to maintain the turgor pressure of the cells. These compounds are secreted by the mycelium and correspond to metabolites present in the fermented material (Ruijter et al., 2004; Holker et al., 2004).

Another class of metabolites produced in SSF culture is organic acids. The fungus *Aspergillus niger* is a potent producer of citric acid whose production can reach 1 g/kg substrate; *A. oryzae* produces oxalic acid (Biesebeke et al., 2002; Couto and Sanromán, 2005). Recently, the production of lactic acid by the bacterium *Lactobacillus delbrueckii* in SSF culture was described (Rojan et al., 2006).

Expression of proteins can differ during SSF incubation (Aguilar et al., 2004). Glucoamylase B (glaB) from *A. oryzae* is highly induced during SSF (koji) but this enzyme is not found in submerged cultures. Molecular studies have demonstrated that the GC-box is a cis-element of the promoter responsible for the expression of this enzyme under conditions of low a_w, high temperature and physical barriers to hyphal extension (Ishida et al., 2000, 1998). Two proteases that are only expressed in SSF culture also have been identified (Biesebeke et al., 2002).

The production of extracellular enzymes by *Trichoderma viridae* was strongly affected by a_w. Maximum production of polygalacturonase and xylanase was observed at a_w of 0.99, whereas the synthesis of β-glucosidase was increased at a_w between 0.96 and 0.98 (Grajek and Gervais, 1987).

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; pO_2 and pCO_2 should be optimized for each type of medium, microorganism and process (Chahal, 1987). The production of flavor compounds, one of the characteristics of SSF, is related to the low oxygen availability in the medium; this results in production of odor compounds including alcohols, aldehydes and ketones (Feron et al., 1996; Medeiros et al., 2001).

Growth of *A. oryzae* in SSF culture at the levels of individual hyphae was not affected by low oxygen concentrations (0.25%) based on hyphal tip elongation rate. However, the total specific growth rate was reduced as a result of a reduction in the branching frequency of hyphae and in the radial extension rate of colonies on the Petri dish. Production of α-amylase by this fungus (activity per gram dry biomass) was reduced at a low oxygen concentration, suggesting that low oxygen reduced expression of this enzyme (Rahardjo et al., 2005).

Temperature is related directly to 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 SmF (Aguilar et al., 2004). For example, the production of polygalacturonase and pectin lyase 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-González 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; Nandakumar et al., 1999; Rahardjo et al., 2006).

Intrinsic properties of microbial extracellular protein molecules such as temperature and pH optima for activity, thermostability, stability in different pH ranges and substrate affinity are also will be influenced by the type of fermentative process used for their production (Alazard and Raimbault, 1981). Acuña-Argüelles et al. (1995) compared the properties of endo- and exo-polygalacturonases produced by *A. niger* in SSF and SmF. Enzyme stability at different pH values and temperatures and substrate affinity differed with culture conditions. When incubated at 60 °C for 30 min, the endo- and exo-polygalacturonases produced in SSF maintained 90 and 70% of their original activity; these enzymes produced during SmF maintained only 40% of activity. The endo-polygalacturonase was stable over the pH range from 2.6 and 6.0 when produced by SSF, but only between 6.0 and 7.0 when produced by SmF. The exo-polygalacturonase was stable between pH 3.5 and 5.0 when produced by SSF but only between pH 4.0 and 5.0 when obtained by SmF. The endo-polygalacturonase produced in SSF culture also had greater substrate affinity although no difference between the two fermentative processes was observed for substrate affinity of exo-polygalacturonase. Diaz et al. (2006) suggested the presence of non-protein compounds produced in SSF medium might be responsible for kinetic differences including higher specific activity and

increased thermostability for lipases produced by *Rhizopus homothalicus* in SSF. Several investigators also have observed lower production of protease in SSF culture (Battaglino et al., 1991; Auria et al., 1990). Presence of proteases in the medium is associated with faster decline in the activity of the target enzyme during the fermentative process in liquid medium (Viniegra-González et al., 2003).

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; Saiz-Jimenez, 1995).

4.2. Utilization of agricultural residues for enzyme production by solid-state fermentation

Enzyme use has increased markedly in the pharmaceutical, food, paper and textile industries. Potential of depolymerizing enzymes for use in animal feeding also has been recognized for partial degradation of agroindustrial residues that serve as feed ingredients (Roopesh et al., 2006; Pandey et al., 2000b). Among the processes used for enzyme production, SSF is attractive because of its numerous advantages, particularly the possibility of using solid agricultural and agroindustrial residues as a medium for microbial growth (Couto and Sanromán, 2006; Panagiotou et al., 2003; Pandey, 2003; Pandey et al., 2000b; Hesseltine, 1972). Such residues have yielded good results in the production of enzymes including pectinases (Martins et al., 2002), cellulases and xylanases (Carmona et al., 2005; Kang et al., 2004; Kalogeris et al., 2003), amylases, ligninases (Couto and Sanromán, 2005), inulinases (Mazutti et al., 2006), chitinases (Binod et al., 2005), and phytases (Roopesh et al., 2006; Chantasartrasamee et al., 2005; Papagianni et al., 1999). This technique also may become economically advantageous for application to cattle production. Moreover, SSF processes present an increased opportunity for underdeveloped or developing countries where current economical difficulties and globalization of the world economy do not permit farming and cattle raising sector to accompany the biotechnological evolution.

Lignocellulose-decomposing microorganisms can be selected as producers of enzymes under specific growth conditions using substrates of low commercial value including agricultural and agroindustrial residues (Hsieh and Yang, 2004). The search for enzymes that attack the lignocellulolytic complex with characteristics adequate for different biotechnological processes has been the focus of many studies (Schimidt et al., 2001; Sonia et al., 2005).

Many microorganisms possess enzyme systems capable of converting lignocellulose into a metabolite essential for their growth (Table 4).

Table 4
Accepted and systematic names of enzymatic complexes capable of hydrolyzing lignocellulosic materials

Cellulases
Cellulase EC 3.2.1.4 (1,4-(1,3;1,4)-β-D-glucan 4-glucano hydrolase)
Cellulose 1,4-b-cellobiosidase EC 3.2.1.91 (1,4-β-D-glucan cellobiohydrolase)
Glucan 1,4-β-glucosidase EC 3.2.1.74 (1,4-β-D-glucan glucohydrolase)
β-Glucosidase EC 3.2.1.21 (β-D-glucoside glucohydrolase)
Hemicellulases
Endo-1,4-β-xylanase EC 3.2.1.8 (1,4-β-D-xylan xylanohydrolase)
Xylan-1,4-β-xylosidase EC 3.2.1.37 (1,4-β-D-xylan xylohydrolase
α-*N*-Arabinofuranosidase EC 3.2.1.55 (α-L-arabinofuranoside arabinofuranohydrolase)
Acetylesterase EC 3.1.1.6 (acetic-ester acetylhydrolase)
Ligninases
Laccase EC 1.10.3.2 (benzenediol:oxigen oxidoreductase)
Manganese peroxidase EC 1.11.1.13 (Mn(II):hydrogen-peroxide oxidoreductase)
Lignin peroxidase EC 1.11.1.14 (1,2-bis(3,4-dimethoxy phenyl) propane-1,3-diol:hydrogen-peroxide oxidoreductase)
Pectinases
Pectinesterase EC 3.1.1.11 (pectin pectylhydrolase)
Pectin lyase
Pectate lyase EC 4.2.2.2 ((1 → 4)-α-D-galacturonan lyase)
Pectate disaccharidelyase EC 4.2.2.9 (1 → 4)-α-D-galacturonan reducing-end-disaccharide-lyase)
Polygalacturonase
Polygalacturonase EC 3.2.1.15 (poly (1,4-α-D-galacturonide) glycanohydrolase)
Galacturan 1,4-α-galacturonidase EC 3.2.1.67 (poly (1,4-α-D-galacturonide) galacturonohydrolase)

Source: IUBMB (2006), Kang et al. (2004), Blanco et al. (1999), Da Silva et al. (1997), and Fogart and Kelly (1983).

Several studies have been conducted to identify white-rot fungi that can degrade lignin bonds but preserve cellulose fibers and thereby improve the quality of lignocellulosic substrates for ruminant feeding (Karunanandaa and Varga, 1996). Results indicate that degradation of the lignocellulosic matrix is a complex phenomenon controlled by a large number of variables that limit the use of such fungi. However, most of these studies were preliminary and used lignin degradation and improvement in the *in vitro* digestibility of dry matter as indicators for assessment of the efficacy of the biological treatment (Zadrazil et al., 1991). Other reports have evaluated addition of enzyme preparations ensiling for the hydrolysis of cell wall components to ensiled products to improve the nutritive value and to increase ruminal cell wall digestion (Schimidt et al., 2001; Yang et al., 2001; Hill et al., 2001; Colombatto et al., 2004a,b). However, few studies testing direct addition of these enzymes to ruminant diets are available and *in vivo* results may not parallel *in vitro* findings. Table 5 summarizes the literature available on by-products that can be used in enzyme production to apply on animal nutrition.

In vitro methods are less expensive, less time consuming, and allow more control of experimental conditions than *in vivo* experiments. Furthermore, *in vitro* systems can accommodate a large number of enzyme candidates (Eun et al., 2007). However, *in vitro* data not always can be directly extrapolates as *in vivo* digestibility, considering the rumen ambient peculiar, the degradation of enzyme by proteolysis and the retention time of the aliment in the rumen.

Table 5
Bibliographic revision of enzymes produced using by-products by fungi for animal nutrition use

Substrate	Microorganism(s)	Enzyme(s)	Author(s)/year
Rice straw	*Trichoderma reesei*	Cellulase Hemicellulase	Eun et al. (2006)
	Bacillus licheniformis	Protease	
Barley	*Talaromyces emersonii*	Endo-β-glucanase	McCarthy et al. (2005)
Corn silage	*Thermoascus aurantiacus*	Xylanase	Colombatto et al. (2004b)
Corn stalk	*Fusarium oxysporum*	Endoglucanase Celobiohydrolase β-Glucosidase	Panagiotou et al. (2003)
		Xylanase β-Xylosidase	
Corn straw	*Penicillium decumbens*	CMCase β-Glucosidase Xylanase	Yang et al. (2001)
Alfalfa	*Gliocladium* spp.	Cellulase, xylanase	Schimidt et al. (2001)
	Orpinomyces joyonii	Endoglucanase	Hodrová et al. (1998)
	Caecomyces communis	β-Glucosidase	
Forage silage	*Streptomyces Achromogenes*	Cellulase, hemicellulase	Hill et al. (2001)
Wheat straw	*Neurospora crassa*	Cellulase	Romero et al. (1999)
	Several fungi	Cellulase, hemicellulase Lignin peroxidase	Peiji et al. (1997)
Sugarcane bagasse	*Trichoderma reesei*	Cellulase	Kansoh et al. (1999)
	Phanerochaete chrysosporium *Coriolus versicolor* *Streptomyces viridosporus*	Ligninase	El-Gammal et al. (1998)
	White rot fungi	Ligninase	Zadrazil and Puniya (1995)
Sugarcane bagasse, wheat straw, corn cobs, rice husks, peanut shells, *etc.*	*Phanerochaete chrysosporium*	Cellulase, xylanase	El-Nasser et al. (1997)
	Coriolus versicolor	Glucanase	
Soy husks		Cellulase	Jha et al. (1995)

There are some limitations concerning value of enzymes fed to animals. The complete characterization of enzyme products before their use is important, as it should lead to the development of more effective, science-based enzyme additives (Colombatto et al., 2003c). In the other hand, enzyme-feed specificity presents a major dilemma for formulating new ruminant feed enzymes products because most commercial ruminant diets contain several

types of forages and concentrates. Therefore, to achieve maximal benefit, a number of different enzyme sources would need to be used in a typical diet (Beauchemin et al., 2003).

Micotoxin production can be another limiting factor when added microorganisms without "generally recognized as safe" or "GRAS". However, differences in activation of secondary metabolism in SSF and SmF have been reported. The growing of *Penicillium viridicatum* in wheat straw, orange bagasse and sugarcane bagasse decrease aflatoxin and ochratoxin levels to low quantity, less than minimum acceptable. Graminha analyzed two strains of *Thermomyces lanuginosus*, one of *Rhizomucor pussillus* and *Rhizomucor* sp. growing in cotton bran and were not detected aflatoxin, ochratoxin and zearalenone (unpublished data).

Colombatto et al. (2004a,c) examined benefits from adding extracellular enzymes derived from thermophilic microorganisms to ruminant diets in view of the advantages of thermostable enzymes, called thermozymes, compared to their mesophilic counterparts. For example, enzymes produced by mesophilic fungi such as *Trichoderma* and *Aspergillus* spp. normally have a temperature optimum between 45 and 60 °C, which is lower than temperatures found during the more advanced stages of silage processing. Ideally, enzymes must act rapidly during the crucial first stage of ensilage before they become thermally inactivated (Margesin and Schinner, 1994). Nevertheless, silages with high temperatures are not desired because high temperatures have been associated growth of undesired microorganisms such as *Clostridium* sp. (Flythe and Russell, 2004).

Enzymes require an optimal activity and stability for a prolonged period of time to be effective. Colombatto et al. (2004c) demonstrated that mesophilic enzymes preserve at least 70% of their activity for 48 h at 39 °C at a pH ranging from 4.0 to 6.8; enzymes from thermophilic microorganisms often have greater stability than enzymes from mesophilic microbes. By increasing enzyme stability, the yield of products to be used in ruminant diets can be increased. In addition, direct use of enzymes as additives in rations or food supplements to act inside the rumen is may prove practical. Such enzymes must be stable under the physicochemical conditions of the rumen, such as a pH of about 6.0 and temperatures of up to 40 °C as well as resistance to salts and proteases. Such characteristics have been described by Vieille and Zeikus (2001) for thermozymes. Compared to mesozymes, these enzymes have a broad tolerance to pH variation, greater resistance to denaturing agents, and increased stability and activity at elevated temperatures.

Most commercially available enzymatic products that have been tested as food additives for ruminants were not designed specifically for this purpose. Instead enzymatic preparations containing cellulases and xylanases destined for use in the food, pulp, paper, textile, fuel and other chemical industries have been used (Beauchemin et al., 2003). Several other products containing fibrolytic enzymes evaluated as additives for ruminant diets were developed originally as silage additives (Feng et al., 1996).

Martins et al. (2006) evaluated supplementation with fibrolytic enzymes (cellulase and xylanase, Fibrozyme®) on the efficiency of microbial synthesis and enzymatic activity of β-1,4-endoglucanase (cellulase) in animals fed on diets containing corn silage and Tifton 85 hay (*Cynodon* spp.). No effect on the ruminal variable studied was detected, except for greater β-1,4-endoglucanase activity that was associated with addition of these enzymes to the hay. According to Morgavi et al. (2000a), commercial preparations of enzymatic complexes normally are standardized according to their capacity to degrade cellulose or

xylan. However, commercial products contain multienzyme compounds that present different fibrolytic activities. Greater quality control is needed over other complement enzymes that may alter the performance of the enzyme complex.

In the examples mentioned above, the enzymes tested often have pH and temperature optima that differ markedly from conditions in the rumen where the temperature is 39 °C and pH ranges from 5.5 to 6.7 (Van Soest, 1994). Enzymatic activities of most commercial products as specified by the manufacturer are markedly higher than would be observed under conditions found in the rumen. This complicates prediction of the efficacy of a product as a supplement for ruminant diets (Beauchemin et al., 2003).

One final aspect regarding use of enzymes as diet additives is the commercial cost, because the fermentation medium is one of the important components that determine the final price of the product, low cost substrates are desired as substrates. Economic analysis of the production of lipase by *Penicillium restrictum* in SSF and SmF cultures showed that the investment capital necessary for SmF was 78% higher than that required for SSF; consequently, the price of the SSF product was 47% lower. The investment return from the SSF process would reach 68% within 5 years (Castilho et al., 2000).

The mechanism whereby enzymatic additives might improve fiber digestibility by ruminants is not well understood. Direct hydrolysis in the rumen is one a potential mode of action. However, ruminal activity to be quantified due to the low activity of added exogenous hydrolases when compared to the total enzymatic activity present in the rumen (Morgavi et al., 2000a). Furthermore, extent of ruminal digestion may be limited by factors other than enzyme activity such as substrate accessibility associated with large particle size of masticated forage and short ruminal retention time.

According to Morgavi et al. (2000b), particular enzyme activities may, however, be more labile than others. If such an activity were a key component of the mode of action of feed-additive enzymes for ruminants, it would appear likely that enzyme activity might be protected by simple means. Applying enzymes as feed supplements promises to be the simplest technology for achieving such amplification in the immediate future.

Beauchemin et al. (2003) extensively discussed the application of exogenous enzymes on revision in respect of the research on enzyme selection, the animal responses to feed enzymes, and the mechanisms by which these products improve nutrient utilization. One of the factors that also should be understood is enzyme level. What the relation of enzymatic unit per kilogram dry matter that the animal intake? Nsereko et al. (2002) speculated that application of a moderate level of enzyme to ruminant feeds caused some beneficial disruption of the surface structure of the feed either before or after ingestion. In other studies, some authors demonstrated that high levels of enzyme addition can be less effective than low levels, and the optimal level may depend on the diet (Beauchemin et al., 2003).

4.3. Enhance of nutritional value of agricultural by-products by SSF

The use of SSF for protein enrichment of lignocellulosic residues has been a focus of attention due its direct applicability of the fermented product for ruminant feeds purposes. This material has low protein content and the fungal growth can improves it in around 10–15% (Shojaosadati et al., 1999) further on increases its digestibility (Iluyemi et al., 2006). On the other hand, the degradability of dietary protein in the rumen (which is the

percentage of a dietary protein or N that is degraded in the rumen by the microorganisms) from fermented lignocellulosic by-product was higher compared with unfermented (Iconomou et al., 1998). Besides protein, an increase of total lipid and fatty acids was observed after solid-state fungal fermentation of agricultural residues (Abu et al., 2000).

The SSF have been reported too as protein enhancement factor of cereal grain and potato residues. Gélinas and Barrete (2007) obtained an increase of 7–8% of protein content of potato processing waste by SSF using yeasts.

Fungal growth showed decrease in the neutral detergent fiber (NDF), acid detergent fiber (ADF) and hemicellulosic contents of lignocellulosic fibers, increase the bioavailability of nutrients, decrease the antinutritional factors such as phytic acid, polyphenols and tannins (Manmdebvu et al., 1999; Elyas et al., 2002). Thus, two nutritionally important contributions can be obtained from fermentative process: depolymerizing enzyme and a protein-enriched and high degradability of feed material. In addition, this process can convert residues widely available in the field into products of higher aggregated value (El-Nasser et al., 1997; Sabu et al., 2005).

5. Conclusions

Agricultural by-products typically vary so widely in their chemical composition and nutritional value, and sometimes are also too high in low-quality fiber, that a specific enzyme complex is required to break it down in order to be used in ruminant feed. Their nutritional value could be increased by biodegradation methods of fiber in the rumen, through efficient delignification. Agroindustrial residues can also be utilized as substrate for the production of enzymes by solid-state fermentation that can be used as feed additives for ruminants. Fiber-degrading enzymes produced by thermophilic fungi can have characteristics more suitable to act inside the rumen than those from mesophilic fungi. More detailed studies to quantify the mode of action of these enzymes can enhance our understanding of ruminal fiber digestion as well as production of nutritionally improved feeds and feed ingredients for ruminants.

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