Industrial Applications of Rumen Microbes*
- Review -

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ABSTRACT : The rumen microbial ecosystem is coming to be recognized as a rich alternative source of genes for industrially useful enzymes. Recent advances in biotechnology are enabling development of novel strategies for effective delivery and enhancement of these gene products. One particularly promising avenue for industrial application of rumen enzymes is as feed supplements for non-ruminant and ruminant animal diets. Increasing competition in the livestock industry has forced producers to cut costs by adopting new technologies aimed at increasing production efficiency. Cellulases, xylanases, β-glucanases, pectinases, and phytases have been shown to increase the efficiency of feedstuff utilization (e.g., degradation of cellulose, xylan and β-glucan) and to decrease pollutants (e.g., phytic acid). These enzymes enhance the availability of feed components to the animal and eliminate some of their naturally occurring antinutritional effects. In the past, the cost and inconvenience of enzyme production and delivery has hampered widespread application of this promising technology. Over the last decade, however, advances in recombinant DNA technology have significantly improved microbial production systems. Novel strategies for delivery and enhancement of genes and gene products from the rumen include expression of seed proteins, oleosin proteins in canola and transgenic animals secreting digestive enzymes from the pancreas. Thus, the biotechnological framework is in place to achieve substantial improvements in animal production through enzyme supplementaion. On the other hand, the rumen ecosystem provides ongoing enrichment and natural selection of microbes adapted to specific conditions, and represents a virtually untapped resource of novel products such as enzymes, detoxificants and antibiotics.
(Key Words : Rumen Microbes, Enzymes, Gene Source, Genetic Engineering, Detoxificants, Review)

INTRODUCTION

The global industrial enzyme market was estimated in 1994 to be worth US$ 400 million per year (Hodgson, 1994). This market was more recently valued at US$ 1.4 billion and was suggested to be increasing at 4 to 5% annually (Cowan, 1996). Enzymes such as cellulases, xylanases, proteases, lipases, amylases, phosphatases, and pectinases are widely used in the pulp and paper, textiles, detergent, food and beverage, and pharmaceutical industries. Industrial enzymes also have application in the livestock industry as feed additives. The potential of enzyme supplementation for improving the efficiency of feed utilization by non-ruminant livestock is widely recognized. The merits of using β-glucanase in barley-based poultry diets and phytase in swine and poultry diets are well documented, and feeding these enzymes has become common practice in some areas of the world. Unlike non-ruminant animals, ruminants have an extensive array of microbial enzymes produced in the rumen, and these enzymes play an important role in the ruminant digestive process. The most extensively studied enzyme systems of the rumen are those involved with the digestion of fibre and other associated or related plant cell wall polymers. Fibrolytic activity in the rumen is estimated to be 10 times higher than that in any other known fermentation system. Notwithstanding this impressive array of endogenous enzymes, recent evidence suggests that enzyme supplementation can also increase

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the production performance of ruminants (Feng et al., 1992a, b; Beauchemin et al., 1995; Beauchemin and Rode, 1996). However, the expense of production using traditional large-scale fermentations and downstream processing has prevented the widespread use of enzymes as livestock feed additives. Manufacturers and researchers have therefore sought more powerful enzymes and alternative systems for enzyme production and delivery. Recombinant DNA technology has recently enabled manufacturers to increase the volume and efficiency of enzyme production, and to create new products. Characteristics of the original source organism no longer dictates limits to commercial enzyme production. Genes encoding superior enzymes can be transferred from organisms such as anaerobic bacteria and fungi, typically impractical for industrial production, into well characterised industrial microbial production hosts (e.g., *Aspergillus* and *Bacillus* spp.). As well, these genes may be transferred to novel plant and animal expression systems. The rumen ecosystem also represents a virtually untapped resource for novel new products (e.g., antibiotics, chemicals, detoxificants) and provides opportunities to define the processes of nutrient degradation.

RUMINAL MICROBES AS A SOURCE OF ENZYMES FOR INDUSTRY

The rumen microbial population represents a rich and, until recently, underutilized source of novel enzymes with tremendous potential for industrial application. The enzyme activities confirmed to exist in the rumen are diverse, and include plant cell wall polymer-degrading enzymes (e.g., cellulases, xylanase, β-glucanase, pectinase), amylases, proteases, phytases and specific plant toxin-degrading enzymes (e.g., tannases). The variety of enzymes present in the rumen arises not only from the diversity of the microbial community, but also from the multiplicity of specific enzymes produced by individual microbial species (Doerner and White, 1990; Malburg and Forsberg, 1993; Flint et al., 1994; Ali et al., 1995; Yanke et al., 1995).

Cellulases and xylanases

The rumen is increasingly being recognized as a promising source of superior fibrolytic enzymes. Cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described to date (Trinci et al., 1994), thus these anaerobic fungi and (or) their enzymes have sparked interest for a number of biotechnological applications. These include development of probiotics and as feed additives for silages and total mixed rations, for saccharification of lignocellulosic residues, and for production of polysaccharide-hydrolysing enzymes. Of note, specific activities of 6,000 and 3,500 μmol xylose released/min/mg protein have been reported for xylanases from ruminal fungi (Gilbert et al., 1992; and Li et al., 1996).

Rumen microbial enzymes could find ready application as feed enzymes, as the trend is growing in the non-ruminant livestock industry to supplement diets with glycanolytic enzymes. Amending rations with fibrolytic enzymes such as cellulase, xylanase and β-glucanases have been shown to increase the efficiency of feed conversion by non-ruminant livestock. Enzymatic hydrolysis of cellulose and xylan to simple sugars provides the non-ruminant animal with carbon sources not normally made available by intestinal enzymes. Moreover, these degradative enzymes eliminate certain forms of the polymers (e.g., arabinoxylan in wheat and rye, and β-glucan in barley and oats) that may interfere with nutrient absorption and promote intestinal disturbances by pathogenic enteric microorganisms. Recent research suggests that fibrolytic enzyme supplementation can also dramatically increase the growth rate and(or) production performance of ruminants (Feng et al., 1992a, 1992b; Beauchemin et al., 1995; Beauchemin and Rode, 1996).

β-Glucanases

The prevalence of glycans in certain cereal grains confers some antinutritional properties on these feedstuffs. In barley and oats, a mixed-linkage (1→3, 1→4) β-D-glucan predominates in endosperm cell walls, whereas in wheat and rye, arabinoxylan is the major polysaccharide (Selvendran, 1983; Wood, 1986; Pettersson and Aman, 1989). β-Glucan in barley and oats accounts for 3 to 5% of the kernel weight, and contains approximately 30% (1→3) linkages and 70% (1→4) linkages. Arabinoxylan in rye contains a 2:1 xylan to arabinose ratio, and it constitutes up to 10% of this cereal grain by weight (Antoniou et al., 1981). These glycans increase digesta viscosity and cause sticky feces in poultry. Furthermore, because the birds lack glycanase activity, the dietary energy in these glycans is lost to them. Sticky feces syndrome causes problems for poultry producers with respect to animal hygiene, fouling of eggs and promoting spread of enteric disturbances. Supplementary xylanases and β-glucanases are effective for counteracting increases in digesta viscosity and the problems associated with it (Edney et al., 1989; Pettersson and Aman, 1989; Bedford et al., 1992; Campbell and Bedford, 1992). β-Glucanases and fibrolytic enzymes can also be used to improve feed for non-ruminant and ruminant animals (e.g.,

silage for cattle) by converting a portion of the indigestible fibre into sugars, and they have potential application in such diverse industries as food processing and manufacturing of chemicals, clothing, paper and detergents. Ruminal fungal enzymes may be especially advantageous for industrial applications because of their high activity, extracellular location and overall stability, the mildness of their reaction conditions, and absence of substrate loss due to chemical modifications.

Phytase

In areas of the world where swine and poultry production is intensive, phosphorus excretion is becoming a major problem. Up to 90% of the phosphate in cereal and oilseed diet constituents occurs as phytate (Graf, 1986), which passes through the digestive tract intact. Diets for poultry and swine must therefore be supplemented with inorganic phosphate, even though large amounts of phosphorus (as phytate) are excreted in the manure and contribute significantly to eutrophication of surface waters (Van Gorcom et al., 1995). Efforts to minimize this problem have focused on using enzyme feed additives (i.e., phytase) to liberate the phosphorus in phytate, and make it available to the non-ruminant animal. At present, commercially available phytase supplements are produced using genetically modified strains of the soil fungus *Aspergillus niger*. Three genes coding for phytases have been characterized from this organism (Ehrlick et al., 1993; Piddington et al., 1993; Van Hartingsveldt et al., 1993), and using the charac- terization information, phytase production has been increased by gene amplification and promoter substitution (Piddington et al., 1993; Van Hartingsveldt et al., 1993; Van Gorcom et al., 1995).

In contrast to non-ruminant animals, ruminants readily utilize the phosphorus in phytic acid. This ability is attributable to the phytase activity of the rumen microbial population which, although recognized for many years, has only recently become the focus of intensified study. Recent research in our laboratory at the Lethbridge Research Centre has been directed toward phytase production by a number of ruminal microbes. *Selenomonas ruminantium* JY35 was identified as having the highest phytase activity of the 335 strains (representing 22 species) studied. The phytase was purified from a recombinant *Escherichia coli* clone, and was found to have a specific activity four to eight times higher (400 to 800 μmol phosphate released from phytate/min/mg protein) than the commercially available *A. niger* phytase. Although the process of phytate degradation has been thoroughly characterized, study of the genetics governing this process is relatively new. Whereas fibre degradation in the rumen is complex, and involves many enzymes, phytate degradation is considerably more simple, and can be conferred upon an organism by substitution of a single gene (Piddington et al., 1993; Van Hartingsveldt et al., 1993; Van Gorcom et al., 1995; our unpublished observations). Techniques developed during research with fibrolytic genes and enzymes from the rumen may well prove easily transferable to applications with phytases.

RUMINAL MICROBES AS A SOURCE OF GENETIC MATERIAL FOR INDUSTRY

The cost of enzymes for enhancing livestock production performance can be reduced by selecting more effective expression and delivery systems. Improvements in this area require establishment of a larger battery of genes from which to choose. Ongoing study of the mechanisms of fibre digestion, the quest to find more efficacious enzymes for industrial purposes, and recent technological developments which now enable genetic manipulations of ruminal microorganisms previously not achievable, all have contributed to the growing number of genes from ruminal bacteria, protozoa and fungi which have been cloned and characterized.

At least 100 different genes, the majority of which encode enzymes involved in fibre degradation, have been cloned from ruminal microbes. Most of these have been isolated from a small group of bacterial species, including *Ruminococcus albus, Ruminococcus flavefaciens, Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Prevotella ruminicola* (for review, see Hespell, 1989; Forsberg et al., 1993; Flint, 1994; Wallace, 1994), and Actinomyces (Min et al., 1994) and researchers have only recently turned to studying the genetics of anaerobic fungi and protozoa from the rumen.

The powerful fibrolytic activity of ruminal fungi and their ability to utilize plant cell wall polymers generally recalcitrant to bacterial degradation have motivated researchers to isolate almost 40 genes coding for fibrolytic enzymes from four fungal species. These include four cellulases (Xue et al., 1992a, b; Zhou et al. 1994; Denman et al., 1996) and three xylanases (Gilbert et al., 1992; Tamblyn Lee et al., 1993; Black et al., 1994; Selinger et al., 1995) from *Neocallimastix patriciarum*, four cellulases (Li et al., 1996, 1997a, b; Liu et al., 1996) and a xylanase (Li et al., 1996) from *Orpinomyces* spp., eight cellulases, four xylanases and seven mannanases from *Piromyces* spp. (Ali et al., 1995; Fanutti et al., 1995; Millward-Sadler et al., 1996), and an endoglucanase (Fujino et al., 1995) and two xylanases (Durand et al.,

1996) from *Neocallimastix frontalis*. To date, there have been no reports of genes cloned from anaerobic fungi belonging to the genera *Anaeromyces* or *Caecomyces*, but this will likely change, as the search for novel genes continues. During construction and screening of cDNA libraries for *Orpinomyces joyonii* SG4 and *Piromyces rhizinflata* 2301 at the Lethbridge Research Centre, 229 fibrolytic clones were recovered. Preliminary sequence analyses of clones from the *O. joyonii* SG4 library has revealed five novel cellulases (X. Qiu, A. Takenaka, L. B. Selinger and K.-J. Cheng, unpublished results).

Ruminal protozoa have received even less attention than the fungi. Although protozoa have been implicated in fibre digestion, there have been no reports in the literature regarding genes cloned from these organisms. Recently, however, four genes (encoding two cellulases and two xylanases) from *Epidinium ecaudatum* and *Polyplastron multivesiculatum* were recently cloned and characterized as part of a collaborative research project between the Japanese National Institute of Animal Industry and the Lethbridge Research Centre (A. Takenaka, X. Qiu, L. B. Selinger and K. -J. Cheng, unpublished results). The genes were isolated from cDNA libraries of the two species, and analysis of the DNA sequences has identified 5' regions encoding catalytic domains related most closely to enzymes produced by other ruminal microorganisms.

Genetically modified ruminal microbes

Manipulation of digestion in ruminants through genetic modification of ruminal bacteria is currently being investigated in many laboratories, and numerous review have been written on this subject. Strategies proposed for manipulating the bacteria include expression of heterologous genes encoding fibrolytic enzymes, artificial peptides, bacteriocins, detoxification agents and antiprotozoal factors. In the past, the lack of functional gene transfer systems for anaerobic bacteria has impeded progress toward expression of foreign genes. However, conjugal and electroporation gene transfer systems have now been developed and refined for several prominent species of ruminal bacteria, including *B. fibrosolvens, Eubacterium cellulosolvens, P. ruminicola, R. albus, Streptococcus bovis* and *S. ruminantium*. Genetic transfer into a rumen bacterial species was first demonstrated by Teather (1985). The conjugal plasmid RP4 was transferred from *Escherichia coli* to *B. fibrisolvens*. Application of gene transfer technologies to construction of recombinant anaerobic bacteria designed to modify the ruminal environment is illustrated in the following examples. Whitehead and Hespell (1989) cloned an endoxylanase gene from *Bacteroides* (now *Prevotella*) *ruminicola* 23 and used the vector pVAL-1 to introduce it into *Bacteroides fragilis* and *Bacteroides uniformis*, dramatically increasing enzyme activity. Endoxylanase activities of the newly constructed xylanolytic strains were up to 50 times higher than the activity measured in the original *B. ruminicola* cultured on xylan (Whitehead and Hespell, 1990). The xylanase gene has since been integrated into the chromosomal chondroitin lyase II gene of *Bacteroides thetaiotaomicron* for stable endoxylanase production in the absence of selective agents (Whitehead et al., 1991). In *B. thetaiotaomicron*, the enzyme activity was 17 times higher than that in *B. ruminicola* 23, and remained stable in *B. thetaiotaomicron* over more than 60 generations of growth in a carbon-limited chemostat culture. Introduction of the new strain into the rumen to increase xylan degradation was proposed, but it remains to be determined whether or not the modified *B. thetoiotaomicron*, which is not a naturally occurring species in the rumen, will persist in numbers high enough to substantially increase hemicellulose digestion. At the Lethbridge Research Centre, *B. fibrisolvens* has been selected as a host for heterologous gene expression. This species is an ubiquitous species native to the rumen, and as such is an ideal candidate for introducing novel genetic information into the ruminal environment. Introduction and expression of an amylase-encoding gene from *S. bovis* in *B. fibrisolvens* H17c was accomplished in our laboratory (Clark, 1994; Clark et al., 1992, 1995). The gene was cloned onto an *E. coli/B. fibrisolvens* shuttle vector, and the resulting construct (pUBLRSA) was introduced into *B. fibrisolvens* using electroporation. Expression of the *S. bovis* amylase was confirmed by zymogram analysis, and the amylolytic activity of *B. fibrisolvens* (pUBLRSA) grown with glucose as the sole carbon source was 2.5 times higher than that of wild type *B. fibrisolvens* H17c. This was the first reported expression of a foreign polymer-degrading enzyme in a bacterium indigenous to the rumen. Research directed toward constructing a more effective expression system and introducing additional genes encoding fibrolytic enzymes into native ruminal species is currently underway.

Genetically modified silage inoculants

Enzymes added to low soluble carbohydrate forages prior to ensiling to effect release of soluble sugars from plant cell walls can improve silage quality and livestock growth performance. The cost and inconvenience of adding these enzymes could be circumvented if the bacteria in standard silage inoculants were also capable of producing the enzymes typically supplemented. To date, attempts to isolate microorganisms suitable as inoculants

and possessing desired enzyme activities have been unsucessful. Recent research efforts have therefore been directed toward developing genetically engineered strains of lactobacilli which produce fibrolytic enzymes. *Lactobacillus* strains expressing heterologous cellulase and xylanase genes have been developed (Bates et al., 1989; Scheirlinck et al., 1989, 1990; Baik and Pact, 1990) and have, in at least one case, exhibited competitive growth in silage (Sharp et al., 1992). Those researchers also determined that the heterologous plasmid pM25 (a pSA3 derivative containing a *Clostridium thermocellum* cellulase gene) was maintained at high levels by the rifampin-resistant host cells. Present as a chromosomally integrated element, it was maintained by 100% of host cells; as an autonomously replicating plasmid element, by 85% of host cells. Although cellulase and xylanase genes have been introduced into *L. plantarum* (Bates et al., 1989; Scheirlinck et al., 1989, 1990; Baik and Pact, 1990; Sharp et al., 1992), development of efficacious recombinant silage inoculants has been confounded by persistently low levels of heterologous gene expression (Hols et al., 1994; Oosting et al., 1995). Construction of hybrid genes, consisting of homologous expression-secretion signals fused with structural sequences of heterologous fibrolytic enzyme-encoding genes, may improve levels of gene expression. Hols et al. (1994) demonstrated the effectiveness of this technique by isolating promoter signal sequence regions from *L. plantarum* and using them to drive expression and secretion of foreign amylase and levanase genes in *L. plantarum*. Expression of the heterologous levanase enabled the modified *L. plantarum* isolates to use inulin as a carbon source. Application of recently developed gene expression and integration technologies will expedite development of genetically modified *Lactobacillus* strains for use as silage inoculants.

Transgenic plants

Using plants as hosts for expression of enzymes for industrial use would provide an alternative, more cost effective method of enzyme production and, in many cases, delivery. The recent advances in plant biotechnology which make such a scheme feasible may well revolutionize the commercial enzyme industry. Large quantities of plant biomass can be produced inexpensively via existing agricultural infrastructure. Production of enzymes for animal feed by plant species commonly fed to livestock will greatly reduce requirements for downstream processing, packaging and delivery, as the whole or parts of the enzyme-producing plants can be fed directly to livestock. Phytase has been expressed in tobacco and soybean plants (Pen et al., 1993; Pen 1994; Russell, 1994), as well as a xylanase in tobacco plants (Herber et al., 1995). At the Lethbridge Research Centre, we are using a plant expression system for production of superior fibrolytic enzymes from ruminal fungal isolates at costs significantly reduced over traditional fermentation. In collaboration with researchers from the University of Calgary, we have produced a gene fusion of oleosin (oil body membrane protein, Van Rooijen and Moloney, 1995) and *N. patriciarum* xylanase, and introduced it into canola via *Agrobacterium*-mediated transformation (J. -H. Liu, M. M. Moloney and K. -J. Cheng, unpublished data). Xylanase activity has been detected in crude oil body protein preparations from the transgenic canola seeds. Yield of the *N. patriciarum* xylanase has yet to be determined, but yields of recombinant protein in excess of 1% of total seed protein are computed to be possible with this system. We have also obtained positive results with expression of a β-glucanase gene from *F. succinogenes* in transgenic potato (L. M. Kawchuk, J. D. Armstrong and K. -J. Cheng, unpublished data), which attests further to the promise and versatility of plant expression systems for enzyme production. In addition to providing an efficient alternative to traditional microbial systems, transgenic plants offer the added advantage of a safe and stable storage and delivery system, in the form of the seed (Pen, 1994). Recombinant enzymes were found to be stable in seeds stored for up to one year at 4°C or at room temperature (Pen et al., 1993; Pen 1994; Van Rooijen and Moloney, 1995).

Transgenic animals

Technological developments enabling introduction of genetic material into domestic animals (Ward et al., 1989; Briskin et al., 1991) provide validity of the concept of direct expression of microbial enzymes by the animal itself, rather than adding them to feed for livestock. To benefit the animal, heterologous expression must be targeted for the appropriate tissue, and the enzyme(s) must be secreted into the lumen of the gastrointestinal tract, resist degradation by proteases, and exhibit activity in the prevailing environmental conditions (e.g., pH, temperature, osmolarity) of the digestive tract (Forsberg et al., 1993; Hall et al., 1993). Expression of fibrolytic enzymes in a non-ruminant animal was first demonstrated by Hall et al. (1993). A truncated endoglucanase E gene from *C. thermocellum* under the control of the elastase I gene promoter was expressed in the exocrine pancreas of transgenic mice. Carboxymethycellulase activity was detected in small intestinal contents, demonstrating secretion of the recombinant enzyme from the pancreas.

In related studies, synthesis and secretion of a bacterial glucanase and a bacterial xylanase in Chinese hamster ovary cells has been demonstrated (Zhang et al., 1995) using an SV40 early enhancer/promoter and mouse *amy*-2.2 signal peptide sequence. Weak but definite synthesis and secretion from an acinar pancreatic cell line has also been achieved, using the amylase gene promoter-enhancer sequence, as well as pancreatic expression of the glucanase in transgenic mice. Preliminary findings indicate that a major challenge of this research will be attainment of sufficiently high expression levels of the glycanase genes in pancreatic cells to effect significant hydrolysis of the glycans in the intestine. The obvious potential of this method of enzyme delivery sparks continued research in this area.

RUMINAL MICROBES AS A SOURCE OF DETOXIFICANTS FOR INDUSTRY

Enzymatic detoxification

Many plants produce astringent or toxic secondary metabolites as a defence against predation by herbivores. Exposure of livestock to these compounds arises from weed infestation of pastures, contamination of feed, and inclusion of toxic plants in feed rations (Gregg, 1995). This exposure results in multimillion dollar annual losses to producers, manifested both as animal deaths and as compromised growth performance. The most widely studied plant toxins include mimosin T-2 toxins, nitrotoxins, pyrrolizidine alkaloids, *trans*-aconitate and tannins. These toxins reduce animal productivity by reducing feed palatability, feed intake, enzyme activity, ruminal fermentation rates, nutrient availability and wool growth, or by inducing toxicosis (Hammond, 1995). In many areas of the world, indigenous ruminants have evolved the ability to digest native plants that are toxic to non-ruminant animals (Jones, 1981; Allison et al., 1992). Often this ability can be traced to activities of the ruminal microbes, which convert toxic components to harmless or even beneficial compounds (Jones and Lowry, 1984; Gregg, 1995). Well documented examples of this phenomenon include resistance to *Leucaena* toxins by ruminants in Hawaii (Jones, 1981; Hammond, 1995) and ovine resistance to pyrrolizidine alkaloids (Hooper, 1978; Craig et al., 1992). *Leucaena lecocephala* is a legume grown in the tropics for a variety of uses, including feed for livestock. This plant produces mimosine, a non-proteic free amino acid which is toxic to non-ruminants and to ruminants in which ruminal degradation of mimosine itself or its degradation product, 3-hydroxy-4-(1H)-pyridone (3,4-DHP) does not occur (Hammond, 1995). Involvement of ruminal microorganisms in detoxification of mimosine was demonstrated by Jones and Lowry (1984) who transferred *Leucaena* tolerance to mimosine-sensitive Australian goats by infusing ruminal fluid from -tolerant Indonesian goats. Resistance of sheep to the toxicity of *Senecio jacobaea* (tansy ragwort) hepatotoxic pyrrolizidine alkaloids which affects cattle and horses, is also attributable to detoxification activity (i.e., hydrolytic transformations) carried out by ovine ruminal microbes (Hooper, 1978; Wachenheim et al., 1992). Identification of ruminal detoxifications of plant metabolites have prompted more detailed examinations of the bacteria accomplishing the degradations. Enrichment and selection techniques have been utilized to isolate anaerobic bacteria able to degrade 3,4-DHP, tannins, *trans*-aconitate and nitrotoxins. Characterization of the biochemical and genetic bases of plant toxin degradation by these microbes may facilitate development of strategies involving toxin-degrading enzymes, or the genes encoding them, for improving livestock production.

Dissimilatory metal reduction

Microbes in nature can typically reduce a variety of metals in metabolic processes not related to metal assimilation. Microbes that use metals as terminal electron acceptors, or that reduce metals in detoxification mechanisms, exert an important influence on the geochemistry of ecosystems such as aquatic sediments, submerged soils and the terrestrial subsurface. Recognition of the potential for exploiting microbial metal reduction for remediation of environments and waste streams contaminated with metals and certain organics is increasing. Until recently, metal reduction in sedimentary environments was generally considered to result primarily from non-enzymatic reactions, but it is now accepted that much of this reductive activity arises from enzymatic processes attributable to microbes in anaerobic conditions (Lovley et al., 1991). The intrinsic activities of dissimilatory metal-reducing microorganisms that use Fe^{3+}, Mn^{4+}, U^{6+} or Se^{6+} as terminal electron acceptors can have a substantial impact upon the fate of these metals in aquatic sediments and groundwater. Dissimilatory reduction of U^{6+}, Se^{6+}, Cr^{6+}, Hg^{2+}, Tc^{7+}, V^{5+}, Au^{3+} and Ag^{1+} is a potential mechanism for removing these metals from contaminated environments or waste streams. Pure culture models for reduction of these metals have been developed, but substantive information about the microbes important in catalysis of metal reduction in the natural environment is lacking. Some microbial species employing anaerobic respiration are able to use as terminal electron acceptors various metals (via reduction of ions), nitrate (via

denitrification and dissimilatory nitrate reduction), sulfate (reduction) and carbon (via methanogenesis). The concept of identifying and exploiting the microorganisms and (or) the enzymes involved in these processes for industrial purposes is intriguing. Although attempts to date to isolate or enrich ecosystems for suitable microbial candidates have not been successful (Lovley, 1992), the potential for valuable research in this area of microbial manipulation, and the contributions that may be made by the ruminal anaerobic population, remain to be investigated.

RUMINAL MICROBES AS A SOURCE OF USEFUL PRODUCTS FOR INDUSTRY

Microbes worldwide are used to produce hundreds of commercial products valued in the tens of billions of dollars annually (Steele and Stowers, 1991). Products manufactured through microbiological means include, in addition to enzymes, chemicals (e.g., citric acid, ethanol, dextran), vitamins and amino acids (e.g., vitamin B_{12}, L-lysine) and other pharmaceuticals (e.g., antibiotics, insulin, steroid transformations). Products produced by naturally occurring microbes have the advantage of being considered natural themselves and are therefore more easily approved for industrial applications than are those produced by genetically manipulated organisms. For this reason, selection of naturally occurring microorganisms for production purposes is favoured in many industries, and viable and efficient screening programs remain a necessity. The diversity of the microbial world holds much promise for new technologies to improve day-to-day operations. Over the past 40 to 50 years, the screening of industrially important microbes has evolved steadily. Increasingly, investigators are seeking out natural enrichments in the environment, such as thermal springs, glacier ice, or industrial waste-treatment facilities, where specialized populations of microbes proliferate under physical or chemieal selective pressures. These environments provide ongoing enrichments and natural selection of organisms adapted to specific conditions. The rumen microbial ecosystem represents a wealth of microbial resources - cellular, enzymatic and genetic - with tremendous potential for exploitation in industrial applications. A vast array of potentially useful organisms probably still await discovery and(or) recognition of their applicability to particular challenges. The idea is enticing - and was stated succinctly by Perlman (1980) in the laws of applied microbiology that microbes are capable of any task.

REFERENCES

Ali, B. R. S., L. Zhou, F. M. Graves, R. B. Freedman, G. W. Black, H. J. Gilbert and J. P. Hazlewood. 1995. Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and are encoded by multigene families. FEMS Microbiol. Lett. 125:15-22.

Allison, M. J., W. R. Mayberry, C. S. Mcweeney and D. A. Stahl. 1992. *Synergistes jonesii*, gen. nov., sp. nov.,: a rumen bacterium that degrades toxic pyridinediols. Syst. Appl. Microbiol. 15:522-529.

Baik, B. -H. and M. Y. Pack. 1990. Expression of *Bacillus subtilis* endoglucanase gene in *Lactobacillus acidophilus*. Biotechnol. Lett. 12:919-924.

Bates, E. E. M., H. J. Gilbert, G. P. Hazlewood, J. Huckle, J. I. Laurie and S. P. Mann. 1989. Expression of a *Clostridium thermocellum* endoglucanase gene in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 55:2095-2097.

Beauchemin, K. A. and L. M. Rode. 1996. Use of feed enzymes in ruminant nutrition. *In*: Rode, L.M. (ed) *Animal Science Research and Development - Meeting Future Challenges*, pp. 103-130. Proc. Can. Soc. Anim. Sci. Lethbridge, AB.

Beauchemin, K. A., L. M. Rode and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Axie. 75:641-644.

Bedford, M. R., J. F. Patience, H. L. Classen and J. Inborr. 1992. The effect of dietary enzyme supplementation of rye- and barley-based diets on digestion and subsequent performance in weanling pigs. Can. J. Anim. Sci. 72:97-105.

Black, G. W., G. P. Hazlewood, G. P. Xue, C. G. Orpin and H. J. Gilbert. 1994. Xylanase B from *Neocallimastix patriciarum* contains a non-catalytic 455-residue linker sequence comprised of 57 repeats of an octapeptide. Biochem. J. 299:381-387.

Briskin, M. J., R. -Y. Hsu, T. Boggs, J. A. Schultz, W. Rishell and R. A. Bosselman. 1991. Heritable retroviral transgenes are highly expressed in chickens. Proc. Nat. Acad. Sci. 88:1736-1740.

Campbell, G. L. and M. R. Bedford. 1992. Enzyme applications for monogastric feeds: A review. Can. J. Anim. Sci. 72:449-466

Clark, R. G. 1994. The development of vectors and transfer systems for the rumen bacterium *Butyrivibrio fibrisolvens*. Ph.D. Dissertation, pp. 1-128. University of Alberta, Edmonton, AB.

Clark, R. G., K. -J. Cheng, L. B. Selinger and M. F. Hynes. 1995. Expression of a *Streptococcus bovis* amylase in the rumen bacterium *Butyrivibrio fibrisolvens*. Proceedings of the 95th Ann. Meet. Amer. Soc. Microbiol. p 526. Washington, DC.

Clark, R. G., Y. -J. Hu, M. F. Hynes, R. K. Salmon and K. -J. Cheng. 1992. Cloning and expression of an amylase gene from *Streptococcus bovis* in *Escherichia coli*. Arch. Microbiol. 157:201-204.

Cowan D. 1996. Industrial enzyme technology. Trends in Biotechnol 14:177-178.

Craig, A. M., C. J. Latham, L. L. Blythe, W. B. Schmotzer and O. W. O Connor. 1992. Metabolism of toxic pyrrolizidine alkaloids from tansy ragwort (*Senecio jacobaea*) in ovine ruminal fluid under anaerobic conditions. Appl. Environ. Microbiol. 58:2730-2736.

Denman, S., G. -P. Xue, and B. Patel. 1996. Characterization of a *Neocallimastix patriciarum* cellulase cDNA (*celA*) homologous to *Trichoderma reesei* cellobiohydrolase H. Appl. Environ. Microbiol. 62:1889-1896.

Doemer, K. C. and B. A. White. 1990. Assessment of the endo-β-1,4-glucanase components of *Ruminococcus flavefaciens* FD-1. Appl. Environ. Microbiol. 56:1844-1850.

Durand, R., C. Rascle and M. Fevre. 1996. Molecular characterization of *xyn*3, a member of the endoxylanase multigene family of the rumen anaerobic fungus *Neocallimastix frontalis*. Curr. Genet. 30:531-540.

Edney, M. J., G. L. Campbell and H. L. Classen. 1989. The effect of β-glucanase supplementation on nutrient digestibility and growth in broilers given diets containing barley, oat groats or wheat. Anim. Feed Sci. Technol. 25:193-200.

Ehrlich, K. C., B. G. Montalbano, E. J. Mullaney, H. C. Dischinger and A. H. Ullah. 1993. Identification and cloning of a second phytase gene (*phyB*) from *Aspergillus niger* (*ficuum*) Biochem. Biophys. Res. Comm. 195:53-57.

Fanutti, C., T. Ponyi, G. W. Black, G. P. Hazlewood and H. J. Gilbert. 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. J. Biol. Chem. 49:29314-29322.

Feng, P., C. W. Hunt, W. E. Julien, K. Dickinson and T. Moen. 1992a. Effect of enzyme additives on *in situ* and *in vitro* degradation of mature cool-season grass forage. J. Anim. Sci. 70 (Suppl.):309 (Abstr.).

Feng, P., C. W. Hunt, W. E. Julien, S. C. Hacnny and G. T. Pritchard. 1992b. Effect of enzyme additives to cool-season grass forage on voluntary intake and digestive function in mature beef steers. J. Anim. Sci. 70(Suppl.):310(Abstr.).

Flint, H. J. 1994. Molecular genetics of obligate anaerobes from the rumen. FEMS Microbiol. Lett. 121:259-268.

Flint, H. J., J. X. Zhang and J. Martin. 1994. Multiplicity and expression of xylanases in the rumen cellulolytic bacterium *Ruminococcus flavefaciens*. Curr. Microbiol. 29:139-143.

Forsberg, C. W., K. -J. Cheng, P. J. Krell and J. P. Phillips. 1993. Establishment of rumen microbial gene pools and their manipulation to benefit fibre digestion by domestic animals. Proc. VII World Conf. Anim. Prod. pp. 281-316. World Assoc. Anim. Prod. Edmonton, AB.

Fujino, Y., K. Ushida, K. Miyazaki, Y. Kojima, T. Nagamine, K. Ogata, M. Matsumoto and H. Itabashi. 1995. Gene Bank Accession Number U38843.

Gilbert, H. J., G. P. Hazlewood, J. I. Laurie, C. G. Orpin and G. P. Xue. 1992. Homologous catalytic domains in a rumen fungal xylanase - evidence for gene duplication and prokaryotic origin. Molec. Microbiol. 6:2065-2072.

Graf, E. 1986. Chemistry and applications of phytic acid: an overview. *In*: Graf, E. (ed) *Phytic Acid, Chemistry and Applications*. pp. 1-22. Pilatus Press. Minneapolis, MO.

Gregg, K. 1995. Engineering gut flora of ruminant livestock to reduce forage toxicity: progress and problems. Trends Biotechnol. 13:418-421.

Hall, J., S. Ali, A. Surani, G. P. Hazlewood, A. J. Clark, J. P. Simons, B. H. Hirst and H. J. Gilbert. 1993. Manipulation of the repertoire of digestive enzymes secreted into the gastrointestinal tract of transgenic mice. Bio/Technol.y 11:376-379.

Hammond, A. C. 1995. *Leucaena* toxicosis and its control in ruminants. J. Anim. Sci. 73:1487-1492.

Herbers, K., I. Wilke and U. Sonnewald. 1995. A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified. Bio/Technol. 13:63-66.

Hespell, R. B., 1989. Problems and progress with the genetics of ruminal bacteria. Dev. Ind. Microbiol.30:13-33.

Hodgson, J. 1994. The changing bulk biocatalyst market. Bio/Technology 12:789-790.

Hols, P., T. Ferain, D. Garmyn, N. Bernard and J. Delcour. 1994. Use of homologous expression-secretion signals and vector-free stable chromosome integration in engineering of *Lactobacillus plantarum* for α-amylase and levanase expression. Appl. Environ. Microbiol. 60:1401-1413.

Hooper, P. T. 1978. Pyrrolizidine alkaloid poisoning - pathology with particular reference to differences in animal and plant species. *In*: Keeler, R. F., Van Campen, K. R. and James, L. F. (eds) *Effects of Poisonous Plants on Livestock*, pp. 161-176. Academic Press, Inc., New York, NY.

Jones, R. J. 1981. Does ruminal metabolism of mimosine explain the absence of *Leucaena* toxicity in Hawaii? Aust. Vet. J. 57:55.

Jones, R. J. and J. B. Lowry. 1984. Australian goats detoxify the goitrogen 3-hydroxy-4(1H) pyridone (DHP) after rumen infusion from an Indonesian goat. Experientia 40:1435-1436.

Li, X. -L., H. Chen and L. G. Ljungdahl. 1996. Cloning, sequencing, and over-expression of a xylanase cDNA in *Escherichia coli* of the polycentric anaerobic fungus *Orpinomyces* sp. strain PC-2. Proc. 96th Ann. Meet Amer. Soc. Microbiol. p. 361. New Orleans, LA.

Li, X. -L., H. Chen and L. G. Ljungdahl. 1997a. GenBank Accession Number U63837.

Li, X. -L., H. Chen and L. G. Lungdahl. 1997b. GenBank Accession Number U63838.

Liu, J. -H., L. B. Selinger, Y. J. Hu, M. M. Moloney, K. A. Beauchemin and K. -J. Cheng. 1996. Cloning and characterization of an endoglucanase gene from *Orpinomyces joyonii*. Proc. 96th Ann. Meet. Amer. Soc. Microbiol. p. 505. New Orleans, LA.

Lovley, D. R. 1992. Microbial oxidation of organic matter coupled to the reduction of Fe (III) and Mn (IV) oxides. Catena. 21:101-114.

Lovley, D. R., E. J. P. Phillips, Y. A. Gorby and E. R. Landa. 1991. Microbial reduction of uranium. Nature 350:413-416.

Millward-Sadler, S. J., J. Hall, G. W. Black, G. P. Hazlewood and H. J. Gilbert. 1996. Evidence that *Piromyces* gene family encoding endo-1,4-mannases arose through gene duplication. FEMS Microbiol. Lett. 141:183-188.

Min, H. K., Y. S. Choi, J. K. Ha, K. K. Cho, Y. M. Kwon, Y. H. Chang and S. S. Lee. 1994. Isolation and identification of anaerobic rumen baeterium, actinomyces sp. 40 and enzymatic properties of β-1,4-endoglucunase. Asian-Australasian J. Anim. Sci. 7:373-382.

Oosting, S. J., L. B. Selinger, T. A. McAllister and K. -J. Cheng. 1995. Development of genetieally engineered silage inoculants: Need for higher gene expression. Proc. Joint Meet. West. Sect. Amer. Soc. Anim. Sci. West. Branch Can. Soc. Anim. Sci. pp 345-348. Lethbridge, AB.

Pen, J. 1994. Application of industrial enzymes produced in

transgenic plants. Proc. Symp. Production of Recombinant Proteins in Plants. pp. 44-49. University of Leicester, UK.

Pen, J., T. C. Verwoerd, P. C. van Paridon, R. F. Beudeker, P. J. M. van den Elzen, K. Geerse, J. D. van der Klis, H. A. J. Versteegh, A. J. J. van Ooyen and A. Hoekema. 1993. Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. Bio/Technol. 11:811-814.

Perlman, D. 1980. Some problems on the new horizons of applied microbiology. Dev. Ind. Microbiol. 21:15-23.

Pettersson, D. and P. Aman. 1989. Enzyme supplementation of a poultry diet containing rye and wheat. Br. J. Nutr. 62:139-149.

Piddington, C. S., C. S. Houston, M. Paloheimo, M. Cantrell, A. Miettinen-Oinonen, H. Nevalainen and J. Rambosek. 1993. The cloning and sequencing of the gene encoding phytase (*phy*) and pH 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. Awamori. Gene 133:55-62.

Russell, D. 1994. Production of industrial and biopharmaceutical proteins in transgenic plants. Proc. Symp. Production of Recombinant Proteins in Plants, p. 43. University of Leicester, UK.

Scheirlinck, T., J. DeMeutter, G. Arnaut, H. M. Joos, M. Claeyssens and F. Michiels 1990. Cloning and expression of cellulase and xylanase genes in *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 33:534-541.

Scheirlinck, T., J. Mahillon, H. Joos, D. Dhaese and F. Michiels. 1989. Integration and expression of α-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. Appl. Environ. Microbiol. 55:2130-2137.

Selinger, L. B., J. -H. Liu, Y. J. Hu, M. M. Moloney, C. W. Forsberg and K. -J. Cheng. 1995. Characterization of a xylanase gene from *Neocallimastix patriciarum*. Proc. 23rd Biennial Conference on Rumen Function, p. 39. Chicago, IL.

Selvendran, R. R. 1983. The chemistry of plant cell walls. *In*: Birch, G. G. and Parker, K. J. (eds) *Dietary Fibre*, pp. 95-147. Applied Science Publishers, London, UK.

Sharp, R., A. G. O'Donnell, H. J. Gilbert and G. P. Hazlewood. 1992. Growth and survival of genetically manipulated *Lactobacillus plantarum* in silage. Appl. Environ. Microbiol. 58:2517-2522.

Steele, D. B. and M. D. Stowers. 1991. Techniques for selection of industrially important microorganisms. Annu. Rev. Microbiol. 45:89-106.

Tamblyn, Lee, J. M., Y. Hu, H. Zhu, K. -J. Cheng, P. J. Krell and C. W. Forsberg. 1993. Cloning of a xylanase gene from the ruminal fungus *Neocallimastix patriciarum* 27 and its expression in *Escherichia coli*. Can. J. Microbiol. 39:134-139.

Teather, R. M. 1985. Application of gene manipulation to rumen microflora. Can. J. Anim. Sci. 65:563-574.

Trinci, A. P. J., D. R. Davies, K. Gull, M. I. Lawrence, B. B. Nielsen, A. Rickers and M. K. Theodorou. 1994. Anaerobic fungi in herbivorous animals. Mycol. Res. 98:129-152.

Van Gorcom, R. F. M., W. Van Hartingsveldt, P. A. Van Paridon, A. E. Veenstra, R. G. M. Luiten and G. C. M. Selten. 1995. Cloning and expression of phytase from *Aspergillus*. U.S. Patent No. 5,436,156.

Van Hartingsveldt, W., C. M. J. Van Zeijl, M. G. Harteveld, R. J. Gouka, M. E. G. Suykerbuyk, R. G. M. Luiten, P. A. Van Paridon, G. C. M. Selten, A. E. Veenstra, R. F. M. Van Gorcom and C. A. M. J. Van Den Hondel. 1993. Cloning, characterization and overexpression of the phytase gene (*phyA*) of *Aspergillus niger*. Gene 127:87-94.

van Rooijen, G. J. H. and M. M. Moloney. 1995. Plant seed oil-bodies as carriers for foreign proteins. Bio/Technol. 13:72-77.

Wachenheim, D. E., L. L. Blythe and A. M. Craig. 1992. Effects of antibacterial agents on *in vitro* ovine ruminal biotransformation of the hepatotoxic pyrrolizidine alkaloid jacobine. Appl. Environ. Microbiol. 58:2559-2564.

Wallace, R. J. 1994. Ruminal microbiology, biotechnology, and ruminant nutrition: progress and problems. J. Anim. Sci. 72:2992-3003.

Ward, K. A., J. D. Murray and C. D. Nancarrow. 1989. The insertion of foreign DNA into animal cells. *In*: *Biotechnology for livestock production*, pp. 17-28. Plenum Press, New York, NY.

Whitehead, T. R. and R. B. Hespell. 1990. Heterologous expression of the *Bacteriodes ruminicola* xylanase gene in *Bacteroides fragilis* and *Bacteroides uniformis*. FEMS Microbiol. Lett. 66:61-66.

Whitehead, T. R., M. A. Cotta and R. B. Hespell. 1991. Introduction of *Bacteroides ruminicola* xylanase gene into the *Bacteroides thetaiotaomicron* chromosome for production of xylanase activity. Appl. Environ. Microbiol. 57:277-282.

Wood, P. J. 1986. Oat -glucan: Structure, location, and properties. *In*: *Oats: Chemistry and technology*, pp. 121-152. American Association of Cereal Chemists, Inc., St. Paul, MN.

Xue, G. P., C. G. Orpin, K. S. Gobius, J. H. Aylward and G. D. Simpson. 1992a. Cloning and expression of multiple cellulase cDNAs from the anaerobic rumen fungus *Neocallimastix patriciarum* in *Echerichia coli*. J. Gen. Microbiol. 138:1413-1420.

Xue, G. P., K. S. Gobius and C. G. Orpin. 1992b. A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities. J. Gen. Microbiol. 138:2397-2403.

Yanke, L. J., L. B. Selinger and K. -J. Cheng. 1995. Comparison of cellulolytic and xylanolytic activities of anaerobic rumen fungi. Proc. 23rd Biennial Conference on Rumen Function, p. 32. Chicago, IL.

Zhang, J. X., P. J. Krell, J. P. Phillips and C. W. Forsberg. 1995. The expression of a *Fibrobacter succinogenes* S85 xylanase gene in mammalian cells. Proc. 23rd Biennial Conference on Rumen Function, p 25. Chicago, IL.

Zhou, L., G. P. Xue, C. G. Orpin, G. W. Black, H. J. Gilbert and G. P. Hazlewood. 1994. Intronless *celB* from the anaerobic fungus *Neocallimastix patriciarum* encodes a modular family A endoglucanase. Biochem. J. 297:359-364.