

**Development of *Pichia pastoris* as a Ruminant Escape Vehicle**

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**by**

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## **Dedication**

This thesis is dedicated to Walter Dixon and Renate Meuser for believing in me and for providing the solid foundation which enabled me to learn molecular biology; and to my parents, Howard and Sherrill, for their love, support, and wisdom throughout my life. Without you this thesis would not exist.

## Abstract

The yeast expression system *Pichia pastoris* was investigated as an encapsulation technology capable of serving as a rumen escape vehicle. Cellularly encapsulated protein is protected from the ruminal environment so long as the cell membrane, which surrounds and isolates the intracellular protein is physically intact. Intracellular expression of Green Fluorescent Protein (GFP) allows for the monitoring of cellular integrity as necessary for the protection of encapsulated protein from ruminal proteases. Upon cellular lysis GFP is exposed to extracellular proteases which result in both the proteolytic degradation of the protein-based GFP chromophore and its associated fluorescence. Visualization of rumen fluid under epifluorescent microscopy revealed a high level of background autofluorescence owing to the fluorescent plant particles, microbes, and fluorescent compounds therein. Visualization of GFP in rumen fluid can be optimized through GFP variant selection, filter set design, and light source selection based on bulb emission spectra. Incubation of intracellular GFP expressing *P. pastoris* in batch culture ruminal *in vitro* simulations demonstrated that 93%, 97%, and 25% of the *P. pastoris* inoculum maintained cellular integrity in clarified rumen fluid, bacterial fraction of rumen fluid, and whole rumen fluid, respectively, when incubated over 36 to 48 h. Continuous fermentation *in vitro* rumen simulations (Rusitec) demonstrated a *P. pastoris* escape rate of 19% when added daily to fully adapted Rusitec vessels having a dilution rate of 0.75 d<sup>-1</sup>. Abomasal *in vitro* simulations demonstrated that 84% of the *P. pastoris* inoculum was lysed within 12 h, as necessary for the release of encapsulated protein. *P. pastoris* may be an effective post-ruminal delivery vehicle, provided that similar results are obtained *in vivo*.

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### **List of Abbreviations**

<b>APP</b>	Abomasal Protected Protein
<b>BMGH</b>	Buffered-minimal Glycerol Medium
<b>BMGY</b>	Buffered-compley Glycerol Medium
<b>BMMH</b>	Buffered-minimal Methanol Medium
<b>BMMY</b>	Buffered-complex Methanol Medium
<b>ERB</b>	Effective Rate of Bypass
<b>GFP</b>	Green Fluorescent Protein
<b>GFPuv</b>	Green Fluorescent Protein variant uv
<b>GMO</b>	Genetically Modified Organism
<b>LSLB</b>	Low Salt Lauria-Bertani Medium
<b>ORF</b>	Open Reading Frame
<b>PCR</b>	Polymerase Chain Reaction
<b>REV</b>	Ruminal Escape Vehicle
<b>SCP</b>	Sigle Cell Protein
<b>SE</b>	Standard Error
<b>YPD</b>	Yeast-peptone-dextrose Medium

Bioactive proteins and peptides fed to ruminants are subject to ruminal proteolysis and may not escape the rumen in an active form (Wallace *et al.*, 1997). Traditional methods of ruminal protein protection such as formaldehyde, tannins, or heat are unsuitable for functional proteins, such as enzymes, because the protection process itself renders the protein biologically inactive. Encasement agents such as kaolin, tristearin and fat dissociate poorly from the encased protein, thereby limiting the availability of active protein in the small intestine.

Cellular encapsulation is another approach under consideration for delivering bioactive proteins to the small intestine of ruminants. Through intracellular production and storage, the protein of interest is protected against microbial degradation. This may allow for the oral administration, ruminal escape, abomasal lysis, and ultimately the effective delivery of intracellular recombinant, biologically active protein to the small intestine of ruminants. Plants, rumen microbial cells, and non-ruminal microorganisms all have potential as recombinant rumen escape vehicles.

*Pichia pastoris* is a potential candidate as a recombinant rumen escape vehicle, being a ruminally exogenous organism previously developed as a high level inducible expresser of biologically active protein. This budding methylotrophic yeast was initially developed by the Phillips Petroleum Company (Bartlesville, OK) for single-cell protein (SCP) applications (Wegner, 1990) in which methanol and oxygen is converted to formaldehyde and hydrogen peroxide by alcohol oxidase (*AOX*) (Veenhuis *et al.*, 1983). A heterologous protein expression system based on the primary alcohol oxidase gene (*AOX1*) was developed by the Salk Institute of Biotechnology (La Jolla, CA) (Ellis *et al.*, 1985). This expression system is now commercially available from Invitrogen Corporation (Carlsbad, CA) as a kit based on zeocin selection of positive recombinants.

Cellularly encapsulated protein is protected from the ruminal environment so long as the cell membrane which surrounds and isolates the intracellular protein is physically intact. As such a means of detecting intact cell membranes is required to facilitate the development and evaluation of *P. pastoris* as a ruminal escape vehicle. Intracellular expression of green fluorescent protein (GFPuv) may allow for the observation of intact cellular membranes when incubated in the presence of ruminal proteases. Cell lysis would expose GFPuv to proteolytic degradation resulting in the loss of both the protein-based GFPuv chromophore and its associated fluorescence. Consequently, this system could serve as an excellent model to monitor cellular integrity and the potential of this strategy as a ruminal escape vehicle.

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## **2.0**

## **Literature Review**

### **2.1. Introduction**

The purpose of animal science is to maximize animal production and animal efficiency simultaneous to economic efficiency. In monogastric agriculture, maximal meat and egg production has been attained through the feeding of high quality feedstuffs and protein supplements. Additionally, significant gains in animal production have been realized through the addition of fibrolytic enzymes to the diet which act by releasing more starch, sugars, and protein from the feedstuff, thereby increasing the amount of protein and carbohydrates available for absorption in the small intestine (Gilbert and Hazlewood, 1991). These enzymes are a major breakthrough in nutritional science and have already shown dramatic increases in feed and economic efficiencies in poultry production and to a lesser extent swine production (Campbell and Bedford, 1992).

Unfortunately, similar gains and achievements have thus far been unobtainable in ruminant production. Through evolution grazers have developed an enlarged forestomach known as the rumen which is a fermentation chamber that is highly adept at converting the nutritionally poor value grasses into microbial protein and volatile fatty acids which have a higher nutritional value to the animal. This is achieved by maintaining a highly active microbial population ideally suited for degrading the significant amounts of cellulose and structural carbohydrate shielding the energy and protein deposits within the consumed forages and grasses. In doing so these microorganisms utilize the released protein and carbohydrates for their own growth, division and energy requirements, effectively transforming the otherwise indigestible grasses and forages into highly digestible microbial protein that constantly flows from the rumen.



## **2.2 Potential of Nutritional Supplements**

While nature and evolution have created an animal well suited for its environment, it has introduced a highly complex digestive system which has proven difficult to manipulate using conventional agricultural practices. While efforts in animal science have dramatically increased the ruminant animal's production of milk, meat, and wool through nutrition, genetics and management, there is still significant room for additional improvement. A series of abomasal infusion studies have shown significant increases in production when high value proteins like casein are directly infused post-uminally. These studies, which were summarized by Clark in 1975, show that milk volume can be increased 1-4 kg per day, and that milk protein content can be increased 10-15% per day. This represents an average increase in producer income of \$0.65 to \$2.60 per cow per day based on the current fluid volume pricing (Alberta Dairy Control Board, personal communication). It should be noted that Alberta prices its milk commodities on the basis of butterfat and fluid volume not on protein content, however this is likely to change within the next decade. Ovine studies have shown that the abomasal infusion of 3 g L-arginine, 2 g L-histidine, 6 g L-isoleucine, 7 g L-leucine, 7 g L-lysine, 3 g L-methionine, 7 g phenylalanine, 4 g L-threonine, 1 g L-tryptophan, and 5 g L-valine can increase wool growth by 86% over modern feeding practices (Reis *et al.*, 1990). This represents a doubling farmers profits, either through gross sales or through production efficiency.

Interestingly, these same proteins and amino acids have no effect when fed to cattle or sheep directly (Nader and Walker, 1970). By the rumen's very nature of being a nutritional modifier these proteinaceous supplements are quickly degraded by the microbial community and incorporated into microbial protein, altering the amino acid profile as compared to the supplement.

### **2.3 Rumen Proteolysis**

The majority of the proteolytic activity in the rumen fluid is directly associated with the microbial fraction (Kopency and Wallace, 1982; Wallace *et al.*, 1997). There are 4 predominant bacterial species involved in proteolytic hydrolysis including *Ruminobacter amylophilus*, *Prevotella ruminicola*, *Streptococcus bovis*, and *Butyrivibrio fibrisolvens*, and while many other species have proteolytic phenotypes their activities are comparatively minimal (Wallace and Brammall, 1985). Like bacteria, the rumen protozoa are well known for their proteolytic function by engulfing and digesting rumen bacteria and particulate matter. This predatory activity is of enormous significance to bacterial protein turnover in the rumen. In 1987, Wallace and McPherson studied bacterial protein turnover in the presence and absence of ruminal protozoa. Their findings revealed that bacterial protein turnover increased from 0.3-2.7% per hour when incubated without protozoa, to 2.7-3.7% per hour in the presence of ruminal protozoa (Wallace and McPherson, 1987; Wallace *et al.*, 1997). This clearly demonstrates the role of protozoa as bacterial predators rather than as scavengers of free ruminal protein. To this end the high lysozyme and chitinase activity of protozoa makes them very effective at degrading bacterial and fungal biomass (Morgavi *et al.*, 1994; Wallace *et al.*, 1997)

### **2.4 Factors Affecting Protein Hydrolysis**

Several factors affect the susceptibility of protein to microbial degradation including increased solubility, structural conformation (linear vs. globular structure), lack of glycosylation (Fontes *et al.*, 1995), the absence of disulphide bonds (Mahadevan *et al.*, 1980; Nugent *et al.*, 1983; Wallace and Kopency, 1983; Wallace *et al.*, 1997), and physical proximity to proteases as defined by free protein versus protein that are encased within structural carbohydrates (Wallace *et al.*, 1997). Additionally *in vitro* proteolytic experiments conducted in 1995 by Fontes *et al.* discovered that cellulases and xylanases

were resistant to proteolytic attack in the presence of their appropriate substrate. They postulated that the binding of a substrate to the enzyme's active site induces steric hindrances to the attachment of proteases, thereby protecting the enzyme from proteolytic cleavage.

Several methods have been developed to combat proteolysis of high value exogenous protein supplements and to preserve the protein value of whole feedstuffs. These treatments include chemicals, heat, and other feed processing techniques. Treatment with formaldehyde forms reversible cross-linkages with amino and amide groups which subsequently decreases the peptides' solubility at ruminal pH (Clark, 1975). Whereas treatment with tannins form hydrogen bonds between hydroxyl groups of the tannin and the peptides carboxyl groups (Clark, 1975). Similar chemical treatments include phosphonitrilic halides, halo-triazines, sulfonyl halides, acrolein acetals, hexamethylenetetramine and acetylenic esters (Chalupa, 1975). These treatments decrease feedstuff solubility, which in turn increases resistance to microbial proteases and deaminases, thereby allowing for an increase in dietary protein escape (Clark, 1975). However, not all feed processing treatments are equally effective in creating escape protein, as each technique differs in its rate of ruminal bypass and more importantly in its rate of post-ruminal absorption and utilization. Formaldehyde for example, if over applied drastically reduces post-ruminal absorption due to protein damage (Clark, 1975). The ideal bypass protein would be very resistant to proteolytic degradation without this characteristic affecting its post-ruminal absorption or ability to be utilized by the body, however as of yet no bypass protein is ideal in all respects as all are limited in these characteristics to some degree.

## 2.5 Amino Acid Analogs

Many researchers have employed artificial techniques to protect synthetic proteins and amino acids in order to create a highly effective bypass protein while retaining its biological value. Several amino acids analogs have been developed in the attempt to alter proteolytic susceptibility. One such synthetic is  $\alpha$ -hydroxy- $\gamma$ -methymercaptoputyric acid (MHA). This methionine analog is functionally absorbed and biologically active (Reis, 1970; Belasco, 1972; Papas *et al.*, 1974; Clark, 1975). However, even though this analog is less soluble than methionine, it is not unable to escape ruminal degradation (Emery, 1971; Salsbury *et al.*, 1971; Gil *et al.*, 1973; Papas *et al.*, 1974; Clark, 1975). A series of additional analogs including: DL-methionine, DL-methionine amide HCl, DL-methionine sulfone, DL-methionine methyl ester HCl, N-benzoyl-DL-methionine amide, and N-lauryl-DL-methionine mirror the above results (Chalupa, 1975). Other analogs which were developed include: N-acetyl-DL-methionine, benzoyl-L-methionine, and N-phthalyl-DL-methionine (Chalupa, 1975). While the latter analogs are successful in bypassing the rumen, for one reason or another, they were either not absorbed or not biologically active within the body (Digenis *et al.*, 1974a; Digenis *et al.*, 1974b; Chalupa, 1975). As of yet, a cost effective amino acid analog that is resistant to ruminal hydrolysis and deamination and that is biologically available has not been developed. Recent work by Volden *et al.*, (1997) has shown that significant amounts of unprotected amino acids can escape the rumen given a high dilution rate (2.28 - 3.12 d<sup>-1</sup>).

## 2.6 Chemical Encapsulation

Several attempts have also been made to artificially coat high value proteins and amino acids with pH sensitive polymers that essentially "pellet" the protein. These polymers are solid at pH ranges typically encountered within the rumen (pH 6-8), consequently at the low pHs encountered in the abomasum (pH 1-3) these polymers

dissociate from the supplement and in doing so release the protein or amino acids for absorption in the small intestine. These coatings include polymers of amino acrylates, methacrylates, cellulose propionate-3-morpholino butyrate, and imidamine polymers (Chalupa, 1975). One of the earliest polymer compounds studied is a kaolin and tristearin mixture developed by Sibbald *et al.* (1968). This polymer consists of a 20% DL-methionine, 20% kaolin, and 60% tristearin pellet of 300 to 1000 microns in diameter. This polymer was a promising development with a ruminal degradation rate of only 30% (70% escape), unfortunately only 60-65% of this tristearin/kaolin encapsulated methionine was absorbed by the small intestine (Neudoerffer *et al.*, 1971; Clark, 1975). Therefore the Effective Rate of Bypass (ERB) for tristearin/kaolin is calculated as 42-45.5% of the application rate (ruminal escape • post-ruminal utilization). Indeed *in vivo* studies with chemically encapsulated products were conducted with varied results with all experimental authors postulating that these results were to be attributed to the low ERB (Broderick *et al.*, 1970; Williams *et al.*, 1970; Mowat and Deelstra, 1972; Chalupa, 1975; Clark 1975). This product, together with many of the other polymer coatings, is currently considered cost prohibitive due to the low ERB when considered in relation to the high cost of the encapsulation materials.

One chemical encapsulation concept however deserves particular mention, that being the encapsulation of amino acids and high value proteins in blood and the subsequent treatment with formaldehyde to create a bypass coating. This concept was proposed by Orskov *et al.* (1980), however there is no information as to whether it was ever developed or tested. Given the recent bovine spongiform encephalopathy outbreak in Britain, followed by the widespread banning of rendered ruminant tissues, it is currently unlikely that such a concept will ever be developed in North America.

## **2.7 Five Characteristics of Successful Bypass Supplements**

This review of bypass proteins reveals that there are five characteristics which a cost effective bypass supplement must possess: a) a cheap source of synthetic protein; b) either i) a high concentration of protein within the polymer pellet or ii) an inexpensive polymer coating such that more pellet can be fed to provide the same amount of protein; c) the bypass technique must not negatively alter rumen fermentation or hinder post-ruminal protein absorption; d) a low rate of rumen degradation; and e) a high rate of intestinal absorption.

Unfortunately, protection of protein by chemical encapsulation and bypass methods developed to date have had only moderate success in enhancing ruminant production (Clark, 1975; Chalupa, 1975). This is due to their inability to meet all five bypass objectives, resulting in sub optimal ERBs, requiring more product to obtain optimal production and thereby increasing costs. In recent years there has been considerable interest in using biologically active cells to produce and protect intracellular proteins from ruminal proteolysis (Smith and Hespell, 1983; Teather, 1985). The protein would be produced by the cell and stored in the cytosol. The cell wall and membrane would then protect the protein from ruminal proteolysis. This is otherwise known as cellular encapsulation. Recombinant plants, ruminal and non-ruminal microorganisms are all possible systems in which to express and encapsulate protein for use as a ruminal escape vehicle (REV). There are however several requirements that all exogenous REVs must fulfill. First, both the biomass and the recombinant protein must be inexpensive to produce. This requires inexpensive media, but more importantly inexpensive inducers. Inducers, typically, are the greatest expense in recombinant protein production (Faber *et al.*, 1995). Secondly, they must be resistant to both lysis and predation in the rumen. Third, they must lyse within the abomasum or duodenum to release the recombinant protein contained within the cytosol. Fourth, the microorganism must be capable of correctly completing the post-translational modifications including peptide folding and

glycosylation in order to create a biologically functional protein. And finally, the genetically modified organism should pose a low environmental risk.

### **2.8 Genetic Modification of Rumen Bacteria**

Since the early 1980's researchers have envisioned the modification of indigenous rumen bacteria to produce and store limiting amino acids and high value proteins (Smith and Hespell, 1983; Teather, 1985). These organisms could then be reintroduced to the rumen environment, either as a transient feed additive or as a colonizing microbe. This native ruminal bacterium would resist extensive lysis and proteolytic degradation and deliver recombinant protein to the small intestine.

### **2.9 *Butyrivibrio fibrisolvens* as a Rumen Colonizing Probiotic**

In 1991, Gilbert and Hazlewood proposed a recombinant lactobacilli as a probiotic that could colonize the intestinal tract, producing digestive enzymes such as cellulases, hemicellulases, xylanases and endoglucanases. However it was not until 1995 that Beard *et al.* published information on the first functioning recombinant expression system in a rumen bacteria. They used a shuttle vector (pBHerm) that was capable of replication and expression in both *Escherichia coli* and *B. fibrisolvens* hosts. Using this vector system Gregg and coworkers modified a strain of *B. fibrisolvens* to produce the enzyme fluoroacetate dehalogenase, previously cloned from *Moraxella sp.* (Gregg *et al.*, 1994; Gregg, 1995). Many native trees and shrubs are found in Australia, Africa and South America which are poisonous because of their ability to synthesize and store toxins within their leaves, stems, and seeds. While some native animals are able to consume this plant material, domestic livestock are frequently killed by ingesting plant toxins, such as fluoroacetate. Not only has this recombinant *B. fibrisolvens* been shown to detoxify fluoroacetate *in vitro* it was also able to colonize and persist in the sheep rumen at  $10^3$ - $10^7$  cells per mL for at least 5 months (Gregg, 1995) and successfully

prevent fluoroacetate poisoning *in vivo* (Gregg *et al.*, 1998). While a modified rumen bacterium capable of co-culturing within the rumen would be the ideal bypass vehicle, many concerns still revolve around the use of a recombinant microbe indigenous to the target system due to the potential to colonize other ruminant hosts such deer, elk, and hind-gut fermenters.

## **2.10 Yeast as a Cellular Encapsulation Technology**

For an endogenous microbe system to work, it must colonize and successfully compete with the native rumen microflora. The establishment, maintenance, and continuous supplementation of an anaerobic population is logistically difficult in a laboratory setting and likely infeasible for commercial cattle production. It is for this reason that we chose to develop an exogenous or non-native aerobic yeast escape system that can be easily delivered as a feed additive.

### **2.10.1 Prevalence of Yeast in Ruminants**

Yeasts have long been used in animal diets, both as palatability amendments and as nutritional supplements (Rose and Harrison, 1987c). In recent years there has been significant emphasis on the production of recombinant proteins in yeasts and given this history they have significant potential for use as recombinant REVs. Yeasts are unicellular fungi which reproduce by budding or fission and includes the divisions ascomycetes and basidiomycetes, which are naturally present in a wide range of environments.

Yeasts have been reported in the rumen contents of several species by Klein and Müller (1941 - as cited by Lund, 1974), Ingram and McGaughey (1948), Rolle and Kolb (1955), Parle (1957), and van Uden *et al.* (1957). The first survey of ruminal yeast flora was conducted by Clarke and di Menna (1961) who reported the yeast population in the rumen of New Zealand cows to be relatively low (80 to  $13 \times 10^3$  CFU/g), of which the



dominant yeast species were not associated with feed particles. Ciarke and di Menna (1961) concluded that the yeast species isolated from the rumen were likely indigenous to the rumen since they were not found in the fodder. This was subsequently disproved by Mehnert (1965) and Gedek (1968) who demonstrated that yeasts are unable to reproduce in the forestomachs of ruminants. Their findings are in agreement with Rolle and Kolb (1955) who presumed that yeasts were introduced into the rumen with the fodder and as such they could be considered as occasional transients in the rumen. It is now known that even facultative anaerobes such as *Saccharomyces cerevisiae* require a series of oxygen dependent sterols (Rose and Harrison, 1987b), thus yeast reproduction is not possible unless these nutrients are provided as byproducts of ruminal digestion. When we consider the prevalence of yeast on the surfaces of feed and fodder, it is surprising that so few species are isolated from the rumen and those populations which are present are found in so few numbers. This is likely a reflection of the highly competitive ruminal microbial population which so strongly resists establishment of foreign organisms.

### 2.11 *Pichia pastoris*

*Pichia pastoris* is a commonly used commercial yeast expression system which was developed by the Salk Institute Biotechnology/Industrial Associates, Inc. (La Jolla, CA) and marketed by Invitrogen Corporation (Carlsbad, CA). *P. pastoris* is an attractive system with potential to serve as a REV due to a system for the stable integration of foreign genes into the chromosome, its high level inducible intracellular expression (up to 10 g L<sup>-1</sup>; Sreekrishna *et al.*, 1989), and high biomass production (450 g L<sup>-1</sup> wet weight; Wegner, 1990). *P. pastoris* is commonly found in tree fluxes and exudates, as was the case when it was first described by Guillermond in 1919. The work by Phaff *et al.* (1964 - as cited by Rose and Harrison, 1969) in California on the yeast flora of *Ulmus carpinifolia* slime flux showed a *P. pastoris* population of 1.8x10<sup>4</sup> to 7.9x10<sup>5</sup>

viable units per gram on a dry weight basis, with the highest counts in the month of March during plant bloom and seed development, but before leaves appeared. To date there are no reports of *P. pastoris* being isolated from the rumen, however *Pichia farinosa*, *Pichia fermentans*, *Pichia kudriavzevii*, and *Pichia membranefaciens* isolates have been reported by van Uden *et al.* (1958) and Lund (1974).

#### 2.11.1 Taxonomy

Yeast systematics employs several criteria in determining a species taxonomy including delineation of sexual and vegetative reproduction characteristics, in addition to physiological and biochemical characteristics (Wickerham, 1951; van der Walt, 1970; van der Walt and Yarrow, 1984; Rose and Harrison, 1987a). *P. pastoris* is a member of the *Saccharomycetoideae* family. The genus *Pichia* which is characterized by budding cells (true mycelium), hat-shaped or saturn-shaped ascospores (round or hemispherical, smooth or warty, generally liberated), and possessing co-enzymes Q7, Q8, and Q9 (Rose and Harrison, 1987a). American Type Culture Collection strain CBS 704 is now regarded as the type strain for this species. A review of *P. pastoris* taxonomic history reveals several previous nomenclatures including: *Zygosaccharomyces pastori* (Guilliermond, 1919), *Saccharomyces pastori* (Lodder and Kreger-van Rij, 1952), *Petaspora pastori* (Boidin and Adedie, 1954), *Zygowillia pastori* (Kudriavzev, 1960), and *Zymopichia pastori* (Novák and Zsolt, 1961) (as cited by Kreger-van Rij, 1984).

#### 2.11.2 Physiology

*P. pastoris* is an ascomycetous budding yeast that most commonly exists in a vegetative haploid state, however under nitrogen limitation mating occurs in this homothallic species, forming diploid vegetative cells that are twice the physical size of the haploid phenotype. Diploids cells can be maintained in their vegetative state, however they are unstable relative to their haploid counterpart as the slightest stresses

will induce meiosis, sporulation and the production of asci containing four haploid spores (Higgins and Cregg, 1998).

It is most likely that *P. pastoris*, expressed in a vegetative haploid state, will maintain haploidy throughout its residency in the ruminal environment. While diploidy cells are twice the size of those in the haploid state, these cells would most likely sporulate when exposed to the stresses of the ruminal environment (Higgins and Cregg, 1998).

### 2.11.3 Development as a Heterologous Protein Expression System

*P. pastoris* was first developed by Phillips Petroleum Company (Bartlesville, OK) in the 1960's to produce single-cell protein (SCP) from methanol via high-cell density fermentations producing 125-150 g/L dry cell weight (Wegner, 1990). In methyltrophic yeast the enzyme alcohol oxidase (*AOX*) catabolizes methanol and oxygen to produce formaldehyde and hydrogen peroxide which are subsequently metabolized by the cell in energy yielding reactions (Koutz *et al.*, 1989). Under conditions of methanol limitation and in the absence of glucose, *AOX* can constitute up to 35% of the soluble cell protein, proving *AOX* to be under the control of a strong promoter (Koutz *et al.*, 1989).

The Salk Institute of Biotechnology and Invitrogen Corporation have further developed *P. pastoris* as an intracellular or extracellular heterologous expression system utilizing the *AOX* system (Cereghino and Cregg, 2000). Extracellular expression is provided by cloning in frame with a *S. cerevisiae*  $\alpha$ -mating factor, an epitope which is cleaved during cellular export. There are two alcohol oxidase genes, *AOX1* and *AOX2*, these genes differ in the rate of methanol utilization and result in a fast methanol utilization phenotype ( $Mut^+$ ), or a slow methanol utilization phenotype ( $Mut^S$ ). Methanol utilization phenotype is determined by the type of strain used, as strain KM71's *AOX1* is disrupted and thus is inherently  $Mut^S$ , whereas strain GS115 contains both genes and is  $Mut^+$  unless the expression cassette interrupts *AOX1* expression creating a  $Mut^S$  strain. In addition to strains KM71 and GS115 there is a peptidase A

deficient strain (Pep4<sup>-</sup>) designated as SMD1168 which is efficient in expressing proteins susceptible to protease degradation. Although it should be noted that current data indicates that SMD1168 is very sensitive to lysis in the laboratory and grows much slower than the other strains (Invitrogen Corp., personal communication). A cloning vector, which is propagated in *E. coli* and utilizes the bleomycin related antibiotic zeocin for selection of recombinant *E. coli* and *P. pastoris* clones, is utilized in the process of integrating the foreign DNA into the *Pichia* chromosome. *P. pastoris* is a very dynamic and flexible expression system given the number of expression factors that can be modified to achieve optimal protein expression.

#### **2.12 Factors Affecting Ruminal Survival of a Yeast REV**

The effectiveness of *P. pastoris* as a REV depends on its ability to maintain cellular integrity in the ruminal environment. There are numerous factors in the rumen that may potentially affect yeast survival. These include protozoal predation, bacterial mediated lysis, and non-cell associated lytic factors such as viruses and bacteriocins. Additionally, there are numerous chemical conditions which can deleteriously affect yeast such as osmotic pressure, urea, nitrate, nitrite and organic acids such as acetic acid (Rose and Harrison, 1969; Rose and Harrison, 1987b), all of which are present in the rumen. Acetic acid is found as a component of volatile fatty acids (VFAs) which the ruminant derives energy from and is typically found as 50-65% of the total VFA pool (Church, 1988). Additionally, there are a series of animal and dietary factors, which affect ruminal and abomasal retention time, thereby determining the length of time the REV is exposed to lytic factors. These include the size of the diet, composition and particle size of the diet, passage rate, water consumption, and many other individual factors. Ultimately, it is the yeast's life cycle and physiology that will determine its resistance to ruminal lysis. Factors such as the composition of the cellular membrane, resistance to toxic compounds, or phases of its life cycle in which it is more or less

resistant to lytic factors may all influence persistence in the rumen.

#### 2.12.1 Effects of Nitrogen Compounds

Yeast cannot fix dinitrogen ( $N_2$ ) from the atmosphere, yet all yeast species are capable of utilizing ammonia and ammonium as a sole nitrogen source. There are however, very few species are capable of utilizing nitrates or nitrites as a sole nitrogen source, unless they are a secondary source that are present in low concentrations (Rose and Harrison, 1969; Rose and Harrison, 1987b). Higher concentrations of these compounds are typically toxic, as at a pH below 6.0 these compounds form toxic nitrous acid (Rose and Harrison, 1969; Rose and Harrison, 1987b).

This is a critical point in relation to ruminal yeast studies as high ammonia, dietary urea, and nitrates are characteristic of the rumen environment. Ammonia is produced as a consequence of amino acid dissimilation and comprises a significant portion of the free nitrogen found in the rumen. The potential negative affects of urea must also be considered due to the fact that urea can compose as much as 33% of the total dietary nitrogen in modern ruminant rations (Church, 1988). Furthermore, ruminants absorb ammonia across the rumen wall into the blood stream where the liver utilizes it to synthesize non-essential amino acids and liberates nitrogen from amino acid catabolism into the bloodstream in the form of urea (Church, 1988). In turn this plasma urea re-enters the rumen via secretion across the ruminal wall and the maxillary glands into the saliva. In this way, the animal is able to regulate the ruminal ammonia pool and provide a continuous source of nitrogen enabling continued ruminal digestion and sustained ruminal function. Although the concentration of these compounds in the rumen can vary depending on the diet, they are typically present at levels below that which causes toxicity in the ruminant animal. Thus, a maximum concentration that a yeast REV would be exposed to is equal to that causing animal toxicity, such as  $1 \text{ g L}^{-1}$  (1% w/v) for ammonia,  $75 - 90 \text{ mg kg}^{-1}$  body weight for nitrate, and  $0.27 - 0.50 \text{ g kg}^{-1}$

body weight for urea, although normally urea is rapidly converted into ammonia by ruminal ureases (Church, 1988).

We must consider if the concentration of ammonia, urea and nitrates found within the rumen have the potential to negatively impact on a yeast cell's ability to serve as a REV. An examination of *P. pastoris* nitrogen metabolism shows that this species is able to utilize ammonia, however it lacks the ability to utilize nitrates, nitrites, or urea (Barnett *et al.*, 1990). The lack of these assimilatory pathways may indicate that *P. pastoris* may be susceptible to these compounds and their effects.

#### 2.12.2 Effects of Carbon Limitations

Yeasts are heterotrophic and therefore are strictly dependent on organic carbon as a carbon and energy source. The types of carbon compounds that can be utilized are typically dependent on the yeast species, and forms the basis to characterize their typical environment. Yeast species such as *Cryptococcus*, *Rhodotorula*, and *Candidia* can utilize a wide variety of carbon compounds, and as such they are widely found in water, soil, and compost (Rose and Harrison, 1969). *Pichia* species, however, are limited in the variety of carbon sources that they are capable of assimilating, and as a result are typically abundant in simple sugar environments such as fruit juices or plant and tree exudates (Rose and Harrison, 1969). Given this limitation in carbon assimilation, it is unlikely that *P. pastoris* will be metabolically active in the rumen.

#### 2.12.3 Effects of Anaerobic Environments

Anaerobic growth of the facultative anaerobe *S. cerevisiae* can only occur in the presence of exogenous sterols (Andreasen and Stier, 1953), an unspecified long-chain unsaturated fatty acid (Andreasen and Stier, 1954), and nicotinic acid (Tyorinoja *et al.*, 1974), as these compounds can only be synthesized in the presence of molecular oxygen. This is of interest due to the fact that these exogenous sterols are directly

incorporated into the yeast cell plasma membrane. Obviously this is of critical importance in the application of yeast as a REV since cellular encapsulation technologies are dependent on the integrity of the cellular membrane.

This begs the question if an aerobic expression system is the best suited as a REV. A yeast cell will be unable to repair cellular damage which occurs in the rumen, whereas an obligate anaerobic expression system would not be limited in this manner. However, there are no yeasts which are obligate anaerobes, perhaps due to their inability to completely synthesize cell walls in an anaerobic environment. Since the application of *P. pastoris* as an REV requires the aerobic expression of recombinant protein, it is unlikely that the plasma membrane will be adversely affected prior to its introduction to the rumen. Assuming that in the rumen there are insufficient carbon sources readily available for *P. pastoris* to be metabolically active, it can be assumed that the three compounds necessary for membrane synthesis are not required as the cells will not be proliferating.

It is, however, interesting to note that in adverse conditions certain yeast species can produce and secrete an extracellular polysaccharide-protein complex which may act as a protein coat (Rose and Harrison, 1969). It should be noted that this polysaccharide-protein complex (if produced) may protect the cell from the rumen's non-biological factors. However, it may not protect the yeast cell from the microbes that may utilize this coating as an energy or nitrogen source, potentially resulting in cellular lysis. It is not known whether *Pichia* species can produce this extracellular coating.

## **2.13 Evaluation of Ruminal Survival**

### **2.13.1 Plate Counts as a Means of Monitoring *P. pastoris* Populations**

Complete evaluation of the adverse effect of ruminal factors can not be achieved without a method of monitoring *P. pastoris* populations over time, such as the traditional technique of plate count assays. The *P. pastoris* expression system is based on zeocin

selection of recombinant clones and thus zeocin can be used as the primary selection agent against non-target microbes. The inclusion of tetracycline and chloramphenicol in the selection media will further select against bacterial growth.

Chloramphenicol is an inhibitor of mitochondrial protein synthesis in yeast, but it does not affect cytoplasmic protein synthesis. This inhibits yeast growth on media containing non-fermentable carbon such as ethanol as the sole carbon source, however, in high glucose media cells obtain energy by substrate level ADP phosphorylation (Rose and Harrison, 1987b). Thus chloramphenicol will not inhibit yeast cultured on media such as yeast-peptone-dextrose medium (YPD) which contains glucose.

#### 2.13.2 GFP as a Means of Monitoring Cellular Integrity

Plate counts however do not enumerate cells that are dead or otherwise not capable of growth on solid media, yet possess an intact cellular membrane. Therefore in order to accurately evaluate cellular encapsulation technologies a means of monitoring cellular integrity must be developed.

The bioluminescent protein, Green Fluorescent Protein (GFP) was isolated from the Pacific NorthWest jellyfish *Aequoria victoria* by Ward and Bokman (1982). The gene was cloned and subsequently expressed in a number of heterologous systems (Chalfie *et al.*, 1994). GFP fluorescence is due to a cyclic tripeptide chromophore which emits green light when excited by ultra-violet light. The chromophore results from the autocatalytic cyclization of the polypeptide backbone between residues Ser<sup>65</sup> and Gly<sup>67</sup> followed by the oxidation of the  $\alpha$ -bond of Tyr<sup>66</sup> by molecular oxygen (Ormo *et al.*, 1996). Intracellular expression of GFP would illuminate the cell when viewed under epifluorescent microscopy. Cellular fluorescence may allow for the visualization of cellular integrity when the cells are incubated in a proteolytic environment such as rumen fluid, due to the fact that the protein-based chromophore is isolated from ruminal degradation. However, upon cellular lysis the chromophore may be exposed to



proteolytic degradation resulting in the loss of the chromophore and its associated fluorescence.

#### **2.14 Conclusion**

In conclusion, proteins that are encased within plant tissues or within microbial cells are degraded at a much slower rate than free protein in rumen fluid. This project intends to capitalize on cellular encapsulation as a means of reducing degradation through the use of an existing microbial expression system as a REV. The yeast expression system of *P. pastoris* was selected because 1) it is a highly efficient expression system with cell yields in excess of 125 g/L dry cell weight (Invitrogen Corp., personal communication), 2) it is foreign to the rumen environment and as such is likely resistant to many anti-microbial factors (e.g. viruses, bacteriocins) present within the rumen, 3) its large cell size (2  $\mu\text{m}$ ) may reduce the level of protozoan predation, and 4) the exterior surface of the yeast cell may be modified via chemical treatments such as formaldehyde to add further protection against ruminal lysis.

The objective of this project is to develop a recombinant *P. pastoris* system that can be used as a REV supplement to protect recombinant enzymes, bio-active proteins, and high-value proteins from ruminal proteolytic hydrolysis, in addition to making them available for duodenal activity and absorption.

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### **3.0 Development of *Pichia pastoris* as a Ruminally Traceable Microbe**

#### **3.1 Abstract**

In order to evaluate cellular encapsulation technologies for use in ruminant nutrition a means of monitoring cellular integrity must be developed. Selective plate count techniques are limited in their detection of cellular integrity as these methods are unable to detect dead or non-culturable cells that may still possess an intact cellular membrane. Intracellular expression of green fluorescent protein variant GFPuv (GFPuv) was investigated for its ability to act as a traceable marker for the visualization of cellular integrity in rumen fluid. In *Pichia pastoris* GFPuv was effectively expressed under aerobic conditions. Fluorescent cells were easily identified in the presence of strained rumen fluid when viewed under epifluorescent microscopy, and intact cells were readily enumerated. GFPuv enumeration via epifluorescent microscopy is an optimal method for the evaluation of aerobic cellular encapsulated ruminal escape technologies.

#### **3.2 Introduction**

Attempts to develop genetically modified organisms (GMO) for use in advanced studies in rumen metabolism and digestion requires detailed cellular and ecological evaluation of the technology's potential when introduced into the rumen. Evaluation of the effect of a modified microbe on ruminal metabolism is related to the introduced genetic material and the resulting metabolic activities of the GMO. The GMO's ecological effect must be evaluated by observation of the initial introduction, colonization, and numerous successional events that occur under various conditions (eg. nutrient load, diurnal fluctuations, animal variability, ect.) that may influence the complex symbiotic rumen microbial ecosystem.

Ecological observation is dependent on the effectiveness of monitoring methodologies. To date only two approaches have been successfully employed in rumen

ecological surveys, these being traditional selective plate count techniques and DNA based detection methods such as nucleic hybridization (Stahl *et al.*, 1988), polymerase chain reaction (PCR) and restriction fragment length polymorphism. Each of these methods are capable of enumerating a recombinant population at a given timepoint or conversely monitoring a population over time. Species do exist, however, which are nonculturable, or whose ability to be cultured can vary depending on a number of stress factors present in the sampled environment such as the availability of nutrients, temperature, and osmotic pressure (Bissenette *et al.*, 1975; Roszak and Colwell, 1987). These factors can further influence an organism's sensitivity to selection agents (e.g. antibiotics) to which it is otherwise resistant (Mossel and van Netten, 1984), and thus cannot be assayed by culture techniques (Duncan *et al.*, 1994). Antibiotic resistance markers are often introduced into recombinant strains in order to differentiate between recombinant and wildtype cells or strains, increasing the danger of spreading antibiotic resistance to other species, especially considering the vast microbial diversity present in the rumen and the potential for genetic transfer (Duggan *et al.*, 2000; Scott *et al.*, 1997; Nikolich *et al.*, 1994). DNA based techniques such as PCR can detect both culturable and nonculturable cells and do not require antibiotic markers.

Culture techniques and DNA based methods are capable of enumerating microbial populations, however both are incapable of observing the interactions of a recombinant strain with other microbes, particles, and fluid-solid phase associations, such as the rumen wall and feed particles as defined by Cheng and McAllister (1997). An observable marker may enhance our ability to survey complex natural communities, events, and effects on microbial structures.

Intracellular expression of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Chalfie *et al.* 1994) is a widely used observable marker which can be efficiently expressed in a wide range of host cells (Cubitt *et al.*, 1995). It has been used to monitor microbial populations such as *Escherichia coli* O517:H7 contamination of

beef carcasses (Delazari *et al.*, 1998), and blackleg infection in potato plants (Pesnyakevich and Lyon, 1998). Additionally GFP has been used to study the efficacy of rinderpest vaccines in ruminants (Walsh *et al.*, 2000) and to facilitate the development of a lactic acid bacterium as a vehicle for live vaccines in non-ruminants (Geoffroy *et al.*, 2000). An enhanced GFP variant (GFPuv) has been developed by Cramer *et al.* (1996) which fluoresces 18 times brighter than the wildtype GFP, when expressed in *E. coli*. GFP may be an effective intracellular expression marker for observation of ruminal lysis as the expressed protein based chromophore would be protected from ruminal proteolysis by the plasma membrane and upon cellular lysis the GFP protein would be rapidly degraded resulting in the loss of GFP associated fluorescence. In the current study we evaluated the potential of GFP as a traceable marker for ecological surveillance and evaluation of a recombinant *Pichia pastoris* expression system in the rumen.

### 3.3 Material and Methods

#### 3.3.1 Microbial Strains and Culture Media

*Escherichia coli* strain DH5 $\alpha$  [ $F^- \phi 80 \Delta lacZ \Delta dm15 \Delta (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44 \lambda^- thi-1 gyrA96 relA1$ ] (Life Technologies Corp., Rockville, MD) was used for the construction of the GFPuv expression cassette (Section 3.3.3). A low salt variant of Lauria-Bertani Medium (LSLB: 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.0) containing the appropriate antibiotic (100  $\mu$ g/mL ampicillin or 100  $\mu$ g/mL zeocin) was used for cultivation of *E. coli* strains carrying plasmids. *E. coli* cultures were grown at 37°C while shaking at 300 rpm.

*P. pastoris* strains GS115 [ $Mut^+, Arg^-, His^-, Ble^-$ ] and KM71 [ $Mut^S, His^-, Arg^+, Ble^-$ ] (Invitrogen Corp., Carlsbad, CA) were transformed with the GFPuv expression cassette. Yeast extract peptone dextrose medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose) was used for propagation of the wildtype yeast strains. After chromosomal

integration of the GFPuv expression cassette, zeocin (100 µg/mL) was added to the medium. *P. pastoris* cultures were grown, as per supplier's instructions (Invitrogen Corp.), in an incubator at 30°C while shaking at 300 rpm.

Recombinant *P. pastoris* GFPuv expression cultures were grown in buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate, 4x10<sup>-5</sup>% biotin, 1% glycerol). After 24 h, cultures were transferred to induction media by centrifuging at 3000 x g for 5 min, decanting the supernatant, and resuspending the cell pellet in buffered methanol-complex medium (BMMY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate, 4x10<sup>-5</sup>% biotin, 0.5% methanol). The culture was incubated at 30°C for a further 16 d, adding absolute methanol to a final concentration of 0.5% every 24 h.

### 3.3.2 General Molecular Biology Techniques

Plasmid DNA isolation from *E. coli*, restriction endonuclease digestion, ligation and *E. coli* transformation were performed according to the procedures described by Sambrook *et al.*, (1989). DNA amplification by PCR was performed using Taq DNA polymerase (Sigma-Aldrich Canada Ltd., Oakville, ON). Sequencing was performed by the University of Calgary Core DNA Sequencing Facility using a DyeDeoxy terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) according to the supplier's instructions.

### 3.3.3 Construction of GFPuv Expression Cassette

Green Fluorescent Protein (GFPuv) was amplified by PCR from pGFPuv (Clontech, Palo Alto, CA) using a sense primer (5' - GGGTACCATG AGTAAAGGAG AAGAACTTTT C - 3') corresponding to the 5' end of the GFPuv open reading frame

(ORF) and an antisense primer (5'- GGCGGCCGCT TTGTAGAGCT CATCCATGCC - 3') corresponding to the 3' end of the GFPuv ORF, designed to be inframe with the 3' *myc* and *6xhis* epitope tags of the pPICZB yeast expression vector (Invitrogen Corp.). The sequences underlined correspond to *KpnI* (sense primer) and *NotI* (antisense primer) restriction sites. The PCR-amplified GFPuv fragment was initially cloned into pGEM-T-Easy (Promega, Madison, WI) and then transferred as a 0.8-kb *KpnI-NotI* fragment from the pGEM-T-Easy vector to the *KpnI-NotI* digested pPICZB to form the GFPuv expression cassette (pPICZB::GFPuv::mycHIS) (Appendix 7.1A).

#### 3.3.4 Yeast Transformation and GFPuv Expression Cassette Integration

The pPICZB::GFPuv::mycHIS (10  $\mu$ g) expression vector was linearized with *BstXI* to facilitate chromosomal integration (Appendix 7.1B). The linearized GFPuv expression cassette was electroporated into *P. pastoris* strains GS115 and KM71 as per the suppliers instructions (Invitrogen Corp.) using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) with a pulse length of 5 msec and a field strength of 7500 V cm<sup>-1</sup>. Zeocin resistant transformants were selected on YPD agar containing 100  $\mu$ g/mL zeocin and 1M sorbitol.

#### 3.3.5 GFP Expression and Screening

Zeocin resistant *P. pastoris* clones were expressed by growing first in BMGY at 30°C, 300 rpm for 24 h. Cells were then transferred to BMMY induction medium by centrifuging at 3000 x g for 5 min, decanting the supernatant, and resuspending the cell pellet in 250 mL BMMY. The culture was incubated for an additional 7 d; adding absolute methanol to a final concentration of 0.5% every 24 h. On a daily basis, 1 mL aliquotes were taken and centrifuged at 12,000 x g for 2 min, immediately frozen in liquid nitrogen and stored at -80°C until Western blot analysis could be completed.

### 3.3.6 GFP Detection

All samples were analyzed under a hand held long-wave (365nm) UV light source (Schleicher & Schuell Inc., Keene, NH) to detect GFP fluorescence indicative of positive expression clones. Putative positive clones were then viewed under epifluorescent microscopy using an Axiovert 135 (Carl-Zeiss, Oberkochen, DE) with a mercury lamp and a Zeiss Fluorosceine filter cube (490 excitation, 525 emission) to further confirm GFP fluorescence.

### 3.3.7 Western Blot Analysis

Induction sample cell pellets were resuspended in *Pichia* breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine tetraacetic acid, 5% glycerol) and an equal volume of acid-washed 0.5 mm glass beads. Cells were disrupted by 7 cycles of vortexing for 30 sec followed by 30 sec on ice. Lysates were centrifuged for 10 min at 12,000 x g, after which the supernatants were transferred to fresh tubes and stored at -20°C. Centrifuged lysates were run on a 15% polyacrylamide SDS-PAGE gel (0.375 M Tris-HCl pH 8.8, 0.1% sodium dodecyl sulfate, 15% bis-acrylamide, 0.5% ammonium persulfate, 0.05% TEMED) with a 4% stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% sodium dodecyl sulfate, 4% bis-acrylamide, 0.05% ammonium persulfate, 0.13% temed) (Laemmli, 1970). Following electrophoresis the gels were transferred to a 0.45 nm nitrocellulose membrane (Schleicher & Schuell Inc.) using an electro-blotter (Bio-Rad Laboratories). Monoclonal GFP antibody (Clontech) was used in combination with an alkaline phosphatase Immun-Blot Assay Kit (Bio-Rad Laboratories) to detect recombinant GFPuv-mycHIS protein.

### 3.3.8 Visualization of GFP Fluorescent Cells in Rumen Fluid

Fluorescent *P. pastoris* cells were introduced into rumen fluid and visualized under epifluorescent microscopy at 400x magnification using a Nikon Optiphot microscope (Nikon Canada Inc., Mississauga, ON) equipped with a 100W mercury lamp (OSRAM Canada Ltd., Mississauga, ON) and a Nikon UV-1A filter cube. Photos were taken using Kodak 800 Gold Max film (Kodak Canada Inc., Toronto, ON).

### 3.3.9 Statistical Field of View Counting Package

A spreadsheet was designed to ensure a statistically valid estimation of the true population using the epifluorescent field of view data. Sample size was calculated based on sample variability using the formula  $n = (z^2 \cdot \sigma^2) \div d^2$ , the  $\sigma$  value was derived from sample variability and the z value provided by an one-tailed probability chart (Thompson, 1992). We elected to set d as a percent of the averaged data set to standardize its effect regardless of a high or low population size. This standardized d value is calculated as:  $(d = 0.1 \cdot \text{data average})$ , which represents 95% confidence interval on an one-tailed probability chart. The completed spreadsheet was formatted to facilitate data entry on a laptop computer sitting adjacent to the epifluorescent microscope (Fig 3.1). A minimum of 10 fields of view to a maximum of 25 fields of view is typically sufficient to achieve a 95% confidence interval on populations between 100 and 5 cells per field of view.

### 3.3.10 Comparison of Plate Count and GFP Epifluorescence Microscope Enumeration Methods

Validation of the GFP assay was performed utilizing a fractionated ruminal *in vitro* technique. *P. pastoris* KM71::GFPuv::mycHIS was cultured on BMGH and induced on BMMH for 16 d. Cultures (250 mL) were harvested by centrifugation at 4°C for 5 min at 3000 x g in an IEC MP4 centrifuge (International Equipment Company, Needham

Heights, MA). The yeast pellets obtained were washed twice with 20 mL ddH<sub>2</sub>O to minimize media carry over and concentrate the culture. The final yeast pellet was resuspended in 20 mL ddH<sub>2</sub>O, enumerated under epifluorescent microscopy, and diluted to a final concentration of  $1.2 \times 10^9$  cells/mL.

A representative sample containing equal volumes of rumen fluid and solids was collected from a fistulated Holstein cow 2 h after the morning feeding, prepared by blending for three 30 sec pulses in a Waring blender and strained through four layers of cheesecloth. All bottles were flushed with reduced carbon dioxide to maintain anaerobicity. Strained rumen fluid was centrifuged twice at 500 x g for 10 min, retaining the supernatant as protozoa-free rumen fluid. Protozoa-free rumen fluid (2 mL) was anaerobically injected into presterilized serum bottles containing 18 mL modified Scott and Dehority medium (Scott and Dehority, 1965) supplemented with 5% (v/v) clarified rumen fluid, 0.2% cellobiose and 0.2% soluble starch. Finally, 100  $\mu$ L of the previously prepared yeast inoculum was injected, achieving a final concentration of  $6.0 \times 10^6$  cells/mL. The bottles were placed on a shaking platform at 150 rpm and incubated in a forced air cabinet at 39°C. At each time point the bottles were opened and placed in a 67 kHz ultrasonic cleaner (Mettler Electronics Corp., Anaheim, CA) for 15 min. Following sonication, samples from each bottle were plated on YPD agar containing 100  $\mu$ g/mL zeocin, 12  $\mu$ g/mL tetracycline, 20  $\mu$ g/mL chloramphenicol, and 1M sorbitol. Samples were simultaneously analyzed under epifluorescent microscopy using a Nikon Optiphot microscope (Nikon Canada Inc.) equipped with a mercury lamp (OSRAM Canada Ltd.) and a Nikon V-1A filter cube. Field of view counts were performed using a 0.2 mm deep Petroff 1/5th 3900 cell counting chamber (Hausser Scientific, Horsham, PA) in combination with a Petroff reinforced coverslip to enable consistent field of view counts in addition to minimizing UV light scatter and associated photobleaching. Total cell counts were subsequently derived by relating field of view counts to the total volume under the field of view (Appendix 7.2).



### **3.4 Results**

#### **3.4.1 DNA Sequence Confirmation of GFPuv Expression Cassette**

The GFP expression vector pPICZB::GFPuv::mycHIS was sequenced as described above. Sequence analysis confirmed correct reading frame placement for expression of the GFPuv::mycHIS fusion protein (Fig 3.2). A PCR error detected at nt 1306 resulted in an arginine to serine mutation at residue 109. This mutation did not appear to affect GFP fluorescence or expression.

Fluorescent analysis of ten induced zeocin resistant pPICZB::GFPuv::mycHIS transformed *P. pastoris* KM71 clones revealed 10 positive clones, expressing GFPuv in varying degrees, with clone B1 having the highest levels of expression (Fig 3.3). Observation under ultra-violet light demonstrated that wildtype *P. pastoris* KM71 cells and ddH<sub>2</sub>O exhibits no background autofluorescence. Further examination under epifluorescent microscopy demonstrated that induced *P. pastoris* KM71 exhibited no autofluorescence, whereas KM71::GFPuv::mycHIS cells were clearly defined and illuminated (Fig 3.4). Western blot analysis demonstrated that the 27 kDa GFPuv protein was effectively expressed in both *P. pastoris* KM71 and GS115 and increased with time (Fig 3.5).

#### **3.4.2 Visualization of Fluorescent *P. pastoris* Cells in Rumen Fluid and Comparison to Plate Count Assays**

Under 365 nm excitation rumen fluid contains varying amounts of red, orange, and pinks however, no greens were visible (Fig 3.6). When fluorescent *P. pastoris* cells were present in rumen fluid, individual cells were clearly visible and differentiated from the native rumen microflora (Fig 3.7). Ruminal autofluorescence can however become a significant impediment to GFP detection, particularly plant derived feed particles (Fig 3.8). Viable plate counts of cells cultured in BMGH/BMMH indicated a 91.59% decrease in *P. pastoris* cells by 24 h and a 99.83% decrease by 48 h. In contrast

epifluorescent enumeration revealed 2.89% loss of fluorescent cells over the 48 h (p<0.01) (Fig 3.9).

### 3.5 Discussion

Green fluorescent protein variant GFPuv was effectively expressed in *P. pastoris* with individual cells being clearly visible under epifluorescent microscopy. Western blot analysis demonstrated that the 27 kDa GFPuv protein was effectively expressed and increased with time (Fig 3.5), although GFPuv appeared to dimerize in strain KM71 as represented by the presence of a 54 kDa band (Fig 3.5B). Dimerization of the wildtype GFP protein occurs under high GFP concentrations (6  $\mu$ M) and under high salt conditions (100 mM NaCl), resulting in a suppression of the 470 nm excitation peak (Cubitt *et al.*, 1995). The GFPuv variant has been shown to dimerize at protein concentrations approximately 5-fold lower than that of wildtype GFP protein and form other multimeres (Clontech, personal communication).

Extensive ruminal autofluorescence is observed in whole rumen fluid under epifluorescent microscopy at 400x magnification and 365 nm long pass excitation (Fig 3.6). Of note is the lack of any organism or particle that fluoresces green, thus enabling GFP expressing cells to be easily recognized and counted (Fig 3.7).

Comparison of selective plate counts to epifluorescent GFP counts demonstrated that GFP can be accurately used to enumerate previously undetectable, nonculturable cells. Additionally, the variability of GFP enumeration is markedly lower than that of the plate count assay (Fig 3.9). It should be noted however, that variability in GFP enumeration can occur, especially when attempting to discern individual cells within actively budding yeasts or flocculating phenotypes.

While there have been no previous reports on the use of GFP in ruminal studies, it has been shown that rumen ecological samples must be immediately preserved in formalin or methylformalin to prevent changes in the samples (Ogimoto and Imai,

1981). We have observed that *P. pastoris* samples could not be preserved with these chemicals as epifluorescent counts were significantly altered, thus ruminal samples had to be counted immediately. This required simultaneous statistical analysis to ensure that a sufficient population size is enumerated. Realtime data entry into a simple spreadsheet which calculated sample variance and determined population size to a given confidence interval ensured that statistically accurate data was obtained. Additionally, the black lines of a hemacytometer are not visible under epifluorescent microscopy, thus whole fields of view must be counted as a measurement unit. Total cell counts can subsequently be derived by relating field of view counts to the total volume under the field of view.

The cell membrane protects the intracellular GFP protein from ruminal proteolysis, enabling cellular fluorescence, however upon the loss of membrane integrity, such as in cellular lysis, the chromophore is degraded and fluorescence is lost. DNA based methods would show similar results as the cell and nuclear membranes isolate the nucleic acids from ruminal nucleases and upon lysis the DNA is rapidly degraded. In support of this concept Duggan *et al.*, (2000) has observed the half-life of unprotected DNA to be less than 30 sec in clarified rumen fluid.

GFP is an effective ecological marker, however its functionality is greatly limited within the rumen environment due to the requirement of molecular oxygen in forming the cyclical fluorescent chromophore (Davis *et al.*, 1994). Thus GFP is highly effective for monitoring cells which are aerobically cultured and introduced into the rumen environment. Any GFP protein produced while residing in the rumen will not form a fluorescing chromophore, due to the lack of molecular oxygen, thus the total cellular fluorescence will diminish upon each successive cellular division due to cytoplasmic sharing between daughter cells.

With these qualities in mind we hypothesize that GFP can be used to evaluate the effectiveness of exogenous cellular encapsulation technologies to act as a REV.

Fluorescing cells which flow from the rumen are indicative of undegraded intracellular protein and successful rumen escape. Quantification of total fluorescence relative to total fluorescent cells escaping the rumen would indicate the extent of cellular division occurring in the rumen due to the decreased fluorescence when cell division occurs in an anaerobic environment. However, it should be noted that the overall usefulness of GFP in rumen studies will be greatly enhanced if we optimize epifluorescent microscopy filters to reduce ruminal autofluorescence, in addition to the selection of the optimal GFP variant for enhanced ruminal detection.

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<b>C/R</b>	<b>A</b>	<b>B</b>	<b>C</b>
1	<b>Cell Count</b>	<b>Cell Count</b>	<b>Minimal Sample Size</b>
2	1	FOV data	$=((SC31^2) \cdot VAR(B\$1..B3)) + (((AVERAGE(B\$1..B3)) \cdot \$A\$31)^2)$
3	2	FOV data	$=((SC32^2) \cdot VAR(B\$2..B3)) + (((AVERAGE(B\$2..B3)) \cdot \$A\$31)^2)$
4	3	FOV data	$=((SC33^2) \cdot VAR(B\$2..B4)) + (((AVERAGE(B\$2..B4)) \cdot \$A\$31)^2)$
5	4	FOV data	$=((SC34^2) \cdot VAR(B\$2..B5)) + (((AVERAGE(B\$2..B5)) \cdot \$A\$31)^2)$
6	5	FOV data	$=((SC35^2) \cdot VAR(B\$2..B6)) + (((AVERAGE(B\$2..B6)) \cdot \$A\$31)^2)$
7	6	FOV data	$=((SC36^2) \cdot VAR(B\$2..B7)) + (((AVERAGE(B\$2..B7)) \cdot \$A\$31)^2)$
8	7	FOV data	$=((SC37^2) \cdot VAR(B\$2..B8)) + (((AVERAGE(B\$2..B8)) \cdot \$A\$31)^2)$
9	8	FOV data	$=((SC38^2) \cdot VAR(B\$2..B9)) + (((AVERAGE(B\$2..B9)) \cdot \$A\$31)^2)$
10	9	FOV data	$=((SC39^2) \cdot VAR(B\$2..B10)) + (((AVERAGE(B\$2..B10)) \cdot \$A\$31)^2)$
11	10	FOV data	$=((SC40^2) \cdot VAR(B\$2..B11)) + (((AVERAGE(B\$2..B11)) \cdot \$A\$31)^2)$
12	11	FOV data	$=((SC41^2) \cdot VAR(B\$2..B12)) + (((AVERAGE(B\$2..B12)) \cdot \$A\$31)^2)$
13	12	FOV data	$=((SC42^2) \cdot VAR(B\$2..B13)) + (((AVERAGE(B\$2..B13)) \cdot \$A\$31)^2)$
14	13	FOV data	$=((SC43^2) \cdot VAR(B\$2..B14)) + (((AVERAGE(B\$2..B14)) \cdot \$A\$31)^2)$
15	14	FOV data	$=((SC44^2) \cdot VAR(B\$2..B15)) + (((AVERAGE(B\$2..B15)) \cdot \$A\$31)^2)$
16	15	FOV data	$=((SC45^2) \cdot VAR(B\$2..B16)) + (((AVERAGE(B\$2..B16)) \cdot \$A\$31)^2)$
17	16	FOV data	$=((SC46^2) \cdot VAR(B\$2..B17)) + (((AVERAGE(B\$2..B17)) \cdot \$A\$31)^2)$
18	17	FOV data	$=((SC47^2) \cdot VAR(B\$2..B18)) + (((AVERAGE(B\$2..B18)) \cdot \$A\$31)^2)$
19	18	FOV data	$=((SC48^2) \cdot VAR(B\$2..B19)) + (((AVERAGE(B\$2..B19)) \cdot \$A\$31)^2)$
20	19	FOV data	$=((SC49^2) \cdot VAR(B\$2..B20)) + (((AVERAGE(B\$2..B20)) \cdot \$A\$31)^2)$
21	20	FOV data	$=((SC50^2) \cdot VAR(B\$2..B21)) + (((AVERAGE(B\$2..B21)) \cdot \$A\$31)^2)$
22	21	FOV data	$=((SC51^2) \cdot VAR(B\$2..B22)) + (((AVERAGE(B\$2..B22)) \cdot \$A\$31)^2)$
23	22	FOV data	$=((SC52^2) \cdot VAR(B\$2..B23)) + (((AVERAGE(B\$2..B23)) \cdot \$A\$31)^2)$
24	23	FOV data	$=((SC53^2) \cdot VAR(B\$2..B24)) + (((AVERAGE(B\$2..B24)) \cdot \$A\$31)^2)$
25	24	FOV data	$=((SC54^2) \cdot VAR(B\$2..B25)) + (((AVERAGE(B\$2..B25)) \cdot \$A\$31)^2)$
26	25	FOV data	$=((SC55^2) \cdot VAR(B\$2..B26)) + (((AVERAGE(B\$2..B26)) \cdot \$A\$31)^2)$
27	<b>Average</b>		$= (SUM(B2..B26)) / (COUNT(B2..B26))$
28			
29			
30	<b>D<sup>2</sup> Value</b>	<b>df</b>	<b>95% t value</b>
31	0.1	1	6.314
32		2	2.920
33		3	2.353
34		4	2.132
35		5	2.015
36		6	1.943
37		7	1.895
38		8	1.860
39		9	1.833
40		10	1.812
41		11	1.796
42		12	1.782
43		13	1.771
44		14	1.761
45		15	1.753
46		16	1.746
47		17	1.740
48		18	1.734
49		19	1.729
50		20	1.725
51		21	1.721
52		22	1.717
53		23	1.714
54		24	1.711
55		25	1.708

**Spreadsheet Function Formulae**

VAR represents the function of Variance.

AVERAGE represents the function of Average.

^ represents the function of Exponentiation.

**Figure 3.1** Spreadsheet design for the realtime calculation of statistical valid sample size to achieve a 95% confidence interval for the enumeration of rumen samples containing GFP fluorescent *P. pastoris* cells.

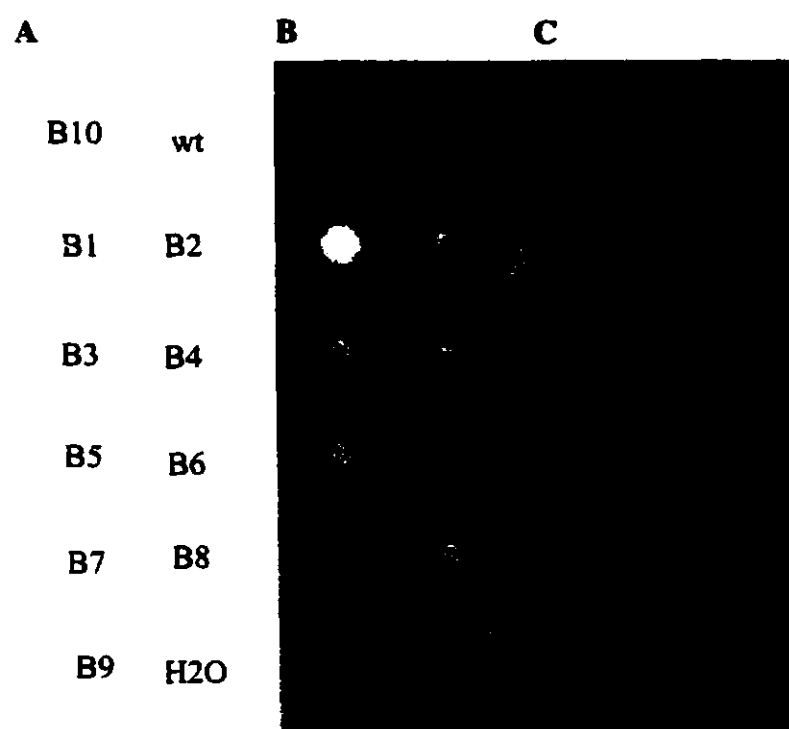
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911  AGATCAAAAA ACAACTAATT ATTCGAAACG AGGAATTCAC GTGGCCCAGC
961  CGGCCGTCTC GGATCGGTAC KpnICATGAGTAAA GGAGAAGAAC TTTTCACTGG
      M S K G E E L F T G
1011 AGTTGTCCCA ATTCTTGTTG AATTAGATGG TGATGTTAAT GGCACAAAT
      V V P I L V E L D G D V N G H K F
1061 TTTCTGTCAG TGGAGAGGGT GAAGGTGATG CAACATACGG AAAACTTACC
      S V S G E G E G D A T Y G K L T
1111 CTTAAATTTA TTTGCACTAC TGGAAAAC TA CCTGTTCCAT GGCCAACACT
      L K F I C T T G K L P V P W P T L
1161 TGTCAC TACT TTCTCTTATG GTGTTCAATG CTTTTCCCGT TATCCGGATC
      V T T F S Y G V Q C F S R Y P D H
1211 ATATGAAACG GCATGACTTT TTCAAGAGTG CCATGCCCGA AGGTTATGTA
      M K R H D F F K S A M P E G Y V
1261 CAGGAACGCA CTATATCTTT CAAAGATGAC GGGAACTACA AGACGAGTGC
      Q E R T I S F K D D G N Y K T S A
1311 TGAAGTCAAG TTTGAAGGTG ATACCCTTGT TAATCGTATC GAGTTAAAAG
      E V K F E G D T L V N R I E L K G
1361 GTATTGATTT TAAAGAAGAT GGAAACATTC TCGGACACAA ACTCGAGTAC
      I D F K E D G N I L G H K L E Y
1411 AACTATAACT CACACAATGT ATACATCACG GCAGACAAAC AAAAGAATGG
      N Y N S H N V Y I T A D K Q K N G
1461 AATCAAAGCT AACTTCAAAA TTCGCCACAA CATTGAAGAT GGATCCGTTT
      I K A N F K I R H N I E D G S V Q
1511 AACTAGCAGA CCATTATCAA CAAAATACTC CAATTGGCGA TGGCCCTGTC
      L A D H Y Q Q N T P I G D G P V
1561 CTTTTACCAG ACAACCATTA CCTGTGCGACA CAATCTGCCC TTTTCGAAAGA
      L L P D N H Y L S T Q S A L S K D
1611 TCCCAACGAA AAGCGTGACC ACATGGTCCT TCTTGAGTTT GTAAGTCTG
      P N E K R D H M V L L E F V T A A
1661 CTGGGATTAC ACATGGCATG NotIGATGAGCTCT ACAAAAGCGGC CGCCAGCTTT
      G I T H G M D E L Y K A A A S F
1711 CTAgaaacaaa aactcatctc agaagaggat ctgaatAGCG CCGTCGACca
      L E Q K L I S E E D L N S A V D H
1761 tcatcatcat catcatTGAG TTTGTAGCCT TAGACATGAG TTTGTAGCCT
      H H H H H .
1811 TAGACACTGT TCCTCAGTTC AAGTTGGGCA CTTACGAGAA GACCGGTCTT

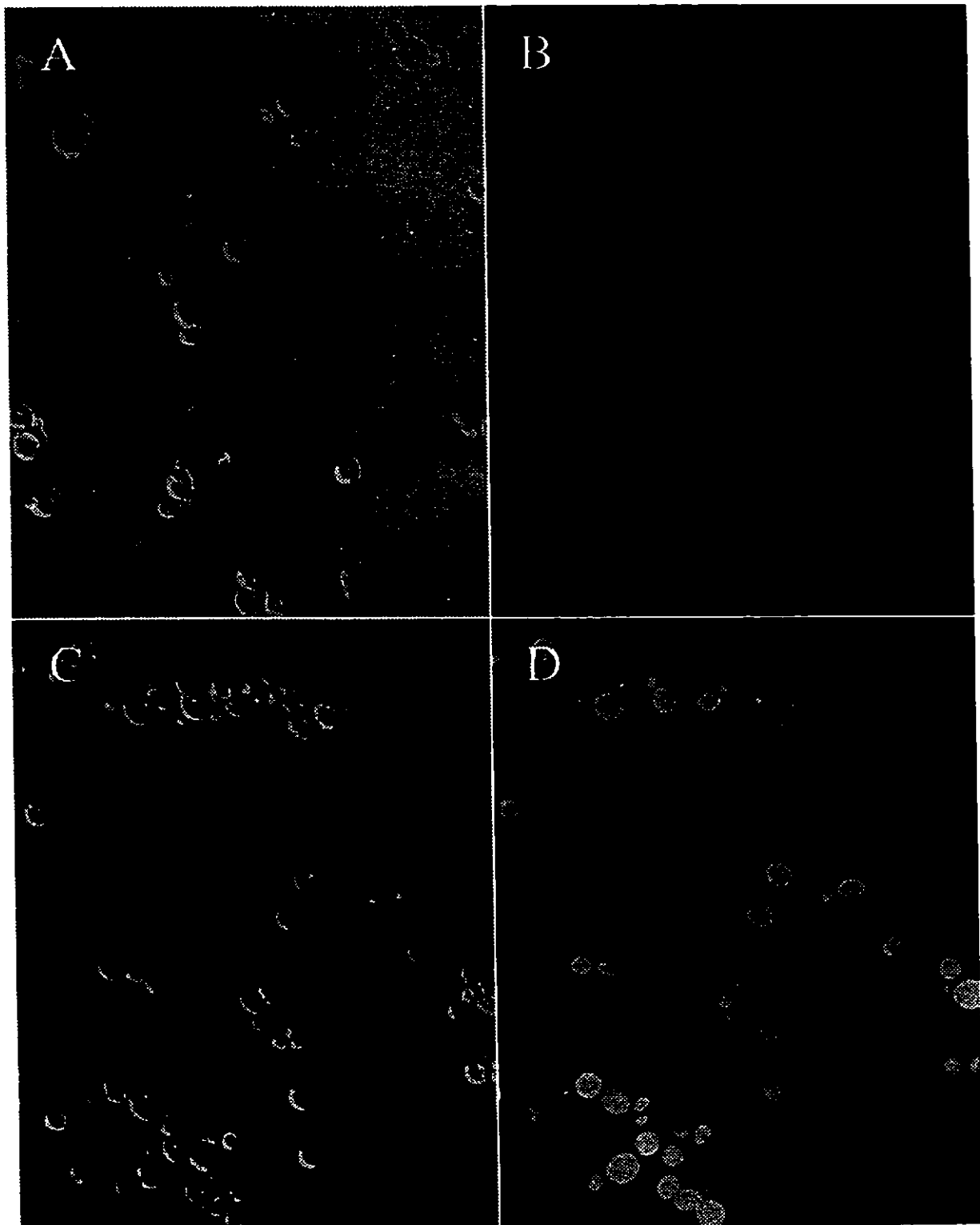
```

**Figure 3.2** Nucleotide sequence of pPICZB::GFPuv::mycHIS construct. GFP primer sequences are underlined and restriction sites are in bold. The GFPuv::mycHIS start and stop codons are indicated by italics. The PCR error at nt 1306 is indicated by stroked through lettering, and the myc and HIS epitope tags are indicated by lowercase lettering.

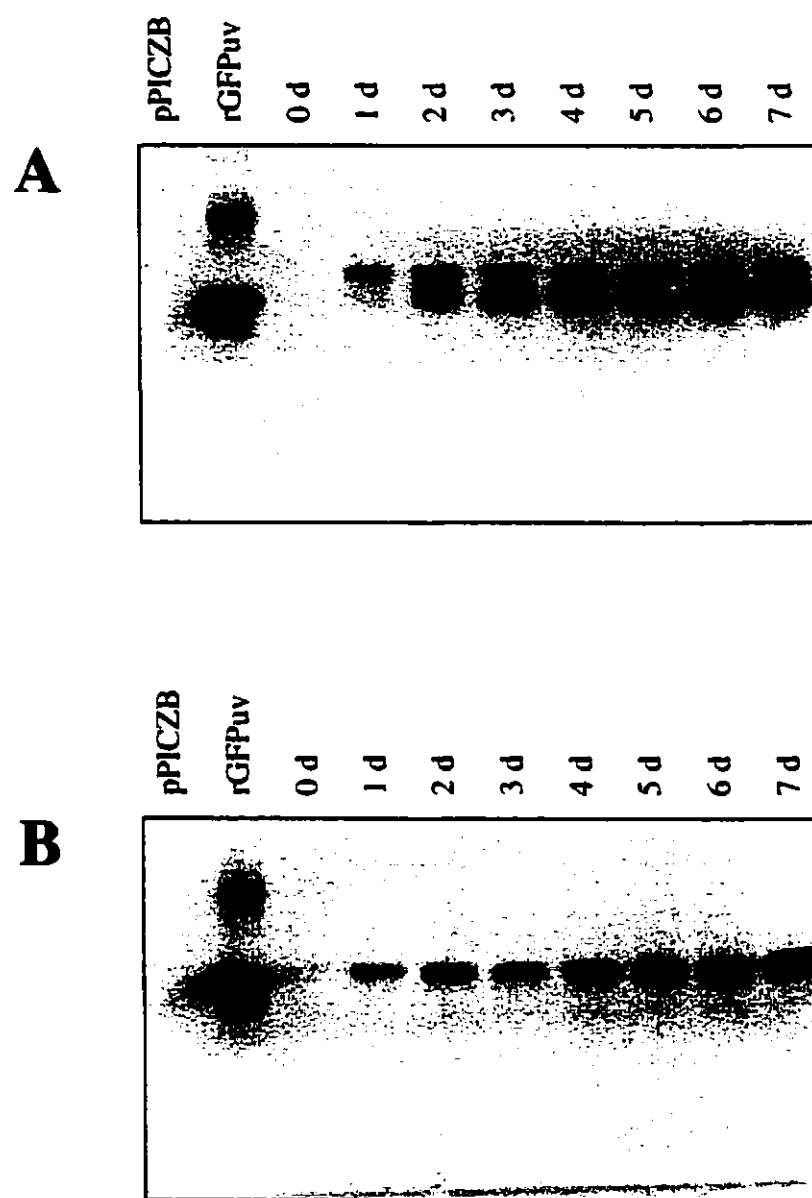




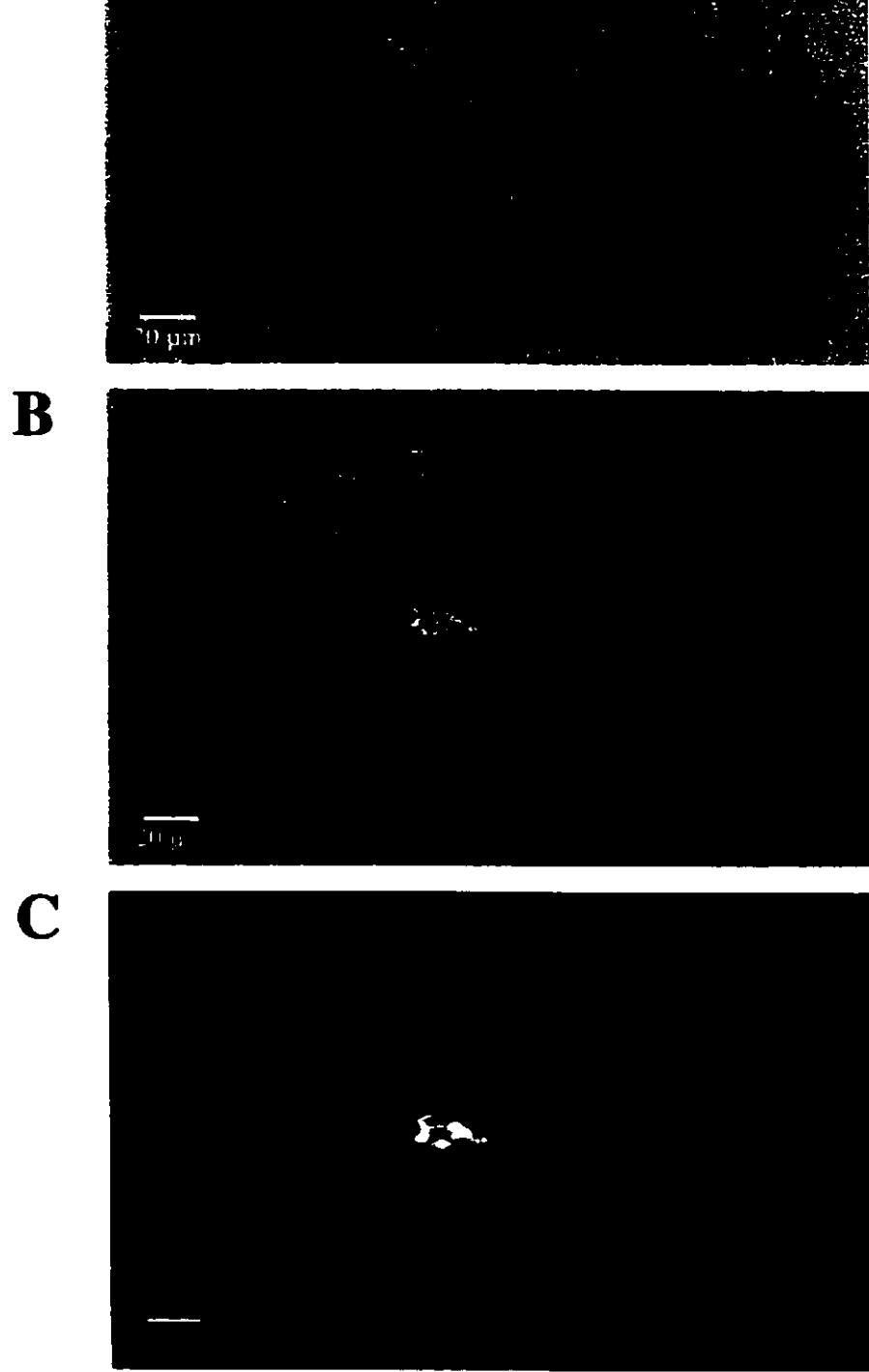
**Figure 3.3** Screening of *P. pastoris* KM71::GFPuv::mycHIS cultures induced 7 days on methanol. Aliquots of cultures (10  $\mu$ l) were placed on a glass plate and illuminated under 365 nm ultraviolet light A) clone identities, B) bright room, and C) dark room. Panels B and C have been digitally enhanced, isolating the green spectrum.



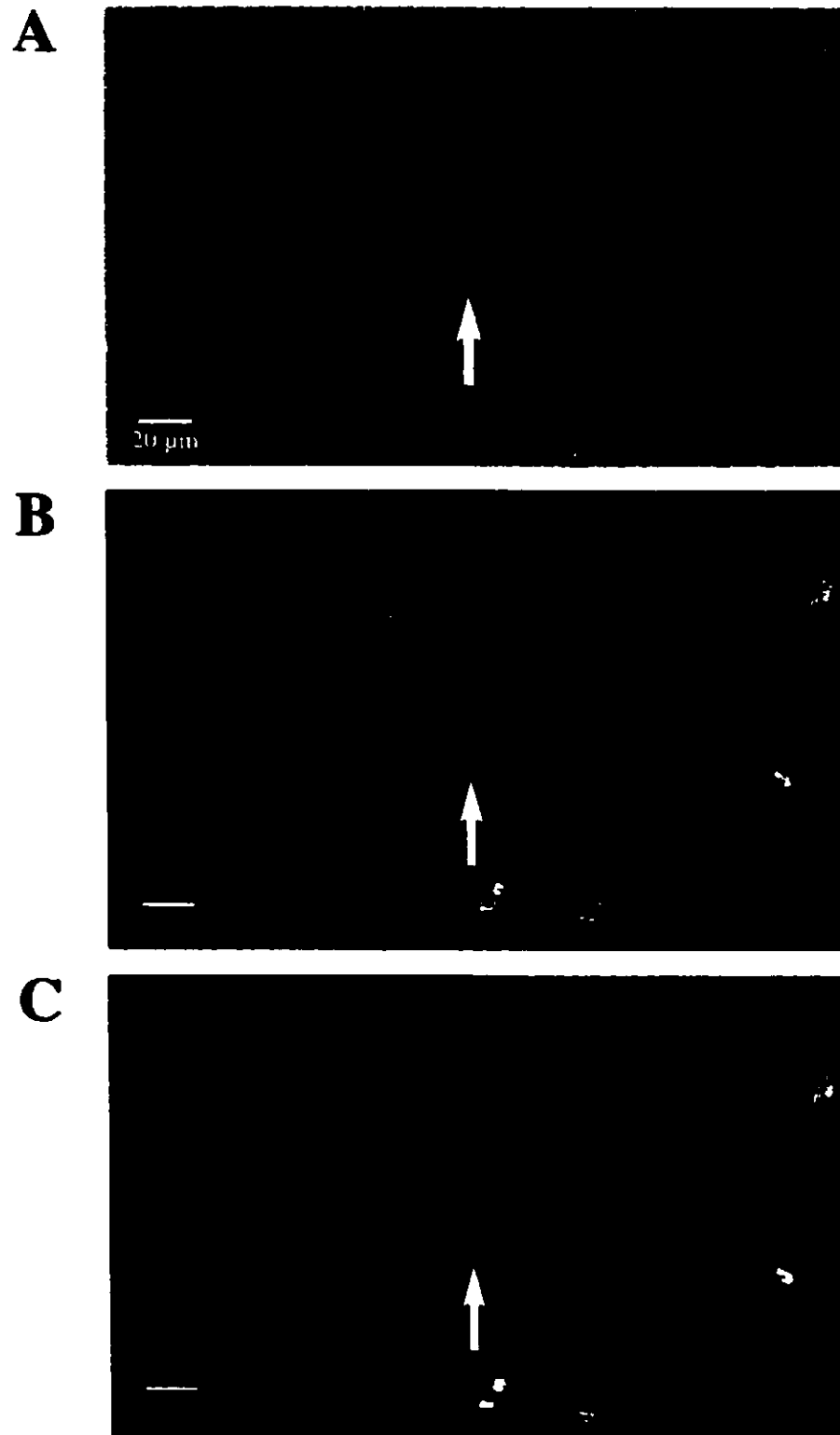
**Figure 3.4** Wildtype (A, B) and recombinant (C, D) *P. pastoris* cells observed under Nomarski (A, C) and epifluorescent (405 nm) microscopy (B, D). Magnification :1600x



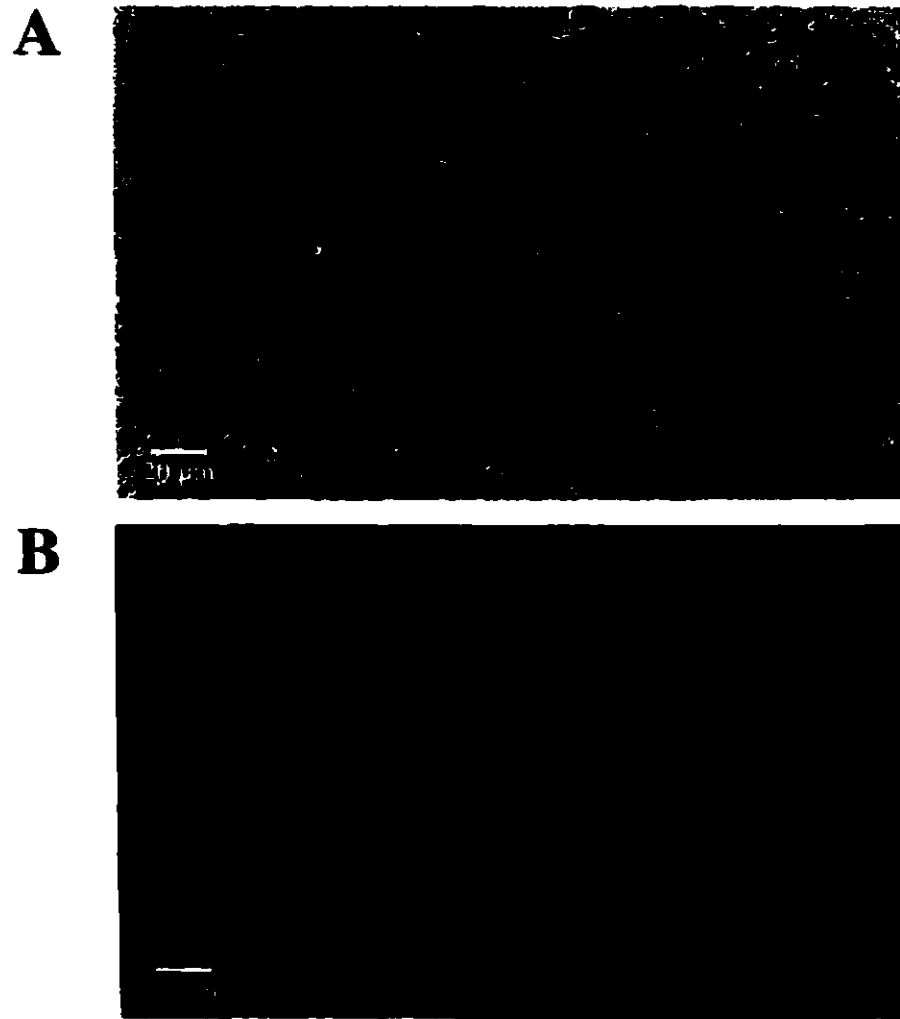
**Figure 3.5** Western blot analysis of GFPuv expression in *P. pastoris* A) KM71 and B) GS115 with methanol induction and sampled daily. Recombinant GFPuv protein (Clontech) and pPICZB transformed *P. pastoris* cells were used as positive and negative controls respectively.



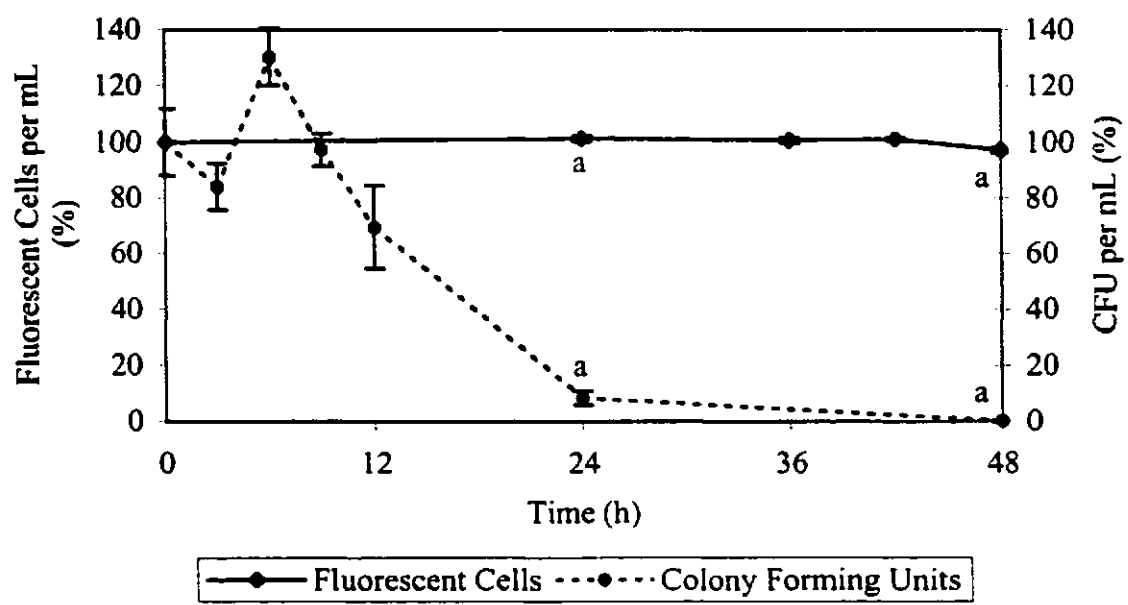
**Figure 3.6** Epifluorescent microscopy visualization of rumen fluid under A) white, B) mixed white and 365 nm, C) 365 nm light. Magnification: 364x.



**Figure 3.7** Epifluorescent microscopy of GFPuv *P. pastoris* cells in rumen fluid illuminated under A) white, B) mixed white and 365 nm, C) 365 nm light. Arrows mark the same cells viewed under the different lighting regimes. Magnification: 364x.



**Figure 3.8** Epifluorescent microscopy of rumen fluid demonstrating extensive autofluorescence interfering with GFP detection, illuminated under A) white and B) 365 nm light. Magnification: 364x.



**Figure 3.9** Comparison of GFP epifluorescent microscopy enumeration to a selective plate count assay. Counts are expressed as percent of original inoculum. The letter “a” denotes  $p < 0.0001$ . Bars represent standard error of the mean,  $n=3$ . In all cases, SE bars are present and may be hidden beneath the symbol.

## **4.0 An Investigation into the Optimal GFP Variant and Detection System for Intracellular *Pichia pastoris* Expression and Observation in Ruminal Fluid**

### **4.1 Abstract**

Visualization of strained rumen fluid under epifluorescent microscopy revealed many autofluorescent plant particles, microorganisms and fluorescent cytochromes. The colors and quantities of ruminal autofluorescence varies depending on the wavelength of the excitation light. Intracellularly expressed Green Fluorescent Protein (GFP) variant GFPuv has been effectively employed in ruminal studies, however it has been hypothesized that other GFP variants may provide enhanced visualization in rumen fluid. Epifluorescent visualization of encapsulated GFP can be optimized through selection of GFP variants and microscope light sources. Selection is best achieved by analyzing the actual excitation spectrum as determined by multiplying the variants' quantum corrected excitation spectrum by the bulb's emission spectrum. Based on emission spectral analysis, filter sets can be designed for the specific excitation spectrum, thereby limiting the excitation of autofluorescent compounds to the greatest extent.

### **4.2 Introduction**

Green Fluorescent Protein (GFP) has the potential to be used as an expressed marker for the monitoring of aerobic microbes introduced into the rumen. However the detection of GFP in rumen fluid is difficult due to the extensive autofluorescence observed at 365 nm (Chapter 3). A survey of the literature shows that microbial autofluorescence is documented in several methanogenic species commonly found in the rumen. In 1972, Cheeseman *et al.* isolated the fluorescent cytochrome F<sub>420</sub> from a *Methanobacterium sp.*, and Mink and Dugan (1977) used epifluorescent microscopy to observe the autofluorescence of cytochrome F<sub>420</sub> to identify methanogenic bacteria. The



first reported uses of epifluorescent microscopy of ruminal samples was in the observation of bacteria adhering to the surface of ruminal ciliate protozoa (Imai and Ogimoto, 1978), and in the observation of methanogenic bacteria (Doddema and Vogels, 1978). Since that time several other investigators have also observed methanogenic autofluorescence in association with feed particles and ruminal protozoa (Vogels *et al.*, 1980; Stumm *et al.*, 1982; Krumholz *et al.*, 1983; and Finlay *et al.*, 1994). However, these reports made no effort to describe the extensive autofluorescence associated with other ruminal feed particles, protozoa and bacteria.

Extensive background autofluorescence interferes with GFPuv detection and enumeration. Thus any reduction in ruminal autofluorescence will enhance the usefulness of GFP in the rumen. Since the initial discovery and cloning of GFP (Chalfie *et al.*, 1994), many mutations have been described with emission and excitation spectra different from the wildtype protein (Fig 4.1 and 4.2). Furthermore, several point mutations are known to result in enhanced fluorescent quantum yield due to increases in the relative intensity of absorption and emission peaks (Table 4.1 and Table 4.2). In the current study we determined the optimal GFP variant, light source and filters which may provide an improvement in the visualization of GFP illuminated cells in rumen fluid. Although this is not intended to be a review paper we must inevitably examine these GFP variants and their characteristic mutations in order to determine the ideal ruminal GFP variant.

### **4.3 Material and Methods**

#### **4.3.1 Yeast Strain and GFP Expression Cultures**

The ruminally traceable *Pichia pastoris* GS115::GFPuv::mycHIS (Mut<sup>+</sup>, Arg<sup>-</sup>, His<sup>-</sup>, Ble<sup>+</sup>) was cultured in extra-deep baffled 500 mL Erlenmeyer flasks (Bellco Glass Inc., Vineland, NJ), containing 250 mL buffered minimal glycerol media (BMGH: 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate

and without amino acids, 1% ammonium sulfate,  $4 \times 10^{-5}$ % biotin, 1% glycerol,  $4 \times 10^{-3}$ % histidine) (Invitrogen Corp., Carlsbad, CA). The culture was inoculated with 1 mL of YPD culture with an  $OD_{600}$  of 1.0 and grown at 30°C while shaking at 300 rpm. After 24 h, cultures were transferred to induction medium by centrifuging at 3000 x g for 5 min., decanting the supernatant, and resuspending the pellet in 250 mL buffered minimal methanol media (BMMH: 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate,  $4 \times 10^{-5}$ % biotin, 0.5% methanol,  $4 \times 10^{-3}$ % histidine). The culture was incubated at 30°C for a further 16 d, adding absolute methanol to a final concentration of 0.5% every 24 h.

#### 4.3.2 Epifluorescent Microscopy

Fluorescent *P. pastoris* cells were introduced into rumen fluid and visualized under epifluorescent microscopy at 400x magnification using a Nikon Optiphot microscope (Nikon Canada Inc., Mississauga, ON) equipped with a 100W mercury lamp (OSRAM Canada Ltd., Mississauga, ON). Nikon UV-1A, V-1A, BV-1A, BV-2B, and B-2A filter cubes were evaluated in addition to a 510/20 nm barrier filter supplied by Chroma Technology Corp (Brattleboro, VT) (Table 4.3). Photos were taken using Kodak 800 Gold Max film (Kodak Canada Inc., Toronto, ON). Field of view counts were performed using a 0.2 mm deep Petroff 1/5th 3900 cell counting chamber (Hausser Scientific, Horsham, PA) in combination with a Petroff reinforced cover slip to enable consistent field of view counts in addition to minimizing UV light scatter and associated photobleaching.

#### 4.4 Results and Discussion

Prior to any discussion of epifluorescence microscopy we must first discuss the limitations of reporting visual colourimetric results. Color films do not have the same

sensitivity to the full light spectrum as the human eye and pose a limitation to the reporting of visual colorimetric results. Color films are a combination of three chemical reactions intended to reproduce common colors, however exposure to ultraviolet light typically results in an image that appears bluer than that seen by the human eye (Eastman Kodak Company, 1999). Additionally, exposure of color film to infrared light produces an effect known as anomalous reflectance in which halo-like artifacts, which were not detectable by the human eye, appear on the image (Eastman Kodak Company, 1999). To date there are no film systems that can accurately report the full spectrum of visible light, and all films are subject to variations in the film development and printing processes. Modern film developing machines are controlled by automated computers that correct the color balance, hue, and saturation of each photo with the intent of providing the most pleasing image. However these alterations drastically affect the images captured from special sources such as epifluorescent microscopy. For this reason, all images presented in this paper have been scanned directly from the negatives with the intention of eliminating variations in the printing process. Only video capture equipment can provide the most accurate image as it can be manually adjusted to present the same visual image as seen with the human eye. Our study is subject to the limitations of photographic film. As a result, there are obvious inconsistencies between the colors observed in the photographs and the colors visually observed during epifluorescence microscopy.

In determining the ideal GFP variant, the first aspect to consider is the desired emission spectrum of the model chromophore, which in the context of the ruminal environment must fluoresce in a color unique from the autofluorescence of native microbial populations and feed particles. Ruminal autofluorescence is not attributed to a single fluorescent compound, but rather is the culmination of potentially hundreds of compounds, including plant phenolics and bacterial cytochromes. Thus, the emission spectrum of ruminal fluid cannot be considered from a single excitation point, but rather

that each excitation wavelength has the potential to produce an unique emission spectrum and visual result. This is demonstrated in Figure 4.3 where rumen fluid is visualized under several discrete excitation wavelengths. Under 365/10 nm excitation (Fig 4.3B) we observed significant levels of the colors blue, yellow, orange, red and pink. A shift of 40 nm to an excitation of 405/10 nm (Fig 4.3C) resulted in a minimal spectral change aside from the loss of the color pink as it transformed to the color yellow. There is an additional benefit to the elimination of the ultraviolet light used in the 365/10 nm excitation filter as the field of view is darker, resulting in crisper images and greater contrast between colors. Examination under 435/10 nm excitation (Fig. 4.3D) and 420/40 nm (Fig 4.3E) excitation revealed a marked reduction in the number of fluorescing particles and their relative fluorescent intensity, as well as a shift in the blues to a more cyan hue. Finally, excitation under 470/40 nm resulted in a dramatic shift of the blue and red colors to a predominately orange color (Fig. 4.3F). This is a dramatic demonstration of the fact that particles which fluoresce a specific color under one excitation wavelength can fluoresce a completely different color when that excitation wavelength is shifted. In the rumen, changes in the fluorescent spectrum of a given particle cannot necessarily be attributed to a shift in the emission of a single fluorescing compound, but rather and more likely are due to the presence of multiple chromophores in a given particle, each with potentially unique excitation and emission spectra. Depending on the particle, multiple chromophores may be simultaneously excited and result in a composite color comprised of several distinct emission spectra.

The rumen is a dynamic environment with its constitutive plant matter constantly changing, both in particle size and its degree of degradation. As such it is not surprising to observe diurnal fluctuations in ruminal fluorescence, both in the colors present, and with respect to their intensities relative to other colors. Perhaps the most significant diurnal observation is that under 365/10 nm excitation ruminal protozoa appear an almost transparent dark blue prior to feeding (Fig 4.3B). However 4-6 h after feeding a

barley-silage diet we have observed that all protozoa species accumulate a multitude of internal colors (data not shown), whereas 4-6 h after feeding a corn-silage diet we have observed that protozoa appear entirely pink in color under 365/10 nm excitation (data not shown).

Choosing an optimal emission color for our model fluorescent GFP variant requires examination of the emission spectra from each of these excitation wavelengths. The optimal emission color for an environmental marker should be a color that is absent under a broad range of excitation wavelengths, despite differences in feedstuffs and diurnal conditions. In the case of bovine rumen fluid from animals fed barley- or corn-silage based diets, the color green is singly absent from the visible spectrum when excited between 365 nm to 470 nm. Thus, despite the existence of many red- and blue-fluorescing GFP variants (Fig 4.1), they are not useful chromophores for application in the rumen given the spectra of ruminal autofluorescence.

Further modification of the visual spectra is possible by blocking all undesired colors with the use of a barrier filter. Given that green is the optimal color for a ruminal environmental marker, a barrier filter which blocks all wavelengths above and below the green portion of the visible spectrum may serve as a method to dramatically reduce the range of wavelengths that interfere with the detection of GFP. A 510/20 nm barrier filter (Chroma Technology Corp.) was evaluated for its potential to reduce interfering ruminal autofluorescence by replacing the emission filters on the Nikon cubes. The 510/20 nm barrier filter is designed to only pass light between the wavelengths of 500 nm to 520 nm and as such effectively blocks all blues, yellows, oranges, and reds. However, particles which previously fluoresced blue now appear as green particles such as the circular particle in the lower left hand corner (Fig 4.4 A-E). This supports the concept of multiple chromogenic compounds which, when excited, emit a combined spectrum yielding the visual color blue. This composite blue color consists of a green fluorescing compound as demonstrated by the 510/20 barrier filter, and perhaps several more

fluorescent compounds which when combined produce the characteristic color under the given excitation wavelength. In the past, the use of absorptive barrier filters could not always block highly fluorescent particles as the intensity would exceed the absorptive capacity of the barrier filter (Chroma Technology Corp., personal communication). Modern barrier filters, such as the one we are using, are designed to reflect the blocked light and thereby eliminate non-specific excess fluorescence (Chroma Technology Corp., personal communication). Barrier filters are designed to limit the emitted light to a specific range of wavelengths, however in doing so they drastically limit the quantity of light which is emitted by the sample. As such the target particle must emit sufficient light energy within the specific spectrum to be detected using a barrier filter. As noted in Fig 4.4, there are very few particles which are visible when using the 510/20 nm barrier filter and it is for this reason that the relative intensity of the GFP variant must also be considered when designing the model GFP ruminal marker.

The relative intensity of a chromophore is dependent on the amplitude and width of the excitation peak, which absorbs energy from the light source. This energy is then emitted by the chromophore at a longer wavelength of lower energy (Appendix 7.3). The quantum efficiency of a chromophore determines the amount of emitted light at a given wavelength and is a measure of the efficiency in energy transfer between the lower and higher wavelengths. The combined influence of the excitation peak and the quantum efficiency of chromophore ultimately determines the amplitude and hence relative intensity of the emission peak. Thus, when determining which GFP variant has the greatest relative intensity, one must first consider the total energy being absorbed by the chromophore. No light source has a constant energy emission across all wavelengths (Ploem and Tanke, 1987). Lasers provide a constant emission along specific wavelengths, characteristic of the elements and pressure used by the laser (Lide, 1999). Recently a number of tunable lasers have become available which emit over a wide range of wavelengths by changing the composition or pressure of the medium, or by

varying the wavelength of the initiating lamp (Lide, 1999). High-pressure elemental bulbs for use in epifluorescent microscopy have a very inconsistent energy spectrum with distinctive energy peaks, characteristic of the type of gas employed by the bulb (Lakowicz, 1988). The most common light sources in epifluorescent microscopy are mercury bulbs which have an energy spectrum between 250 nm and 600 nm (Fig 4.5), whereas xenon bulbs have a much less intense spectrum between 250 nm and 600 nm with its peaks appearing between 850 nm and 1050 nm (Fig 4.6). These energy peaks have a significant influence on the amount of energy absorbed by a chromophore at a given wavelength. Transformation of the GFP excitation spectrum with the mercury spectrum by multiplication of the relative intensities reveals the actual amount of energy the GFP chromophore absorbs. This transformation significantly alters the excitation spectrum of GFPwt (Fig 4.7), GFPuv (Fig. 4.8), and GFP I167T (Fig 4.9). To date GFP I167T is the brightest GFP variant when excited at 475 nm. Examination of the transformed excitation spectra reveals that there are three distinct excitation peaks at 367 nm, 406 nm, and 437 nm, which allows filter sets to be designed based on these specific absorption peaks with the objective of optimizing GFP fluorescence while minimizing ruminal autofluorescence (Table 4.4). The 475 nm region is included for discussion as this is the second excitation peak of wildtype GFP. Considerable effort has been made to develop mutants with enhanced 475 nm absorption as excitation at this wavelength is less prone to photoisomerization and photobleaching (Cubitt *et al.*, 1995). This wavelength is also desirable since fewer ruminal particles autofluoresce at 475 nm as compared to 365 nm. Using the optimal excitation ranges, as defined in Table 4.4, we can integrate the total area under each peak to calculate the total absorbed excitation energy for each variant (Table 4.5). As seen in table 4.5, GFPuv is the brightest variant when excited at 367 nm and absorbs 76.75 times more energy than the wildtype's 475 nm excitation peak and 17.59 times the GFP I167T variant's 475 nm excitation peak. Thus, given the I167T mutation's increased 475 nm peak and increase in absorbed

energy relative to the wildtype, we can hypothesize that any future mutation in the GFP 475 nm peak must provide at least a 67.39 fold increase relative to the wildtype to equal the fluorescence of GFPuv's brightest peak when excited by a mercury light source.

In 1996 Siemering *et al.* developed a GFP variant which is based upon a mutated *mgpf4* cDNA (Haselhoff *et al.*, 1997) yielding GFP<sub>A</sub> which possesses two point mutations (i.e., V163A and S175G). These mutations resulted in a thermotolerant protein capable of fluorescing when expressed at elevated temperatures (37°C), in addition to possessing an enhanced emission spectrum when excited at 475 nm. At this point, it has not been determined whether these mutations act independently or in tandem to produce these characteristics. Siemering (1996), further modified GFP<sub>A</sub> to produce GFP<sub>5</sub>, which included the I167T point mutation creating a chromophore whose 475 nm peak is of equal amplitude to the 395 nm peak. Unfortunately, the emission value of purified GFP<sub>5</sub> protein relative to wildtype has not been reported, and thus we can only speculate that GFP<sub>5</sub> has a reduced emission yield relative to GFP I167T when excited at 475 nm. This can be assumed based on the fact that the 475 nm excitation peak is of equal amplitude to the 395 nm peak, which was not reported to be enhanced relative to the wildtype GFP. Additionally, Siemering described a GFP<sub>5</sub> (S65T) variant which eliminated the 395 nm excitation peak and further increased the 475 nm excitation peak relative to GFP<sub>5</sub>, although again the relative emission intensity at 475 nm excitation was not reported. Table 4.5 demonstrates that GFPuv is a superior variant to GFPwt and GFP I167T, and likely GFP<sub>5</sub> and GFP (S65T) when cultured at 30°C. GFPuv contains the V163A point mutation in addition to the F99S and M153T point mutations that are believed to reduce hydrophobicity and protein aggregation, and result in a significant increase in emission when excited at 395 nm (Cramer *et al.*, 1996). It is interesting to consider the potential of modifying GFP<sub>5</sub> (S65T) with F99S and M153T point mutations, this putative variant would likely have a 475 nm excitation peak of equal amplitude to that of GFPuv's 395 nm peak yet itself lack a 395 nm excitation



peak. This would represent a significant improvement over presently available variants excited at 475 nm.

The selection of an optimal light source may also provide enhanced detection when used in combination with the optimal GFP variant and optimized filter set design. The selection of the optimal elemental gas for high-pressure epifluorescent bulbs may be chosen with the assistance of the tables of “Persistent Line Spectra of the Elements” as published in the CRC Handbook of Chemistry and Physics (Lide, 1999). These tables provide descriptions of the wavelengths and intensities of the line spectra that elements produce when excited by arc currents. The elemental gas should be selected based on the desired excitation wavelength of the chosen chromophore and should provide sufficient intensity to illuminate the sample using the narrowest excitation filter possible. Epifluorescent microscopy bulbs can be custom manufactured to contain the optimal gas, although they may not be recommended for use in all epifluorescent systems (OSRAM Canada Ltd., personal communication).

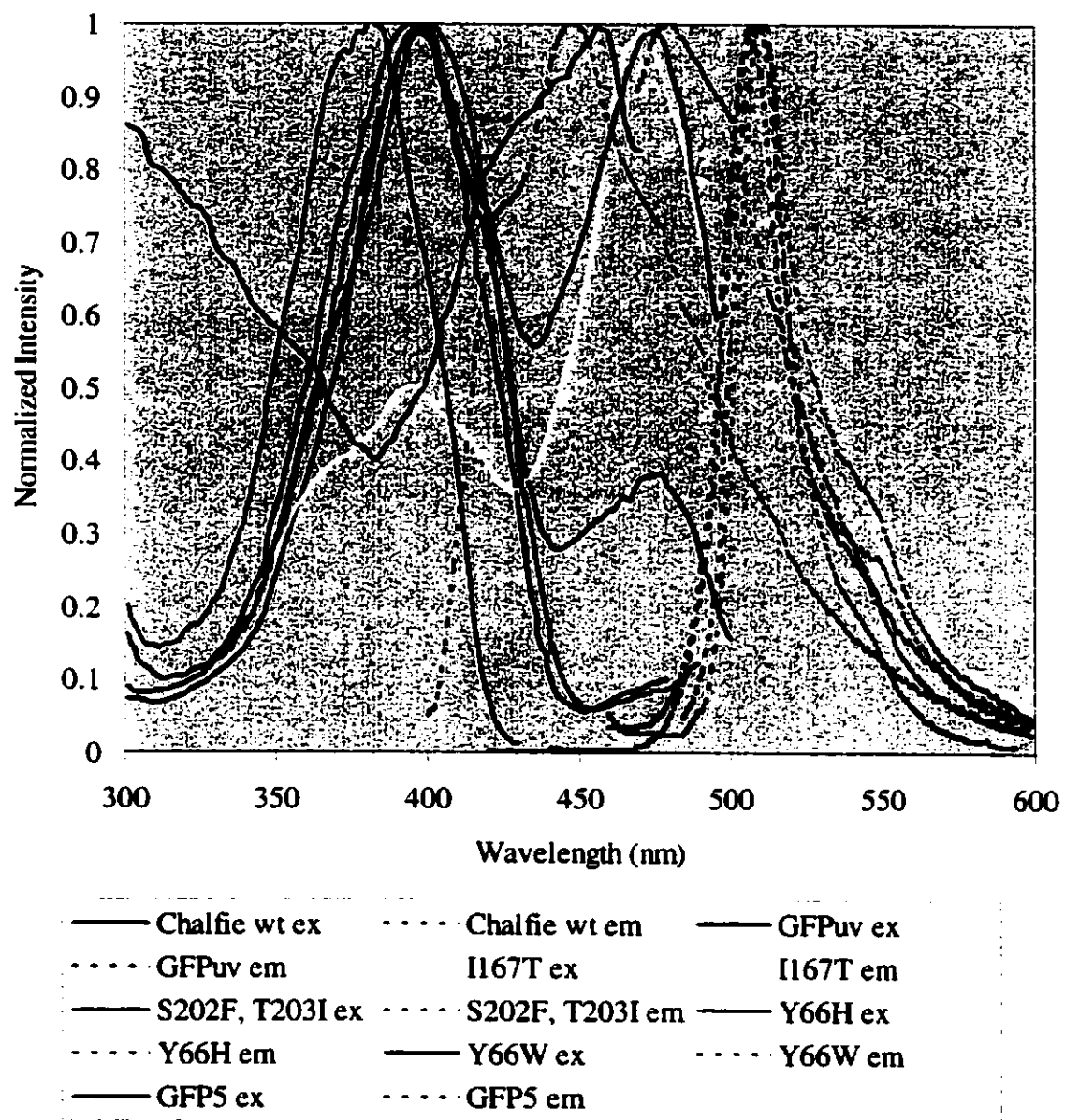
In conclusion, GFPuv is currently the optimal GFP variant to function as an aerobically expressed ruminally traceable marker. It is effectively visualized at both 367 and 406 nm. Transformation of the chromophore’s excitation spectrum with the light source spectrum is an effective means of determining the optimal filter design for visualization under epifluorescent microscopy. The optimal excitation filters for GFPuv illuminated with a mercury light source is 367/25 nm and 410/20 nm, with the 410/20 nm providing the best image when viewing *P. pastoris* expressing GFPuv in ruminal fluid under 400x magnification illuminated with a mercury light source. The use of a 510/20 nm barrier filter is not feasible for visualization of GFPuv in ruminal fluid due to the weak GFP signal relative to the extensive ruminal autofluorescence when excited by a mercury light source. The development of a variant which is more fluorescent than the GFPuv variant, and preferably excited at 475 nm; or the employment of a light source which is more intense than mercury and preferably emitting at 475 nm may allow

for the effective use of a highly absorptive barrier filter to eliminate non-target autofluorescent particles.

#### **4.5 References**

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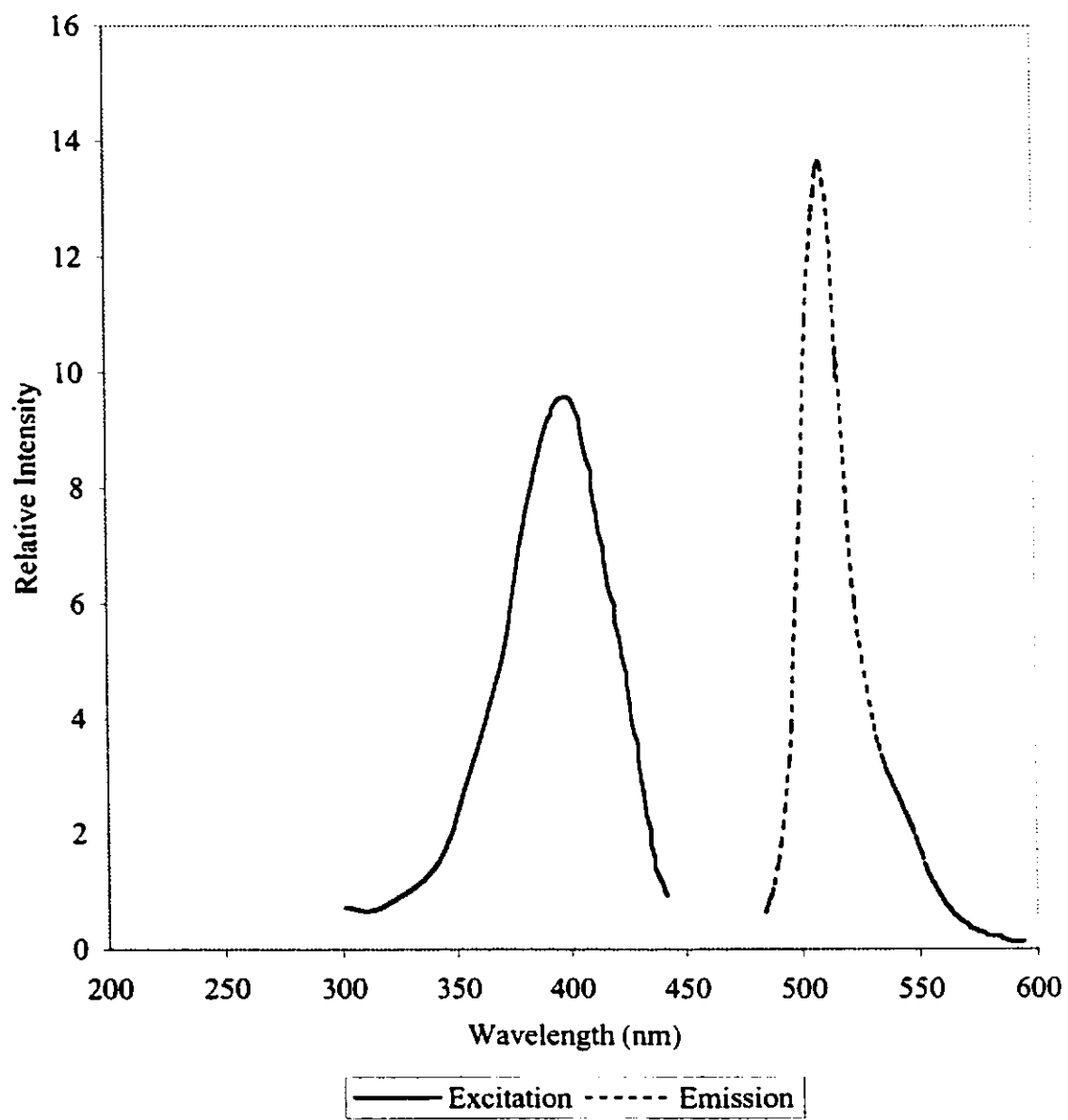
**Figure 4.1** Excitation (ex) and emission (em) spectra of GFP variants normalized to a maximum intensity of 1.0. Adapted from Chalfie *et al.* (1994), Heim *et al.* (1994), Crameri *et al.* (1996), Siemering *et al.* (1996).

**Table 4.1** Description of selected GFP cDNA variants.

<b><u>cDNA Designation</u></b>	<b><u>Author</u></b>	<b><u>Modifications</u></b>
<b>wildtype (<i>Aequorea victoria</i>)</b>	Chalfie <i>et al.</i> , 1994	
<b>wildtype TU#60</b>	Chalfie <i>et al.</i> , 1994	
<b>gfp10 cDNA</b>	Prasher <i>et al.</i> , 1992 modified wt TU#60	Q80R (PCR error) 24 residue LAC promoter “Clontech pGFP”
<b><math>\Delta</math>gfp10(<math>\bullet</math>2A)</b>	Crameri <i>et al.</i> , 1996 modified gfp10	silent codon changes arginine insertion after fMet residues numbered as gfp10
<b>mgfp4 cDNA</b>	Haselhoff <i>et al.</i> , 1997 modified gfp10	cryptic plant intron removed silent codon changes
<b>mgfp5 cDNA</b>	Siemering <i>et al.</i> , 1996 modified mgfp4	thermotolerant mutations

**Table 4.2** Description of selected GFP variants.

<b><u>Mutation</u></b>	<b><u>Author</u></b>	<b><u>Effect</u></b>
<b>Q80R</b>	<b>GFP wt</b> Prasher <i>et al.</i> , 1992 "Clontech pGFP"	silent PCR error
<b>S202F</b>	Heim <i>et al.</i> , 1994 contains Q80R	17% increase in emission @ 395 nm
<b>T203I</b>	Heim <i>et al.</i> , 1994 contains Q80R	17% increase in emission @ 395 nm
<b>I167T</b>	Heim <i>et al.</i> , 1994 contains Q80R	88% increase in emission @ 475 nm
<b>Y66H</b>	Heim <i>et al.</i> , 1994 contains Q80R	43% decrease in emission 395 nm excitation shifted to 382 nm loss of 475 nm excitation peak shift of emission from 510 nm to 488 nm
<b>S65T</b>	Heim <i>et al.</i> , 1994 contains Q80R	17% increase in emission at 475 nm
<b>F99S</b> <b>M153T</b> <b>V163A</b>	<b>GFPuv</b> Cramer <i>et al.</i> , 1996 $\Delta$ gfp10( $\bullet$ 2A)	reduced hydrophobicity reduced aggregation 10x increase in excitation at 395 nm
<b>S175G</b> <b>V163A</b>	<b>GFPA</b> Siemering <i>et al.</i> , 1996 contains mgfp4	enhanced protein folding at 37°C (decreased thermo-sensitivity) increase in 475 nm excitation
<b>V163A</b>	<b>GFPB</b> Siemering <i>et al.</i> , 1996 contains mgfp4	increase in 475 nm excitation
<b>Y66H</b> <b>S175G</b> <b>V163A</b>	<b>GFPA (Y66H)</b> Siemering <i>et al.</i> , 1996 contains mgfp4	loss of 475 nm excitation peak 395 nm excitation shifted to 382 nm 510 nm emission shifted to 488 nm 29x increased em @ 37°C vs. Y66H 3x increased em @ 25°C vs. Y66H
<b>V163A</b> <b>I167T</b> <b>S175G</b>	<b>GFP5</b> Siemering <i>et al.</i> , 1996 contains mgfp4	increased emission at 395 nm excitation increased emission at 475 nm excitation
<b>V163A</b> <b>S175G</b> <b>I167T</b> <b>S65T</b>	<b>GFP5 (S65T)</b> Siemering <i>et al.</i> , 1996 contains mgfp4	loss of 395 nm peak 475 nm excitation shifted to 490 nm increased emission at 475 nm excitation

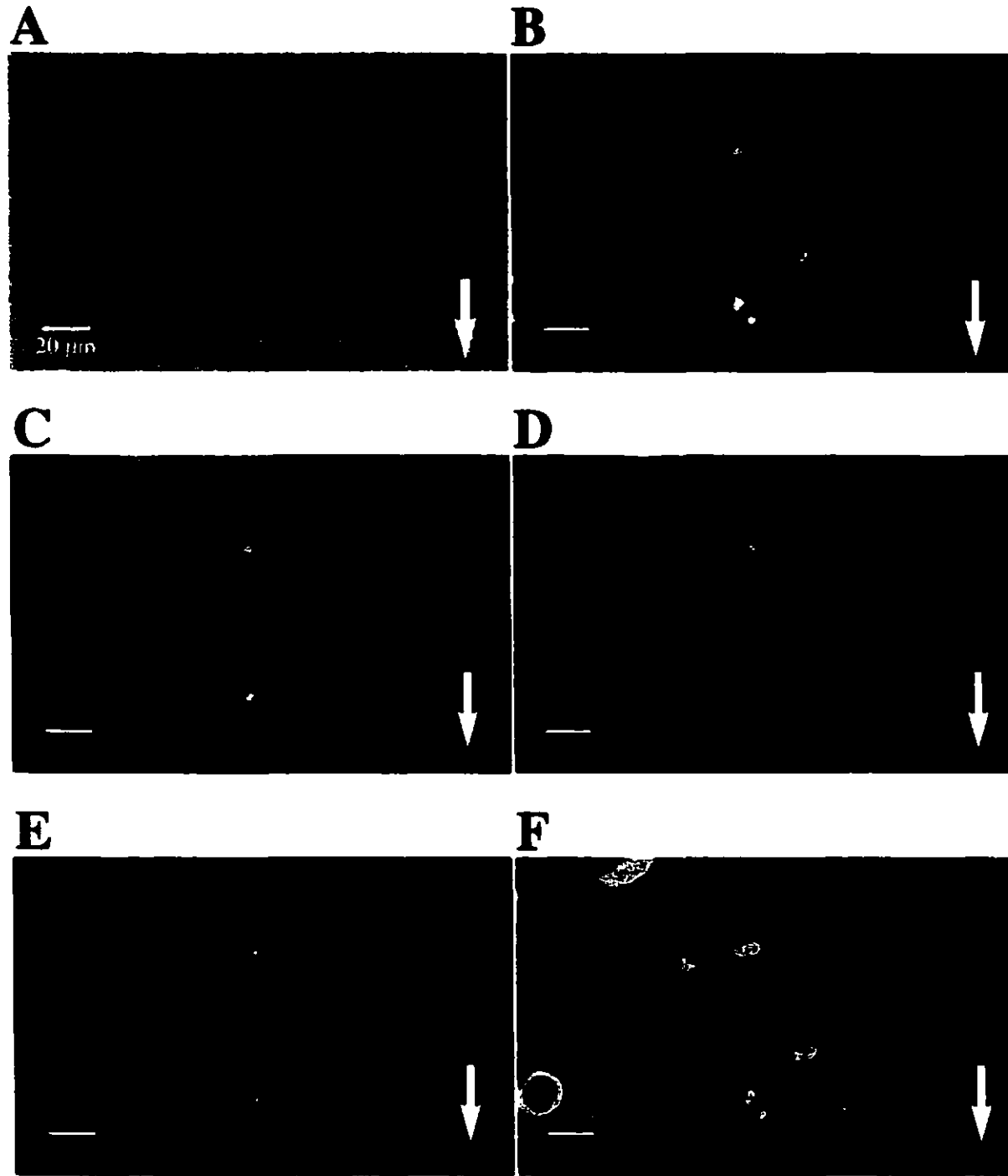


**Figure 4.2** GFPuv spectrum relative to GFPwt. Adapted from Cramer *et al.*, 1996.

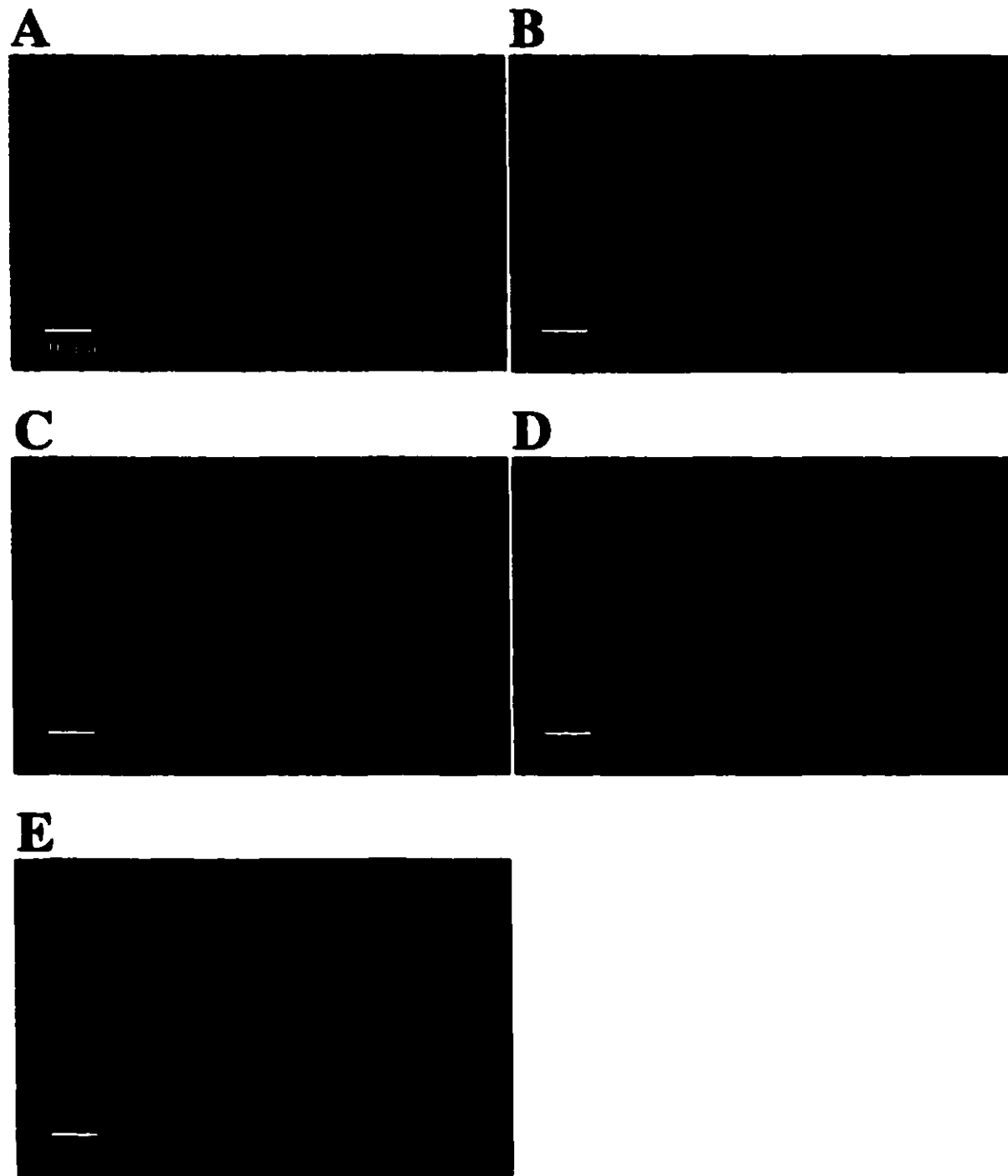
**Table 4.3** Specifications of filter cubes used in this study.

<b><u>Filter</u></b>	<b><u>Excitation (nm)</u></b>	<b><u>Dichroic Mirror (nm)</u></b>	<b><u>Emission Filter (nm)</u></b>
UV-1A	360 - 370	400	400 +
V-1A	400 - 410	430	450 +
BV-1A	430 - 440	455	480 +
BV-2B	400 - 440	455	480 +
B-2A	450 - 490	505	520 +
510/20 nm (Barrier filter)			500 - 520

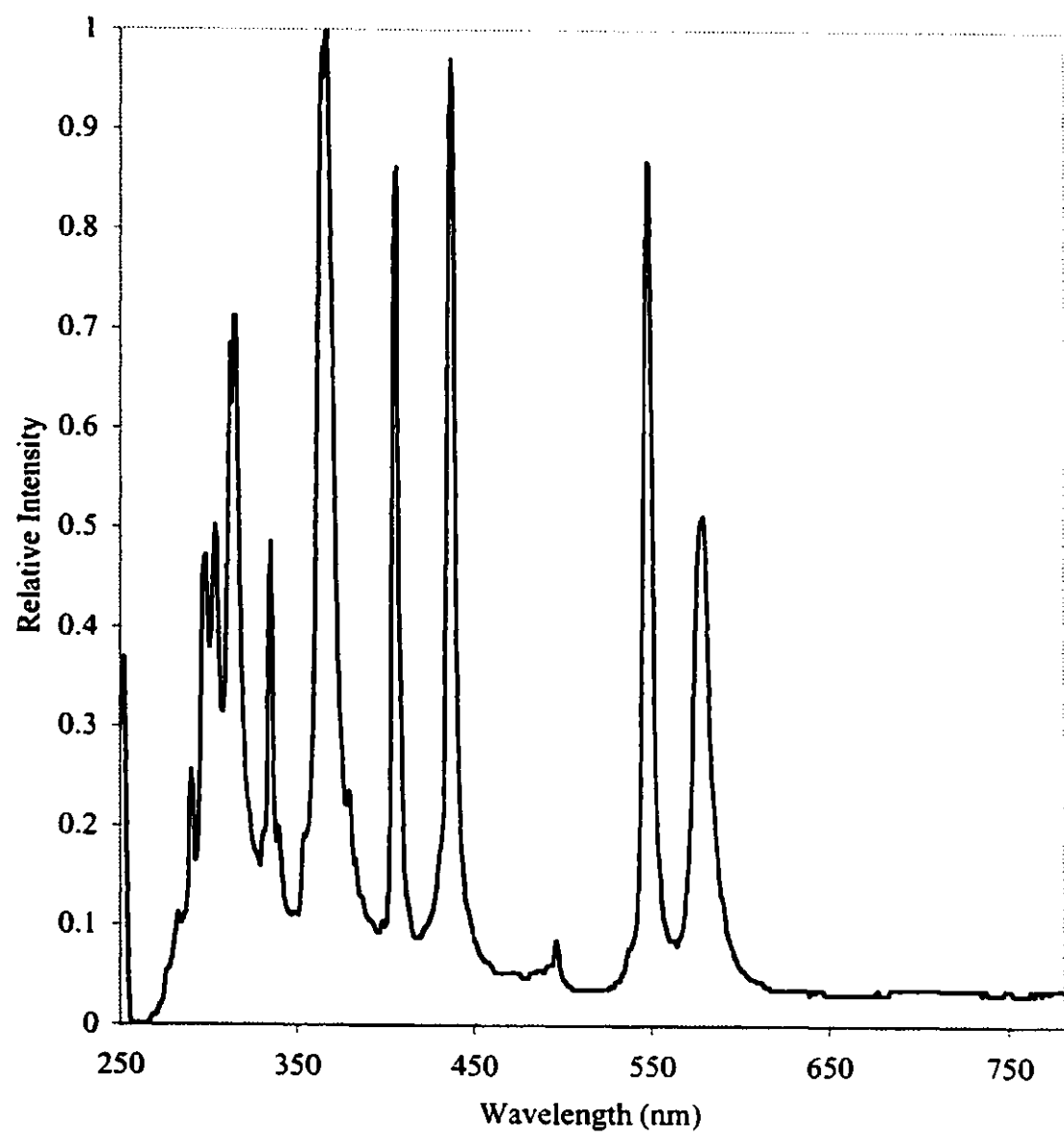




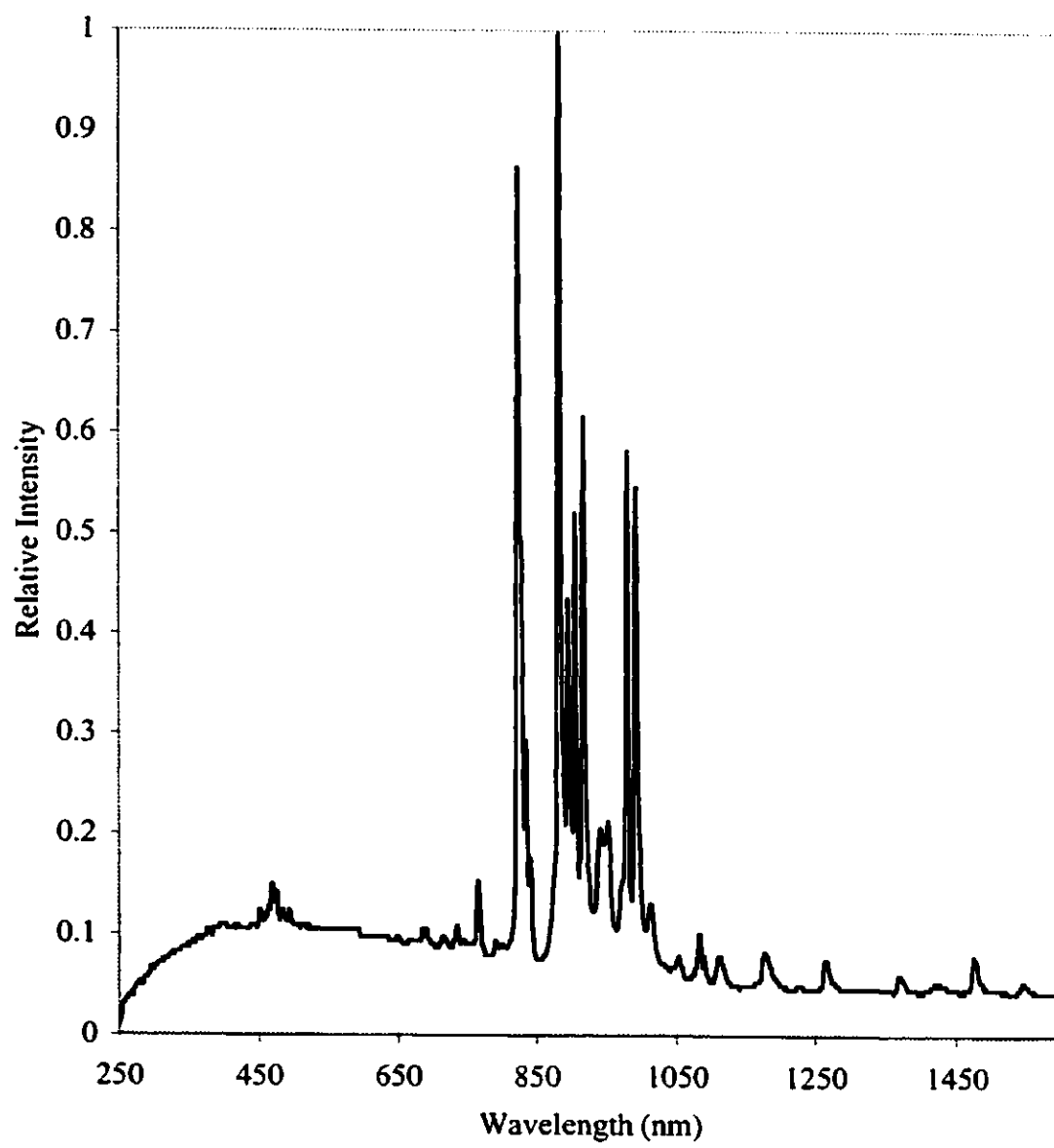
**Figure 4.3** Visualization of rumen fluid under A) white light, and epifluorescent microscopy using filters B) UV-1A, C) V-1A, D) BV-1A, E) BV-2A, F) B-2A. The arrow marks a protozoan. Magnification 364x.



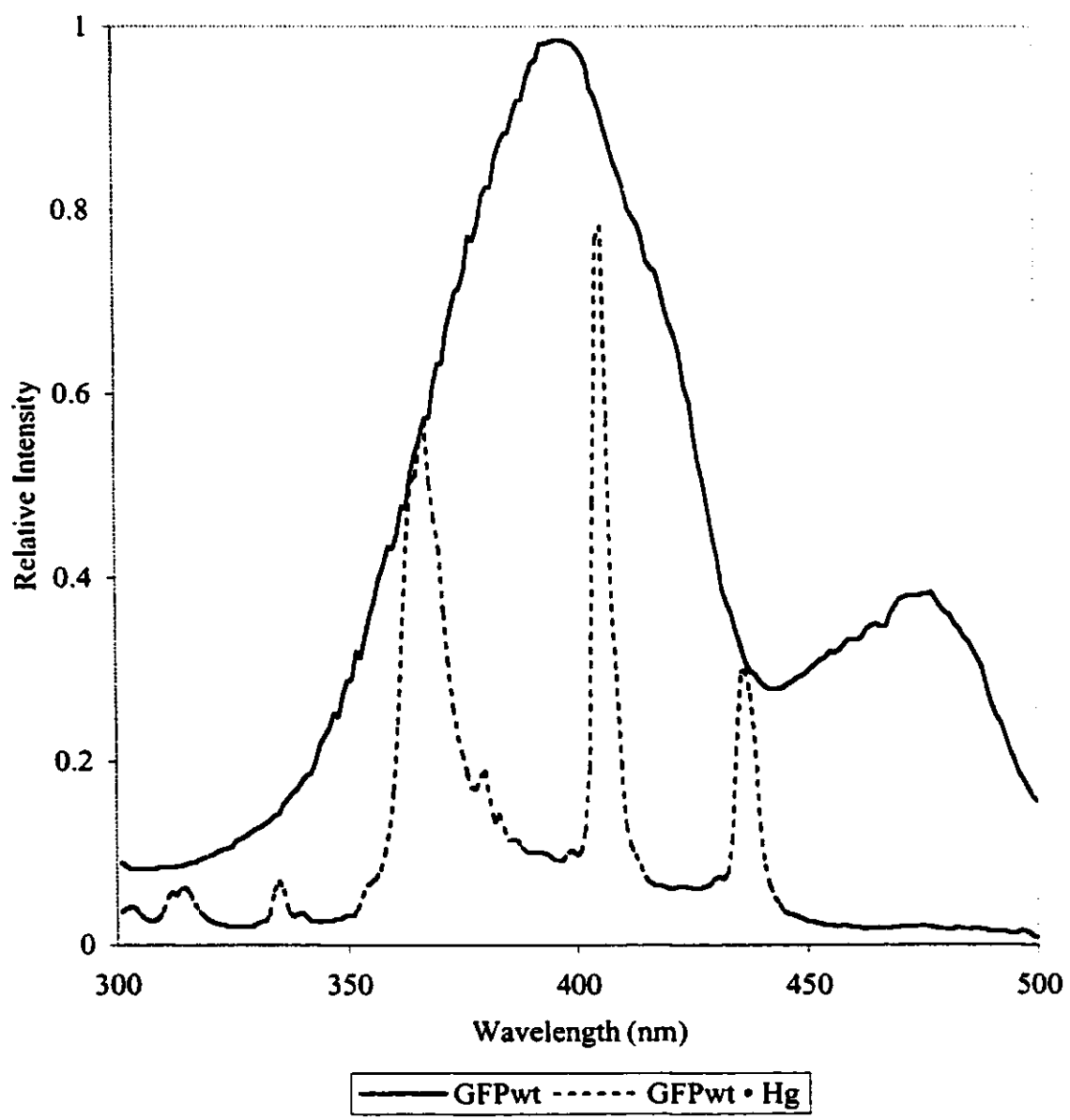
**Figure 4.4** Visualization of rumen fluid under A) white light, and epifluorescent microscopy using filters equipped a with 510/20 nm emission barrier B) UV-1A, C) V-1A, D) BV-1A, E) BV-2A. Magnification 364x.



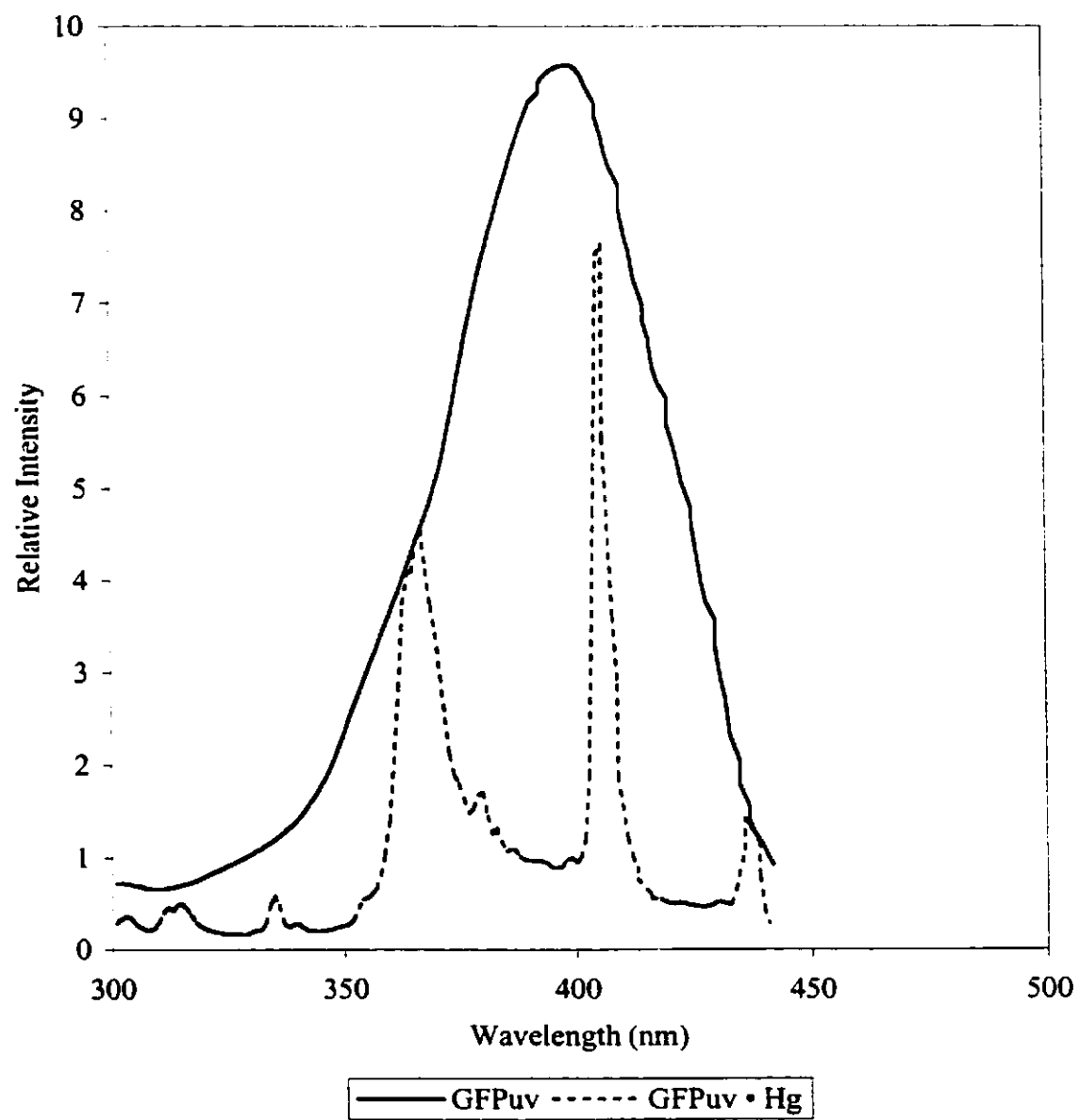
**Figure 4.5** Mercury bulb (100W) emission spectrum. Supplied by OSRAM Canada Ltd., Mississauga, ON.



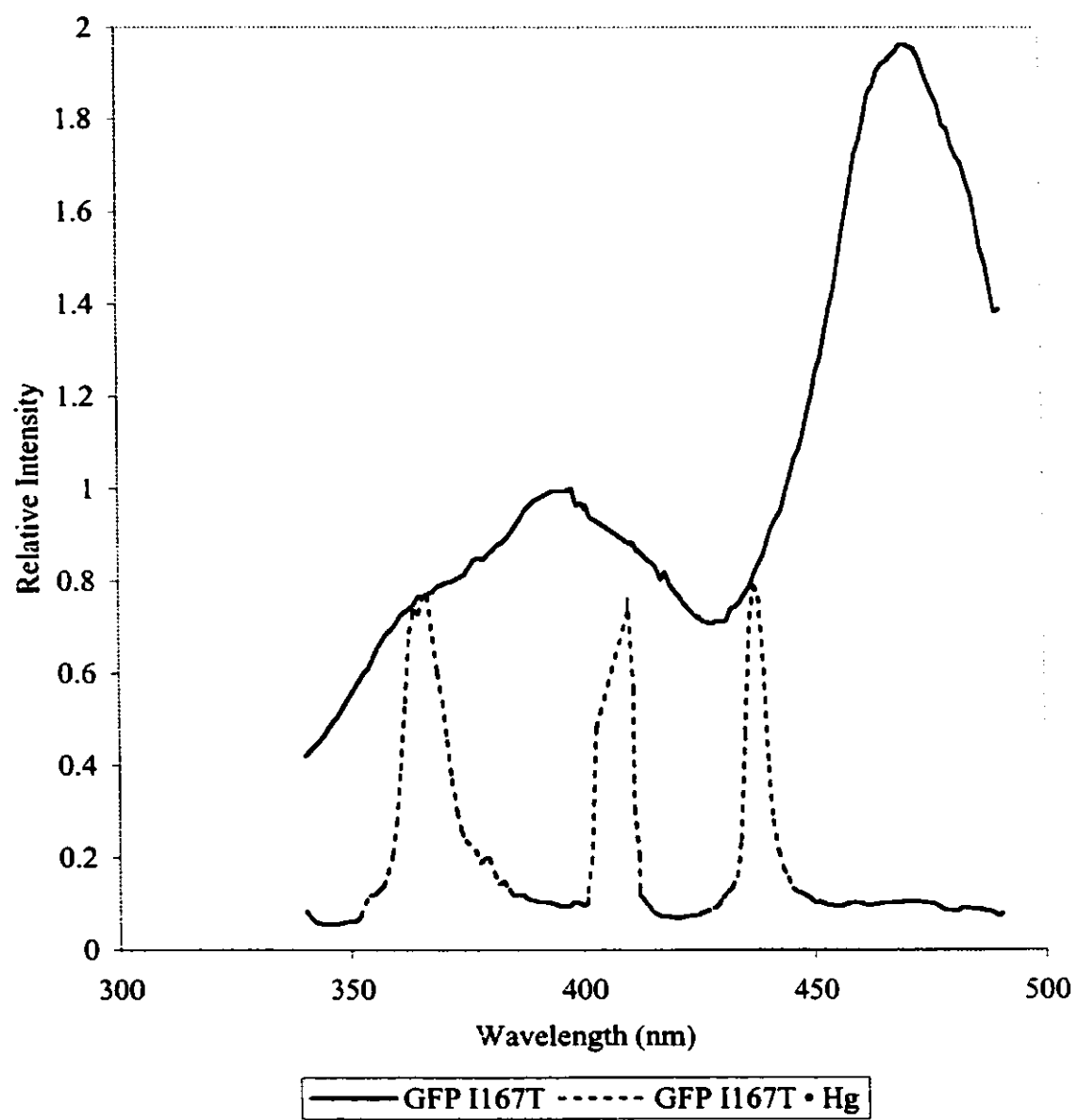
**Figure 4.6** Xenon bulb (100W) emission spectrum. Supplied by OSRAM Canada Ltd., Mississauga, ON.



**Figure 4.7** GFPwt excitation spectrum as transformed by the mercury bulb emission spectrum. GFPwt spectrum adapted from Chalfie *et al.*, 1994.



**Figure 4.8** GFPuv excitation spectrum relative to GFPwt as transformed by the mercury bulb emission spectrum. GFPuv spectrum adapted from Crameri *et al.*, 1996.



**Figure 4.9** GFP I167T excitation spectrum relative to GFPwt as transformed by the mercury bulb emission spectrum. GFP I167T spectrum adapted from Heim *et al.*, 1994.

**Table 4.4** Optimal excitation filters for GFP excitation by a mercury light source.

<b><u>Variant</u></b>	<b><u>367</u></b>	<b><u>406</u></b>	<b><u>437</u></b>	<b><u>475</u></b>
Wild type	370/20	410/20	438/14	477/22
GFPuv	367/25	410/20	438/14	N/A
GFP I167T	367/25	410/20	440/20	477/45

N/A = not available



**Table 4.5** Integrated absorbed energy of GFP variants excited by mercury light as viewed by the optimal filters, defined in Table 4.4.

<b><u>VARIANT</u></b>	<b><u>367</u></b>	<b><u>406</u></b>	<b><u>437</u></b>	<b><u>475</u></b>
Wild type	7.00	4.80	1.89	0.80
GFPuv	61.40	46.12	7.56	N/A
GFP I167T	10.07	4.82	6.05	3.49

N/A = not available

## **5.0 *In vitro* Evaluation of *Pichia pastoris* as a Rumen Escape Vehicle**

### **5.1 Abstract**

Cellular encapsulation may be an effective method for delivering of limiting amino acids, enzymes, and bio-active peptides to the small intestine of ruminants as intracellular protein is isolated from ruminal degradation by the cell's protective membrane. The yeast expression system *Pichia pastoris* was evaluated for its potential to serve as a ruminal escape vehicle through incubations in a variety of *in vitro* simulations. Batch culture rumen *in vitro* simulations demonstrated that 93%, 97%, and 25% of the *P. pastoris* inoculum maintained cellular integrity in clarified rumen fluid, bacterial fraction of rumen fluid, and whole rumen fluid, respectively, when incubated over 36 h to 48 h. In continuous culture *in vitro* simulation (Rusitec) *P. pastoris* demonstrated an 19% escape rate when added daily to vessels having a dilution rate of 0.75 d<sup>-1</sup>. Abomasal *in vitro* simulations demonstrated that 84% of the *P. pastoris* inoculum was lysed within 12 h, a property that is necessary for the release of encapsulated protein. Ruminal and abomasal *in vitro* studies support that *P. pastoris* may be an effective post-ruminal delivery vehicle.

### **5.2 Introduction**

Ruminants have evolved to utilize high-fiber, low-energy, low-protein grasses and forages by relying on microbial fermentation. As a result, orally administered protein supplements are extensively degraded within the rumen such that very little reaches the small intestine intact (Tamminga, 1979). Experimental use of abomasal and duodenal cannulae have demonstrated that ruminant production can be greatly enhanced by providing high-value protein, rich in limiting amino acids, to the small intestine for the animal to utilize directly. Abomasal infusion of casein in cattle resulted in a one to four kg increase in milk volume accompanied by a 10-15% increase in milk protein content

(Clark, 1975). More recently, Schwab *et al.* (1992) demonstrated that lysine is the first limiting amino acid for milk production, of which the extent of limitation changes through the lactation cycles. Reis *et al.* (1990) demonstrated that amino acid infusions of one to seven grams of ten basic amino acids can increase wool growth by 86% in addition to increasing wool quality, as compared to oral administration.

Unfortunately, infusion technology is not feasible for use in modern agricultural production systems unless a non-invasive delivery method is developed. To date, several bypass technologies have been developed with the intent of supplying simple amino acids or nutritional protein for duodenal absorption, including structural modifiers (Chalupa, 1975), amino acid analogs (Belasco, 1972), and encasement agents (Sibbald *et al.*, 1968). These technologies have been reviewed by Clark (1975) and Chalupa (1975) in which they concluded that the perfect bypass technology has yet to be developed. A more recent survey of the literature suggests that at least five factors are required for a ruminal escape technology to be successful. These include: 1) a low rate of rumen degradation, 2) a high rate of post-ruminal protein release, 3) efficient dissociation of the encapsulated protein, 4) that it be inexpensive relative to the production benefit of the delivered protein, and 5) that ruminal fermentation or post-ruminal digestion not be negatively affected.

Cellular encapsulation has been proposed as an alternative technology that produces and stores recombinant protein in a cell, thereby providing protection from ruminal proteolysis. Development of a recombinant endogenous rumen microbe as a cellular protein encapsulation technology was first proposed by Smith and Hespell (1983), but the concept extends to recombinant feeds and ruminally foreign microbes.

Rumen microbes have become highly adapted in their ability to compete in the complex rumen ecosystem and persist despite dramatic diurnal or feed mediated ecological changes such as those experienced during bloat. Many researchers are of the opinion that anti-microbial systems such as antibiotics, toxins, lytic enzymes, and

metabolic byproducts are the most significant tools a microbe has in ensuring ruminal survival (Jack *et al.*, 1995). An exogenous microbe developed as a ruminal escape vehicle (REV) may be less susceptible to ruminal degradation because it may not be a specific target of an endogenous anti-microbial system. Furthermore, an aerobic microbial system is more easily cultured, induced, and produces biomass more efficiently than anaerobic systems.

The yeast *Saccharomyces cerevisiae* has long been used in cattle diets as a feed amendment, although its ability to affect ruminal fermentation is still uncertain (Kmet *et al.*, 1993). Ingledew and Jones (1982) reported metabolically active *S. cerevisiae* in the rumen of cows fed live brewers yeast indicating the presence of intact cells and some degree of ruminal resistance despite the reported loss of cellular viability. These researchers were unable to estimate the size of the *S. cerevisiae* population or if the cells escaped the rumen intact, the first requirement of a REV. The fate of the live yeast feed additive Levucell® Sc I-1077 (*S. cerevisiae* CNCM -1077, Institut Pasteur, Paris, France) in the digestive tract of cows was recently investigated through ruminal and fecal plate counts in which researchers recovered 17% to 34% of the original inoculum in the feces (Durand-Chaucheyras *et al.*, 1998). This estimation of fecal escape does not include dead or otherwise nonculturable cells, thereby underestimating fecal excretion of intact cells. A more thorough examination of Levucell® would be prudent as any intact yeast cells being excreted from the animal are not delivering their intracellular contents and are therefore not serving as a REV. Finally, a lysine accumulating *S. cerevisiae* mutant was developed by Ohsumi *et al.* (1994) and found to be ruminally resistant. Further examination by J. Newbold (personal communication) indicated that this strain was effectively delivering free L-lysine to the small intestine of cattle. This strain has not been developed as an expression system, which would allow it to perform as a more versatile REV; it should be further noted that this strain is not likely to become commercially available (J. Newbold, personal communication).

The yeast expression system *Pichia pastoris* has been developed to produce high levels of biologically active enzymes and peptides (Cregg *et al.*, 1993), and may be a candidate as a REV. To date the only *Pichia* species to be reported in the rumen are *Pichia farinosa*, *Pichia fermentans*, *Pichia kudriavzevii*, and *Pichia membranefaciens* (van Uden *et al.*, 1958; and Lund, 1974). The ruminally foreign *P. pastoris* may not be a target of a specific ruminal lytic system and deserves evaluation as a potential REV.

Traditional selective plate counts cannot detect cells that are viable but nonculturable and those that are non-viable yet still possess an intact cellular membrane and as such are still functioning as a REV (see Chapter 3). Thus a means of monitoring cellular membrane integrity is critical in evaluating the ability of a microbe to serve as a REV. Green Fluorescent Protein (GFP) (Chalfie *et al.*, 1994) can function as a quantitative ruminal ecological marker and a marker for cellular integrity (see Chapter 3). The objective of this study was to determine the potential of the aerobic ruminally exogenous yeast expression system *P. pastoris* expressing GFP variant GFPuv (Crameri *et al.*, 1996) to provide undegraded protein, enzymes or bio-active peptides to the small intestine of cattle, as determined by *in vitro* rumen and abomasal incubations.

### 5.3 Material and Methods

#### 5.3.1 Yeast Strain and Inoculant Culture

The ruminally traceable *P. pastoris* GS115::GFPuv::mycHIS (Mut<sup>+</sup>, Arg<sup>-</sup>, His<sup>-</sup>, Ble<sup>+</sup>) was cultured in extra-deep baffled 500 mL Erlenmeyer flasks (Bellco Glass Inc., Vineland, NJ) containing 250 mL buffered minimal glycerol media (BMGH: 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate, 4x10<sup>-5</sup>% biotin, 1% glycerol, 4x10<sup>-3</sup>% histidine) (Invitrogen Corp., Carlsbad, CA). The culture was inoculated with 1 mL of YPD culture with an OD<sub>600</sub> of 1.0 and grown at 30°C while shaking at 300 rpm. After 24 h, cultures were transferred to induction media by centrifuging at 3000 x g for 5 min,

decanting the supernatant, and resuspending the pellet in 250 mL buffered minimal methanol media (BMMH: 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate,  $4 \times 10^{-5}$ % biotin, 0.5% methanol,  $4 \times 10^{-3}$ % histidine). The culture was incubated at 30°C for a further 16 d, adding absolute methanol to a final concentration of 0.5% every 24 h. In experiments requiring BMGY/BMMY treatments, cells were cultured as above with the exception that BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate,  $4 \times 10^{-5}$ % biotin, 1% glycerol) replaced BMGH, and BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 0.34% yeast nitrogen base without ammonium sulfate and without amino acids, 1% ammonium sulfate,  $4 \times 10^{-5}$ % biotin, 0.5% methanol) replaced BMMH.

### 5.3.2 Preparation of Fluorescent *P. pastoris* Inoculants

Induced cultures (250 mL) were harvested by centrifugation at 4°C for 5 min at 3000 x g in an IEC MP4 centrifuge (International Equipment Company, Needham Heights, MA). The yeast pellets obtained were washed with 20 mL ddH<sub>2</sub>O to minimize media carry over and concentrate the culture. The final yeast pellet was resuspended in 20 mL ddH<sub>2</sub>O, enumerated under epifluorescent microscopy, and diluted to a final concentration of  $1.2 \times 10^9$  cells/mL.

### 5.3.3 Epifluorescent Enumeration of Intact Fluorescent *P. pastoris* Cells

GFP expressing *P. pastoris* cells were enumerated under epifluorescent microscopy at 400x magnification using a Nikon Optiphot microscope (Nikon Canada, Mississauga, ON) equipped with a mercury lamp (OSRAM Canada Ltd., Mississauga, ON) and a Nikon V-1A filter cube. Field of view counts were performed using a 0.2 mm deep Petroff 1/5th 3900 cell counting chamber (Hausser Scientific, Horsham, PA) in

combination with a Petroff reinforced cover slip to enable consistent field of view counts in addition to minimizing UV light scatter and associated photobleaching. The epifluorescent field of view data was immediately entered into a spreadsheet designed to ensure a statistically valid estimation of the true population (Section 3.3.9).

#### 5.3.4 Batch Culture Ruminal *in vitro* Incubations

A representative sample of rumen fluid and solids was collected from a fistulated Holstein cow 2 h after the morning feed. Cows were fed 43.21 kg d<sup>-1</sup> of a forage/concentrate diet consisting of corn silage, chopped hay, barley grain, blood meal, corn gluten meal, canola meal, megalac, sodium bicarbonate, calcium carbonate, dicalcium phosphate, dairy lac mineral mix, urea, dry molasses, canola oil, Altech flavor (64.80, 5.25, 19.88, 0.25, 3.01, 3.54, 1.30, 0.19, 0.12, 0.37, 0.14, 0.12, 0.74, 0.30, 0.02, 0.05 % of diet, respectively). Rumen fluid was prepared by blending for three 30 sec pulses in a Waring blender and strained through four layers of cheesecloth. All bottles were flushed with reduced carbon dioxide to maintain anaerobicity. A portion of strained whole rumen fluid was maintained at 39°C for use in the incubations. Strained rumen fluid was centrifuged twice at 500 x g for 10 min, retaining the protozoa-free supernatant as the bacterial fraction of rumen fluid. The absence of protozoa was confirmed by light microscopy. A portion of protozoa-free rumen fluid was clarified by centrifuging at 20,000 x g for 10 min, retaining the microbial-free supernatant as clarified rumen fluid.

The three rumen fluid fractions (2 mL) were anaerobically injected into presterilized serum bottles containing 18 mL of anaerobic Scott and Dehority medium (1965) supplemented with 5% (v/v) clarified rumen fluid, 0.2% cellobiose, and 0.2% soluble starch. Finally, 100 µL of the previously prepared yeast inoculum was injected, achieving a final concentration of 6.0x10<sup>6</sup> cells/mL. The bottles were placed on a shaking platform at 150 rpm and incubated in a forced air cabinet at 39°C. At each time

point, gas production was measured on a modified water-displacement manometer (Wang *et al.*, 1999), the bottles were opened and pH recorded then placed in a 67 kHz ultrasonic cleaner (Mettler Electronics Corp., Anaheim, CA) for 15 min prior to analysis under epifluorescent microscopy. An ultrasonic cleaner was used to dissociate *P. pastoris* from feed particles and disrupt cell aggregates. This treatment did not affect cellular integrity as determined by analysis of the clarified and bacterial fraction of rumen fluid treatments.

#### 5.3.5 Rusitec Apparatus

The rumen simulation technique Rusitec was used as described by Czerkawski and Breckenridge (1977). Ruminal inocula for the fermentation vessels were collected and pooled from two Holstein cows 2 h after the morning feeding. Cows were fed 43.21 kg d<sup>-1</sup> of a forage/concentrate diet consisting of corn silage, chopped hay, barley grain, blood meal, corn gluten meal, canola meal, megalac, sodium bicarbonate, calcium carbonate, dicalcium phosphate, dairy lac mineral mix, urea, dry molasses, canola oil, Altech flavor (64.80, 5.25, 19.88, 0.25, 3.01, 3.54, 1.30, 0.19, 0.12, 0.37, 0.14, 0.12, 0.74, 0.30, 0.02, 0.05 % of diet, respectively).

Nylon bags (0.45 µm pore size) containing 14 g of the above diet were placed in the fermentation vessels and gently agitated in the liquid phase. Two bags were present in each vessel at any time, with one bag replaced daily to give a 48 h incubation per bag. The nominal volume in each vessel was 850 mL and artificial saliva (pH 7.5) (McDougall, 1948) was constantly infused into the vessels at a rate of 0.75 d<sup>-1</sup>. Vessels were fed the basal diet for the first six days, then supplemented with the fluorescent *P. pastoris* inocula for the remaining eight days. At feeding time vessels were opened under a stream of reduced CO<sub>2</sub>, pH was measured, samples were taken from the liquid phase for analysis under epifluorescent microscopy, and feed bags were changed. Each day four vessels received fluorescent *P. pastoris* GS115::GFPuv::mycHIS cultured on



BMGH/BMMH, while the remaining four vessels received fluorescent *P. pastoris* GS115::GFPuv::mycHIS cultured on BMGY/BMMY. All vessels received  $6.0 \times 10^9$  cells (127.75 mg dry weight) to achieve a final concentration of  $6.0 \times 10^6$  cells/mL. On day eight a 24 h time course on BMGH/BMMH was performed by sampling from both the vessel's liquid phase and effluent every 2 h until fluorescent *P. pastoris* cells were no longer detected.

#### 5.3.6 Abomasal *in vitro* Incubation

Sterile serum bottles were filled with 20 mL simulated gastric juice (SGJ: 2 g/L NaCl, 3.2 g/L pepsin, and 7 mL/L conc. HCl, final pH 1.45) diluted 3:1 with ddH<sub>2</sub>O to simulate gastric fill (Zikakis *et al.*, 1977). Yeast inocula cultured on BMGY/BMMY or BMGH/BMMH were prepared as described in Section 5.3.2 and injected into each bottle, to achieve a final concentration of  $6.0 \times 10^6$  cells/mL. The bottles were placed on a shaking platform at 150 rpm and incubated in a forced air cabinet at 37°C until sampled. At sampling points the bottles were opened and placed in a 67 khz ultrasonic cleaner (Mettler Electronics Corp.) for 15 min prior to analysis under epifluorescent microscopy.

## 5.4 Results

To study the extent of ruminal lysis and the mechanism by which *P. pastoris* is lost from the rumen, survival was evaluated in a variety of rumen fluid fractions. *P. pastoris* GS115::GFPuv::mycHIS had the capacity to persist in clarified rumen fluid, with loss rates of only 7.68% when calculated over 48 h (Fig 5.1). Membrane integrity in whole rumen fluid was maintained for only the first 6 h, followed by a 27.97% loss by 24 h, eventually reaching 74.78% loss by 36 h. In contrast, *in vitro* evaluation demonstrated that *P. pastoris* cultivated in this method exhibits minimal loss rates in the bacterial fraction (500 x g) of rumen fluid as only 2.86% was lost when monitored over 48 h.

Data from pH (Fig 5.2) and gas production (Fig 5.3) shows that acid and gas was produced, demonstrating that fermentation did take place. Additionally, it should be noted that throughout this project more than 10,000 microscope fields of view were counted and no protozoa predation was observed, (i.e., no engulfed fluorescent *P. pastoris* cells were observed).

To study the extent of lysis experienced in a continuously cultured rumen environment *P. pastoris* GS115::GFPuv::mycHIS cells cultured in two different media were evaluated in an eight vessel Rusitec adapted for six days to a corn-silage-concentrate diet. Vessel effluent sampled at the time of feeding demonstrated that 18.78% of the BMGH/BMMH inoculum and 14.28% of the BMGY/BMMY inoculum was recovered the first day, however fluorescent *P. pastoris* cells were not subsequently detected in the remaining six days (Table 5.1). A 24 h time course of a BMGH/BMMH treatment vessel was conducted after day seven and revealed that *P. pastoris* did successfully escape the Rusitec intact, however only for the first 12 h after feeding (Fig 5.4). During that time a cumulative total of 18.92% of the initial inoculum successfully escaped the ruminal environment and is indicative of cells entering the abomasum whereby they would be lysed. Figure 5.4 includes theoretical population curves, based on the 0.75 d<sup>-1</sup> dilution rate, assuming no lysis or predation occurred. Daily pH monitoring of the Rusitec vessels revealed no significant upset in ruminal fermentation and that the vessels maintained very similar pH values throughout the trial (Fig 5.5).

Evaluation of gastric lysis using fresh fluorescent *P. pastoris* cultures demonstrated that 68.32% of the BMGH/BMMH inoculum was lysed within 6 h and 83.67% lysed by 12 h, as indicated by the decrease in fluorescent cells and the presence of cellular debris (Fig 5.6). There was no statistical difference in gastric lysis between BMGH/BMMH and BMGY/BMMY cultured cells.

## 5.5 Discussion

The successful delivery of digestive enzymes, high-value proteins, protein-based vaccines, hormones and probiotics to the small intestine of cattle holds significant promise to enhance agricultural production and optimize herd health. Historically, it was believed that orally administered protein and enzymes were rapidly and completely degraded by ruminal proteases immediately upon entrance into the rumen (Chesson, 1994). Recent studies have shown that the rate of ruminal degradation varies between proteins, and that some proteins are ruminally resistant and that significant levels can be detected in the small intestine (Fontes et al., 1995; Hristov *et al.*, 2000). Resistance to ruminal degradation is largely dependent on the protein solubility as influenced by structural conformation, degree of glycosylation, presence of disulphide bonds and its physical proximity to proteases (Wallace *et al.*, 1997). Ruminal bypass proteins are created by reducing their solubility through treatment with chemical agents such as formaldehyde, thereby decreasing their susceptibility to ruminal deamination. Chemical manipulation of protein typically alters its structural conformation (Chalupa, 1975), which plays a critical role in enzyme activity. Thus, chemical treatment of enzymes or bio-active peptides would likely result in the reduction or complete loss of the associated biological activity. Therefore, unless an enzyme is inherently ruminally resistant, it is unlikely that it can be made to be resistant to proteolytic degradation and still retain its biological activity (Wallace *et al.*, 1997)

Intracellular production and protection of biologically active protein has potential as a ruminal escape system. Evaluation of *P. pastoris* by *in vitro* rumen and abomasal simulations suggest that this organism has potential as a REV when applied as a feed additive. Batch culture ruminal *in vitro* incubations using GFP expressing *P. pastoris* allowed us to systematically define culture conditions that affected membrane integrity and hence the organism's ability to act as a REV. Several culture conditions were found to have an effect on membrane integrity including the initial inoculation rate of the

induction culture (Appendix 7.4) and the type of culture medium (Appendix 7.5), No strain specific differences were observed when cells were cultured on complex media, however strain GS115 appeared superior to strain KM71 when cultured on minimal media. We also observed that the presence of a buffering agent in the media (Appendix 7.6), and slight temperature variation (Appendix 7.7) had no effect on membrane integrity in ruminal fluid. This is a valuable aspect of the *Pichia* escape system as protein expression is affected by pH and temperature (Cereghino and Cregg, 2000). These results suggest that *P. pastoris* protein expression can be optimized without detrimentally affecting the cell's ability to deliver that protein to the small intestine.

Evaluation of *P. pastoris* cells cultured in BMGH/BMMH medium under conditions optimal for ruminal survival demonstrated that there was negligible loss of fluorescent cells in clarified or bacterial fractions of rumen fluid. These fractions represent the non-cell-associated lytic factors and the bacterial-associated lytic factors, while whole rumen fluid represents the combined influence of bacteria and protozoa, and potential synergistic effects. It should be noted that the bacterial fraction, as defined by centrifugation at 500 x g, can exclude large and feed associated bacteria that would limit the evaluation of cell-associated lytic factors. Additionally, feed particles would be excluded that may limit the amount of soluble carbohydrates available for fermentation. In whole rumen fluid *P. pastoris* experienced a loss of 27.97% by 24 h and 74.78% by 36 h. These are acceptable disappearance rates given the average rumen retention time of 4-10% h<sup>-1</sup> for a microbe which associates with the fluid phase or 1-7% h<sup>-1</sup> for a microbe which associates with the particulate fraction of rumen fluid (Church, 1988). We have observed that *P. pastoris* has a tendency to associate with feed particles and clump together, however the extent of association and resulting ruminal retention time for *P. pastoris* has not been evaluated.

A more representative *in vitro* evaluation was performed in a continuous open system fermentation that includes more realistic factors such as altered population

demographics, interactions with feed substrates, diurnal feeding, and the presence of fluid outflow. The Rusitec was first adapted for seven days to develop a stable ruminal population, prior to the introduction of the *Pichia* escape vehicle. Each day the *Pichia* inoculum was added to the vessel at the time of feeding to simulate it appearing as a feed additive and the outflow collection containers were sampled and enumerated (24 h after the previous inoculation) as a quantification of *Pichia* cells escaping the vessel. Green fluorescent *P. pastoris* cells were recovered on the first day, but not on subsequent days when sampled at the time of feeding. This is indicative of a shift in the microbial population or, at very least an increase in predatory organisms most likely due to the abundance of *P. pastoris* as a favorable substrate. Although we have not been able to identify the factors responsible for the loss of *Pichia*, at no time did we observe protozoal predation as would have been indicated by the appearance of engulfed green fluorescent cells. Furthermore, despite not seeing individual cells engulfed within a protozoan, if *Pichia* was actively predated it should have accumulated within the protozoal population and multiple fluorescent *P. pastoris* cells should have been visible. It should be noted that only intact cells would have fluoresced within protozoa as cellular lysis would result in the exposure of the protein-based GFP chromophore to proteolysis and loss of its associated green fluorescence. Thus green fluorescence would not have been transferred to the whole organism.

Enumeration of *Pichia* populations through the 24 h time course demonstrated that 18.92% of the initial inoculum escaped the Rusitec intact prior to loss of the population by 12 h. Failure to detect fluorescent cells in the effluent collection containers at the time of feeding indicates that we were unable to prevent lysis in the effluent collection containers despite attempts to preserve the culture with mercuric chloride. Additionally we must consider that the Rusitec is not an ideal representation of the microbial populations present in the rumen as shown by the fact that many protozoal species are typically lost from the Rusitec (Hillman *et al.*, 1991).

Successful escape of cellular encapsulation REVs from the rumen must be followed by abomasal or duodenal lysis in order to release the intracellular protein. Lysis studies conducted in simulated gastric juice showed that 68.32% of the BMGH/BMMH *P. pastoris* population was lysed within 6 h, reaching 83.67% by 12 h. This rate of lysis may be acceptable when we consider the average abomasal rate of passage is between 5.75% and 39.25% h<sup>-1</sup> (Church, 1988) Maximal enzyme activity is typically achieved when the enzyme is not hindered by any interfering matrix. At this time, we report only the lysis of the *P. pastoris* membrane and the exposure of the GFPuv protein to either diffusion or peptic degradation which results in the loss of visual cellular fluorescence. Dissociation of an intracellular protein from the cell matrix is largely dependent on the characteristics of the given protein, thus the degree of dissociation, resulting activity and ultimate benefit to the animal must be evaluated for each expressed protein. It should be further noted that this gastric simulation was performed using fresh *P. pastoris* cultures that had not been exposed to ruminal degradation. Cell walls and membranes would most likely be partially degraded in the rumen, thereby contributing to a faster abomasal lysis rate. When ruminal and abomasal degradation is combined with duodenal lysis, the result would likely be the complete lysis of all cells escaping the rumen.

We cannot fully evaluate *P. pastoris*'s potential as a REV by only using *in vitro* techniques. The rumen is largely affected by diet, as are other characteristics of the digestive tract such as dilution rates, gastric pH, and rates of intestinal absorption; all of which will have an effect on a REV. Thus only animal trials will ultimately determine its value. The value of a REV is dependent on its rumen escape rate, the production value of the expressed protein, the expression level of that protein within the REV, and cost of producing the REV, which determines the amount that can be economically fed. For example a polysaccharide-degrading enzyme may be required in relatively large amounts in order to achieve a production response (Hristov *et al.*, 1998), whereas a

smaller amount of a protein rich in limiting amino acids may be needed to show an equivalent response (Titgemeyer and Merchen, 1990). Furthermore a bio-active peptide, such as epidermal growth factor, may only require a minute amount to achieve a considerable production response (Buret *et al.*, 1998). Thus if animal trials with *P. pastoris* show an 18.92% ruminal escape rate followed by a 83.67% abomasal lysis rate and the protein is completely dissociated from the cellular matrix, then the effectiveness of the REV would be 15.83% of the inoculum (ruminal escape • abomasal lysis • degree of dissociation = 0.1892 • 0.8367 • 1.0). Depending on the amount fed and the cost of production, this amount may be more than enough to see a profitable production response from some proteins (Appendix 7.8).

There is a secondary benefit to using *P. pastoris* as a REV, that being the length of time it takes *P. pastoris* to lyse within the abomasum. Many proteins are degraded by the abomasum (Campbell and Bedford, 1992), some of which may be beneficial if they reached the small intestine intact or in greater amounts. The time that it takes *P. pastoris* to lyse, effectively protects the intracellular protein from gastric degradation during that time, and may increase the amount of undegraded protein reaching the small intestine, depending on the protein's susceptibility to proteolysis.

There is significant potential for a *P. pastoris* REV to provide abomasal protected protein (APP) to milk replacer calves, especially if the REV contained protein-based vaccines, probiotics, bacteriocins, or growth-promoting bio-active peptides. There is also potential for the use of a REV in providing APP to swine, as many enzymes are inactivated by the gastric environment (Baas and Thacker, 1996). Additionally, the use of a REV in swine nutrition could provide more consistent responses to enzyme supplementation by reducing the length of time enzymes are exposed to gastric hydrolysis, proteolysis, and denaturation, which is believed to be the primary cause of enzyme inactivation (Thacker, 2000).

A REV such as *P. pastoris* has the tertiary benefit of being a species in which many

proteins are currently commercially produced. The direct feeding of the expression vehicle has measurable impact on the production cost of an enzyme cocktail as expensive downstream processing, formulation, and stabilization procedures are eliminated. Protein production and processing is the most expensive aspect of rumen bypass methods such as polymer coating (Clark, 1975).

Cellular encapsulation using the ruminally foreign yeast expression system *P. pastoris* has significant potential to act as a ruminal escape vehicle for the delivery of bio-active peptides, enzymes, and high-value protein to the small intestine of ruminants. *P. pastoris* effectively fills all the requirements as a successful ruminal escape technology, however animal trials must be conducted to confirm this potential still exists *in vivo*. Additionally, the *P. pastoris* REV has potential applications in the protection of protein from abomasal degradation in ruminant and monogastric production. In conclusion, *P. pastoris* may be an economical vehicle to produce high value biologically active proteins and act as a ruminal escape vehicle for their transport to the small intestine.

## 5.6 References

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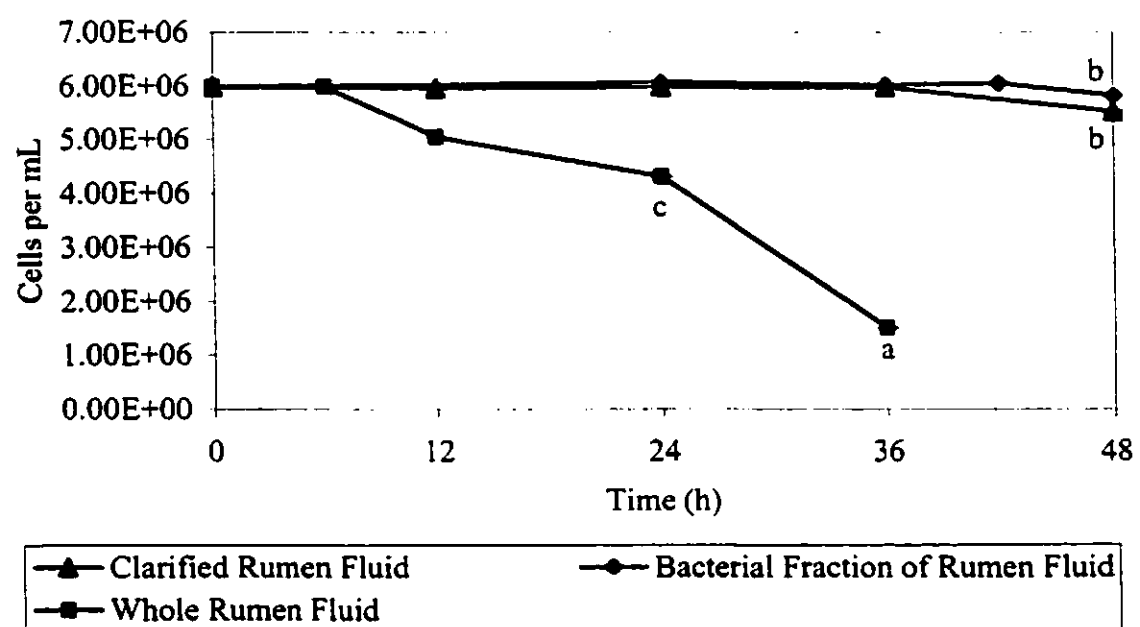


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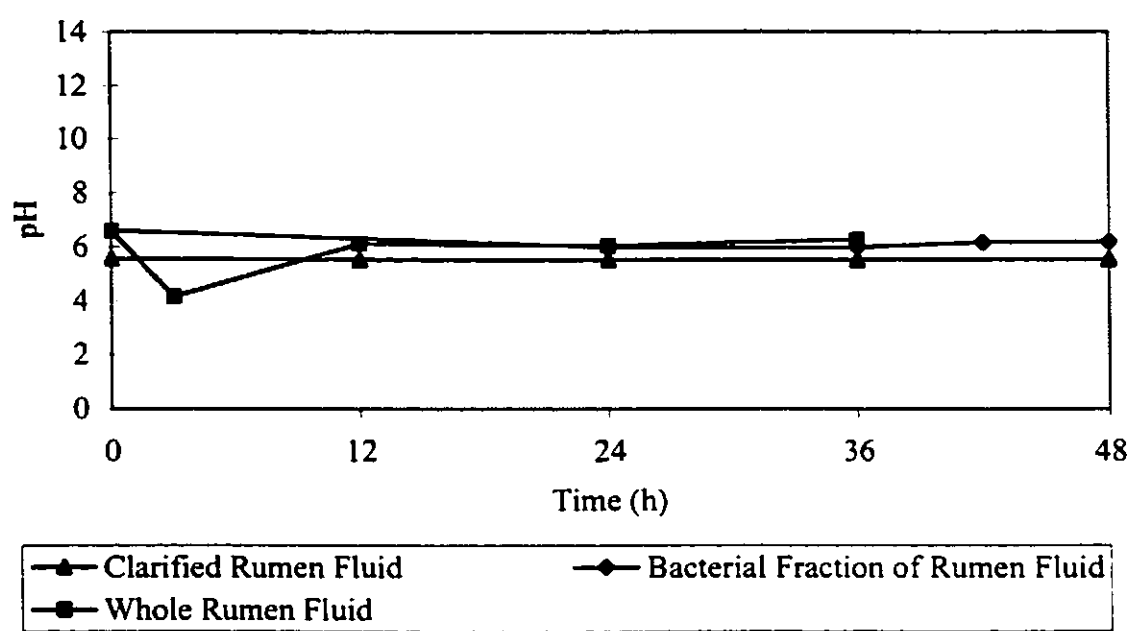
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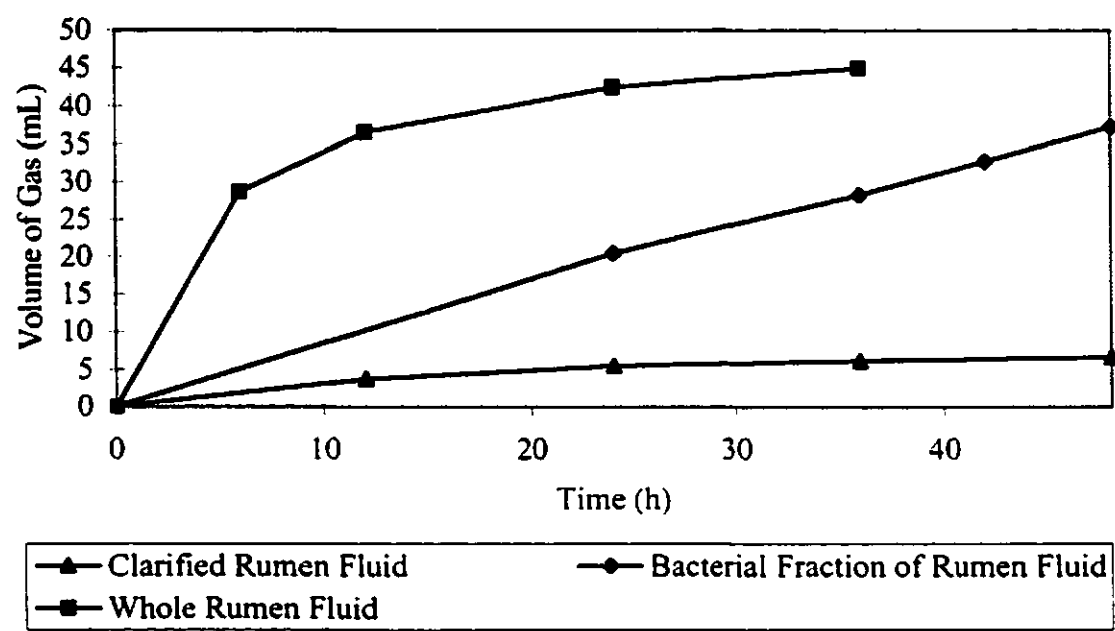
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**Figure 5.1** Survival of BMGH/BMMH *P. pastoris* cells in batch culture fractionated rumen fluid *in vitro* incubations. The letter “a” denotes  $p < 0.0001$ , “b” denotes  $p < 0.001$ , “c” denotes  $p < 0.01$ . Bars represent standard error of the mean,  $n=3$ . In all cases, SE bars are present and may be hidden beneath the symbol.



**Figure 5.2** pH of batch culture fractionated rumen fluid *in vitro* incubations as measured at each time point. Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.

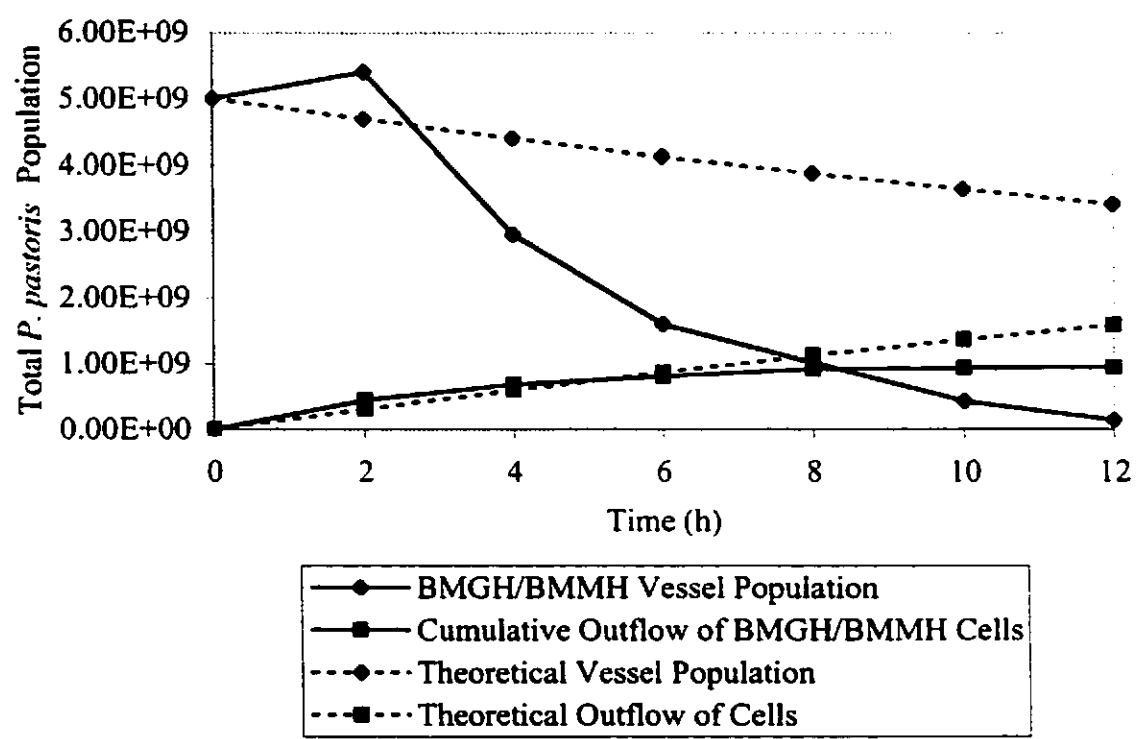


**Figure 5.3** Cumulative gas production of batch culture fractionated rumen fluid *in vitro* incubations as measured at each time point.

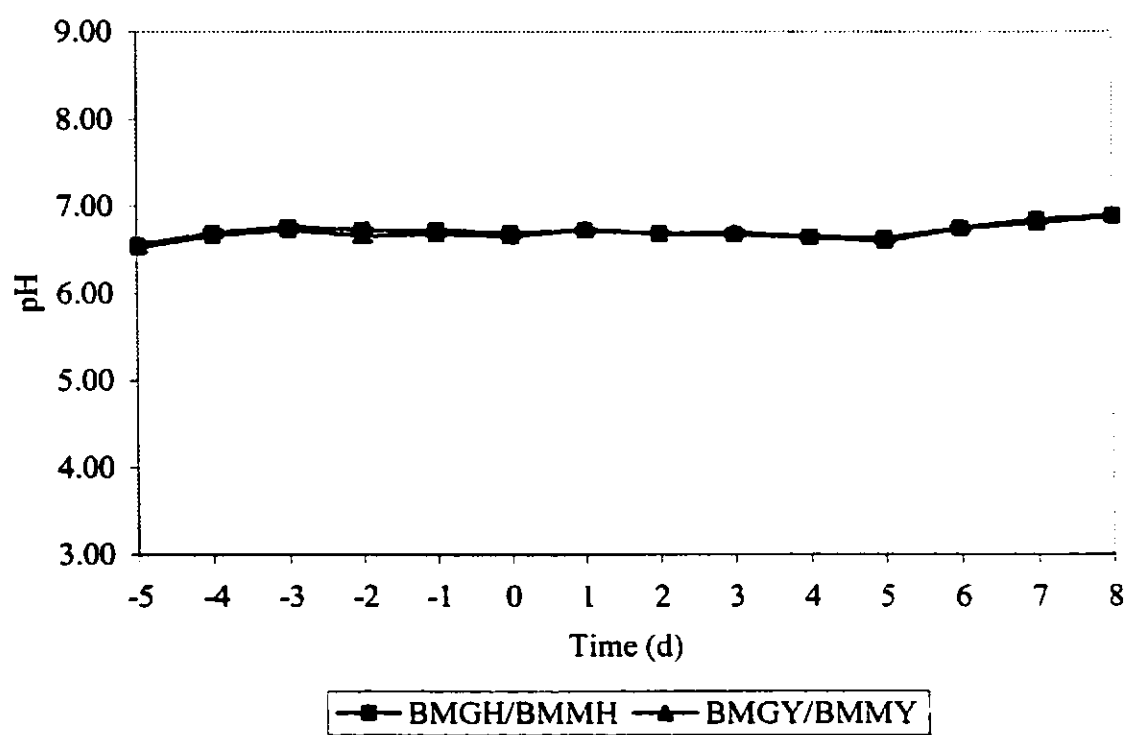
<b><u>Time (d)</u></b>	<b><u>BMGH/BMMH Outflow</u></b>	<b><u>BMGY/BMMY Outflow</u></b>
1	1.12x10 <sup>9</sup>	8.55x10 <sup>8</sup>
2	not detected	not detected
3	not detected	not detected
4	not detected	not detected
5	not detected	not detected
6	not detected	not detected
7	not detected	not detected

**Table 5.1** Outflow of *P. pastoris* (total cells) from the Rusitec vessel as monitored from day one to day seven of supplementation, as sampled at the time of feeding. Data represents n=4.

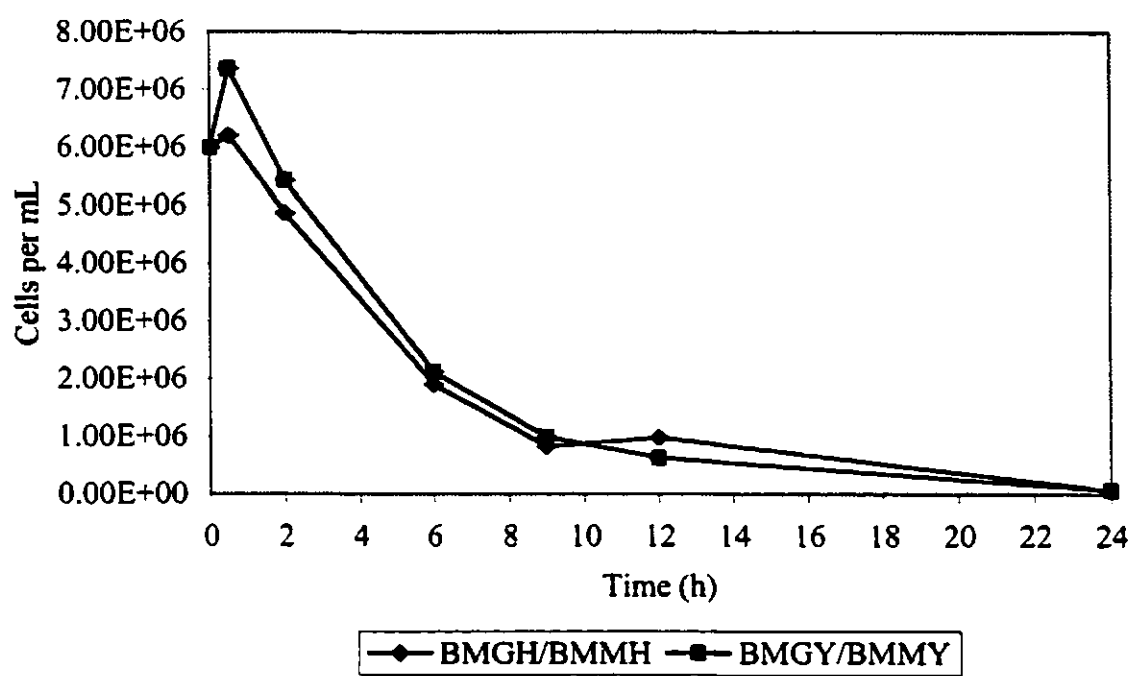




**Figure 5.4** Outflow of fluorescent *P. pastoris* cells from one Rusitec vessel as monitored from 0 h to 12 h post-supplementation on day eight. The theoretical lines are derived from a vessel dilution rate of 0.75 day<sup>-1</sup>.



**Figure 5.5** pH of Rusitec vessels from day one of adaptation to day eight of supplementation, as measured at the time of feeding. Bars represent standard error of the mean, n=4. In all cases, SE bars are present and may be hidden beneath the symbol.



**Figure 5.6** Lysis of *P. pastoris* in abomasal *in vitro* incubations. Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.

Bio-active proteins and peptides fed to ruminants are subject to ruminal proteolysis and seldom escape the rumen in an active form. Traditional methods of ruminal protein protection such as treatment with formaldehyde, tannins, or heat, are unsuitable for bioactive proteins, such as enzymes, because the protection process renders the protein biologically inactive.

Cellular encapsulation is another approach under consideration for delivering bio-active proteins to the small intestine of ruminants (Smith and Hespell, 1983). In this method proteins are produced by the cell, stored in the cytosol, and thereby protected by the plasma membrane from degradation. Recombinant protein can be produced and protected in this manner through intracellular expression in plant or microbial cells. Cellular encapsulation may allow for the oral administration, ruminal escape, abomasal lysis and ultimately the effective delivery of intracellular recombinant protein to the small intestine of ruminants. The ruminally foreign yeast expression system *Pichia pastoris*, as a cellular expression and encapsulation technology, was evaluated for its potential to serve as a ruminal escape vehicle (REV).

For a cellular encapsulation technology to succeed as a REV it must maintain cellular integrity throughout its residency in the rumen, lyse within the abomasum, and dissociate from the intracellular recombinant protein for full duodenal activity. Thus we must employ a means of monitoring cellular integrity in order to evaluate potential encapsulation technologies.

Intracellular expression of green fluorescent protein (GFP) allows for the monitoring of cellular integrity in proteolytic environments as the plasma membrane isolates the protein-based fluorescent chromophore from proteolytic degradation. Upon the loss of cellular integrity, the chromophore is exposed to extracellular proteases resulting in the degradation of the chromophore and its associated fluorescence. Selective plate count

methods are not an accurate measure of cellular integrity as non-culturable cells with an intact membrane cannot be detected. This is confirmed by our work which demonstrated that viable plate counts of *P. pastoris* cells cultured in BMGH/BMMH indicated a 92% loss by 24 h and 99.8% loss by 48 h whereas GFP epifluorescent microscopy confirmed that cellular integrity was lost in only 3% of the population.

Epifluorescent microscopy of rumen fluid samples revealed that the rumen contains many microorganisms and particles that are highly autofluorescent, and as such may interfere with visualization of cells containing GFP. Ruminal visualization of GFP may be enhanced by the selection of a GFP variant that is more conducive to visualization in the ruminal environment. Epifluorescent visualization of encapsulated GFP can be optimized through variant and light source selection, which is best achieved by analyzing the actual excitation spectra as determined by multiplying the GFP variant's quantum corrected excitation spectrum by the bulb emission spectrum. Our study was limited to use of a mercury lamp, thus transformational analysis of GFP variants excited with this light source demonstrated that variant GFPuv was the most suited for visualization in rumen fluid when employing mercury excitation. Based on emission spectral analysis, filter sets can be designed for the specific excitation spectrum, thereby limiting the excitation of autofluorescent compounds to the greatest extent.

Using the model system of intracellular GFPuv expression, *P. pastoris* was incubated in batch culture fractionated ruminal *in vitro* simulations to evaluate the effect of culture conditions of *P. pastoris* survival in rumen fluid. Several conditions were found to have an effect on membrane integrity including the initial inoculation rate of the culture, the type of culture medium, and the *P. pastoris* strain when optimally cultured, although it is not known if this was due to the rate of methanol utilization or other strain specific differences. The presence of a buffering agent and temperatures typical of *P. pastoris* induction did not appear to have an effect on membrane integrity.

Optimally cultured *P. pastoris* cells incubated in rumen fluid fractions experienced loss rates of only 8% in clarified rumen fluid by 48 h, 3% in bacterial fraction (500 x g) of rumen fluid by 48 h, and 75% in whole strained rumen fluid by 36 h. These are acceptable loss rates provided the rumen has a high rate of motility. Incubation in a fully adapted Rusitec with a dilution rate of 75% d<sup>-1</sup> revealed that 19% of the daily inoculum successfully escaped the Rusitec prior to the loss of the population by 12 h.

Cellular encapsulating REV's must be lysed in the abomasum in order to release the intracellular protein for duodenal delivery. Incubation of fresh *P. pastoris* cells in simulated gastric juice indicated that *P. pastoris* was effectively lysed in the gastric environment achieving 68% lysis by 6 h, and 84% by 12 h. It is likely that all *P. pastoris* cells entering the ruminant abomasum will be quickly lysed given that the cell membrane was previously exposed to ruminal degradation. Furthermore, lysis would likely continue beyond the abomasum, however it must occur at or prior to the intended target site of the intracellular recombinant protein. It should be noted that the protein must fully dissociate from the cellular matrix in order to achieve optimal biological activity. The full extent of duodenal delivery and cellular dissociation can only be determined through *in vivo* animal trials with *P. pastoris* expressing a protein with a measurable production response.

One such protein which may achieve a production response is the bio-active peptide epidermal growth factor (EGF), a gastronomic polypeptide. Buret *et al.* (1998) hold a patent for the use of EGF as a gastrointestinal therapeutic agent that is described to have an effect on the weight gain of rabbits. Using the data presented within the patent, it is possible to consider the potential of using an EGF containing REV in cattle diets to increase the rate of gain. EGF has also been proposed as a therapeutic agent for the treatment and prevention of enterococcal infections such as calf scours (Buret *et al.*, 1998). In rabbits EGF was orally administered at a rate of 60 µg/kg (body weight) per day which resulted in a reduction of *Escherichia coli* colonization by 62% and prevented

the occurrence of diarrhea. Treatment of a 75 kg milk-replacer-fed calf at the recommended dose of 60 µg/kg would require 4.5 mg of EGF per day as delivered to the small intestine. Mouse EGF has been expressed in *P. pastoris* by Clare *et al.* (1991) at a rate of 0.45 g/L. Assuming a similar expression rate for bovine EGF and that the 16% of the *P. pastoris* REV actually reaches the small intestine, we can infer that 4.5 mg of EGF can be delivered by  $5.92 \times 10^{10}$  cells (7.96 g dry weight) of the *P. pastoris* EGF REV when provided as a daily supplement at a cost of 19 to 25 cents per day (Appendix 7.9). This may be a highly effective means of treating and preventing intestinal infections in small calves.

Another possibility is the inclusion of an amylolytic or fibrolytic enzymes in the *P. pastoris* REV. It has been postulated that post-ruminal digestion is limited by structural polysaccharides which shield energy and protein deposits from digestion (Orskov, 1991). Enzymes such as xylanase, cellulase and β-glucanase may enhance the degradation of these structural polysaccharides, which putatively limit digestion (Hristov *et al.*, 1998). Post-ruminally, starch is enzymatically digested by α-amylase, which hydrolyzes amylose and amylopectin into oligosaccharides and limit dextrans (Huntington, 1997). Theoretically, enzymatic digestion of starch in the small intestine is 42% more efficient than ruminal digestion of starch (Owens, 1986), however digestion in the small intestine appears limited by the supply of pancreatic amylase (Huntington, 1997). Post-ruminal supplementation of α-amylase, possibly in combination with other fibrolytic enzymes, may provide a means of increasing starch and fibre digestion in the small intestine and enhancing feed efficiency.

While structural polysaccharides limit feed efficiency, production on the other hand is limited by deficiencies of essential amino acids. Rumen bacterial protein is the major source of dietary protein and is insufficient to meet the amino acid demands of high producing dairy cows (Schwab *et al.*, 1992). Microbial protein is deficient in methionine, lysine, threonine, and leucine, as such these amino acids are believed to

limit the quantity and quality of milk synthesis (Munneke *et al.*, 1991; King *et al.*, 1990). Consider the potential of utilizing *P. pastoris* as a REV for the delivery of limiting amino acids to the small intestine for absorption, utilization and fulfillment of amino acid requirements. A novel protein with an enriched amino acid profile was designed by Beauregard *et al.* (1995) to serve as a ruminal bypass protein. This protein, Milk Bottle 1 Protein (MB1) could be expressed in *P. pastoris* to provide additional protection against degradation. Given that 84% of the *P. pastoris* cells do not reach the abomasum intact there is nevertheless an inherent benefit to MB1 survival given that the protein was protected for a portion of its residency time in the rumen and thus has a greater chance of reaching the small intestine than unprotected MB1 protein. Through the use of a ruminally resistant protein, such as MB1, expressed in *P. pastoris* we could exceed 15.83% of the supplemented protein reaching the small intestine. Additionally, it should be noted that MB1 has been redesigned yielding MB15/13 which appears to be more resistant to ruminal degradation than MB1 (R. Teather, personal communication). *P. pastoris* has significant potential to enhance ruminant production through the elimination of amino acid deficiencies by delivering high-value proteins to the small intestine of high-producing dairy cattle.

In addition to providing protection from ruminal degradation, there is potential for *P. pastoris* to protect protein from abomasal degradation. Many proteins, such as cellulases and  $\beta$ -glucanases, are susceptible to peptic degradation (Hristov *et al.*, 1999). Oral administration of these proteins in hogs result in the inactivation of significant amounts of enzyme (Thacker, 2000), thus requiring the feeding of increased amounts, thereby increasing the cost of achieving a production response. Production of these enzymes in *P. pastoris* and delivery in swine diets could substantially decrease the length of time enzymes are exposed to abomasal degradation as the cell would provide protection until it is lysed. The protection that *P. pastoris* provides may allow for sufficient quantities of enzyme to reach the small intestine prior to peptic inactivation.



While we have shown that *P. pastoris* has significant potential as a REV, we must in all fairness address the challenges facing its arrival in the marketplace. The first issue to be resolved is the form which *P. pastoris* is to be distributed as, be that liquid, solid, or crumbled. Fleischmann's Yeast Corp. (Fenton, MO) has developed a technique for the preparation of yeast culture in a dry, loose form which is made by squeezing the culture paste through a rotary drum and air drying to a specified moisture content (Fleischmann's Yeast Corp., personal communication). Yeast culture preserved in this manner remains active for up to three years, however it is unknown if this process is feasible for a yeast REV preparation. In our application it is critical that the intracellular protein is stable and not degraded over time. Additionally the preparation method must not interfere with the yeast's ability to withstand the ruminal environment. Finally, a protocol for the large-scale fermentation must be developed using the culture conditions defined as a result of the *in vitro* experiments. Thus, before *P. pastoris* can be used in large scale trials, processes for the scale-up and product preparation must be developed in such a way that *P. pastoris* maintains the ability to escape the rumen, lyse in the abomasum and retain a suitable shelf-life.

A recombinant cellular encapsulation technology employed as a REV is fundamentally a genetically modified organism (GMO) and as such issues surrounding its safety, ethics of its use, and environmental risk must be considered prior to approval for use in ruminant diets. Historically, *P. pastoris* has been used as a source of single-cell protein for human and animal diets (Wegner, 1990), and thus as an organism should not itself pose a barrier to regulatory approval. The introduction of new genetic material makes *P. pastoris* an unique organism and the potential effect of the expressed protein must be considered both on the animal and on the environment (Bruggemann, 1993). The effect of a *P. pastoris* REV on the animal is dependent on the effect of the expressed protein on the animal and on the protein's effect on *P. pastoris* which may in turn affect the animal. Any live GMO microbe has the potential to escape into the environment

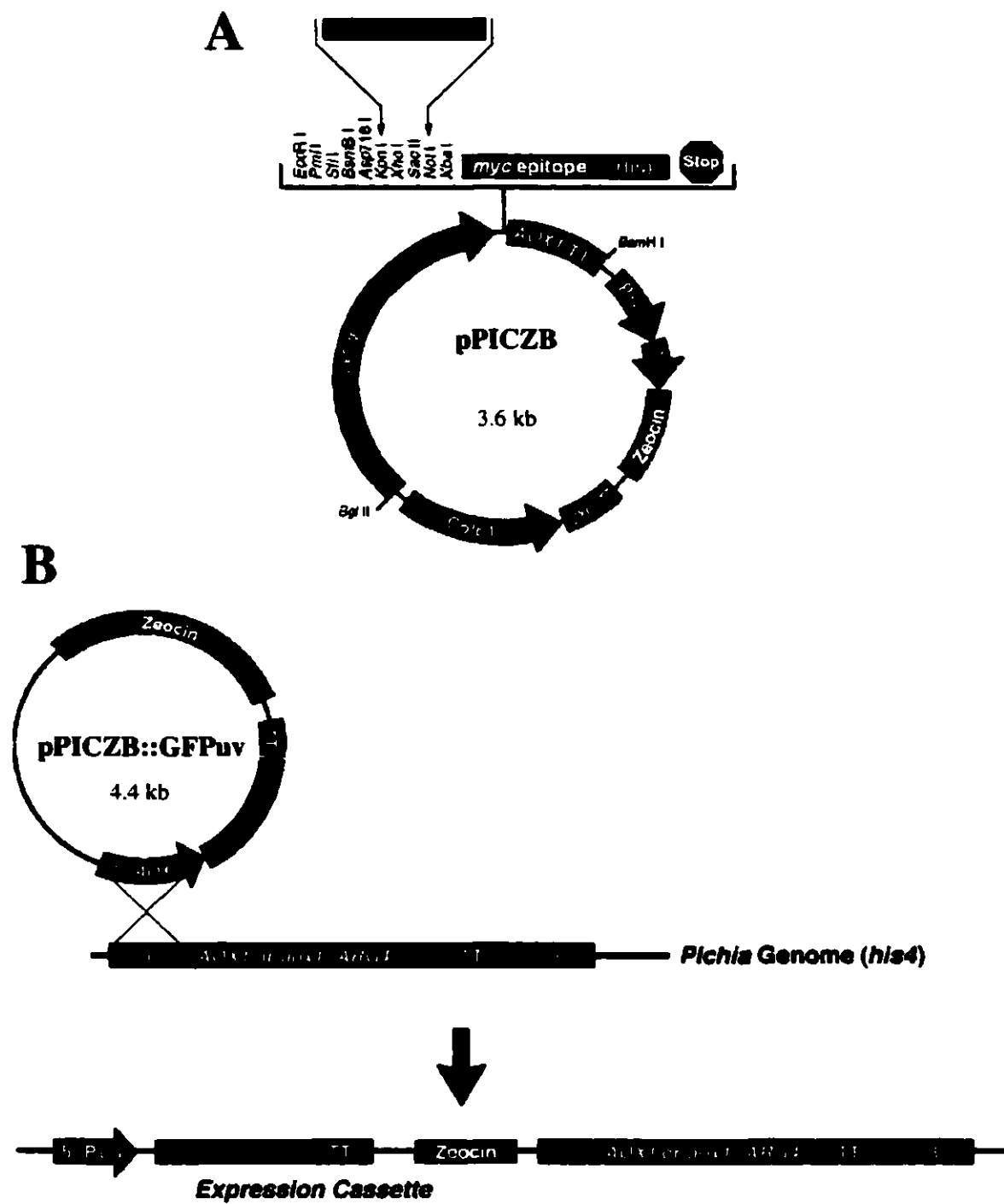
beyond its intended areas, such as through live cells being deposited in the feces, bunk or bedding scrapings, and discarded packaging containers. Therefore the ability of genetically modified *P. pastoris* to persist, multiply, and alter the environment must be evaluated.

Cellular encapsulation using the ruminally foreign yeast expression system *P. pastoris* has significant potential to act as a REV for the delivery of bio-active peptides, enzymes, and high-value protein to the small intestine of ruminants. Additionally, the *P. pastoris* REV has potential applications in the protection of protein from abomasal degradation in ruminant and monogastric production. More work, however, is required to define techniques to produce sufficient quantities of cells for field trials.

#### 6.1 References

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**Given (empirical data):**

**Petroff Hauser Chamber dimensions:**

$$= 0.2 \text{ mm} \cdot 0.2 \text{ mm} \cdot 0.2 \text{ mm}$$

**White light count per hemocytometer square**

$$= 48 \text{ cells/square}$$

**The Field of View (FOV) is the total visible area under the microscope**

$$= 100 \text{ cells/FOV}$$

**Derivation:**

**The number of hemocytometer squares within the Field of View =**

$$= \text{White Light Count} \div \text{Field of View Count}$$

$$= 100 \text{ cells/FOV} \div 48 \text{ cells/square}$$

$$= 2.083 \text{ squares/FOV}$$

**The area of each hemocytometer square =**

$$= \text{hemocytometer square width} \cdot \text{hemocytometer square length}$$

$$= 0.2 \text{ mm} \cdot 0.2 \text{ mm}$$

$$= 0.04 \text{ mm}^2/\text{square}$$

**The area of the Field of View =**

$$= \text{squares/FOV} \cdot \text{area of the square}$$

$$= 2.083 \text{ squares/FOV} \cdot 0.04 \text{ mm}^2/\text{square}$$

$$= 0.083 \text{ mm}^2/\text{FOV}$$

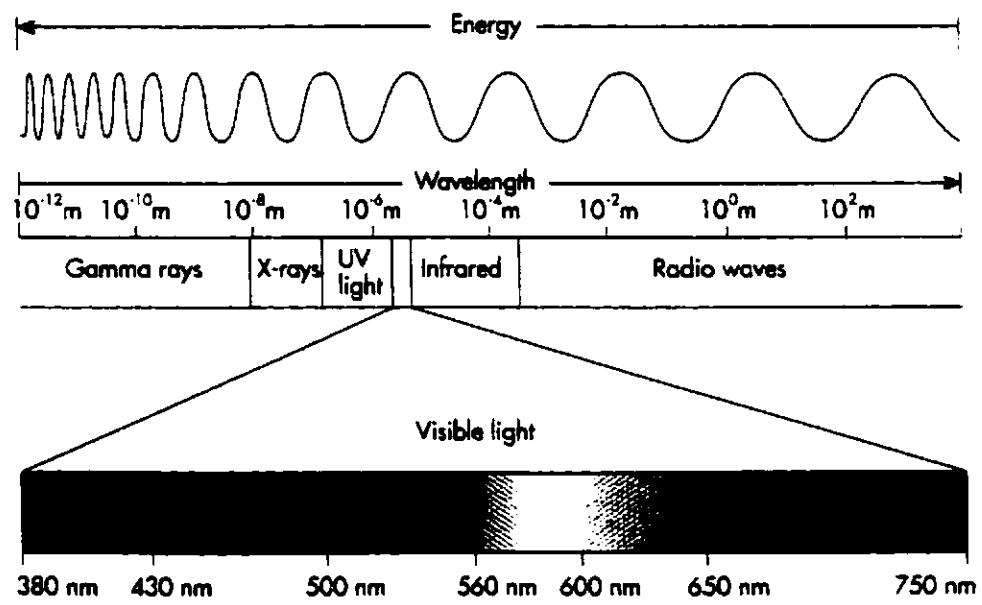
**The volume of the Field of View =**

$$= \text{FOV area} \cdot \text{hemocytometer depth}$$

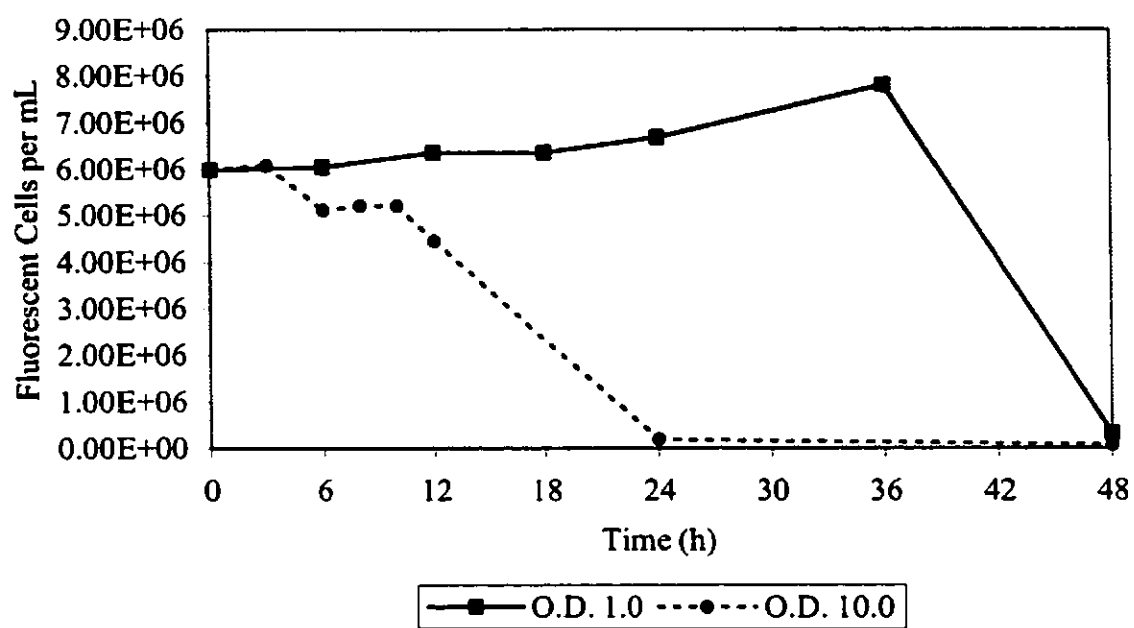
$$= 0.083 \text{ mm}^2/\text{FOV} \cdot 0.2 \text{ mm}$$

$$= 0.0167 \text{ mm}^3 \text{ or } 0.0167 \text{ } \mu\text{l} \text{ or } 1.67 \times 10^{-5} \text{ mL}$$

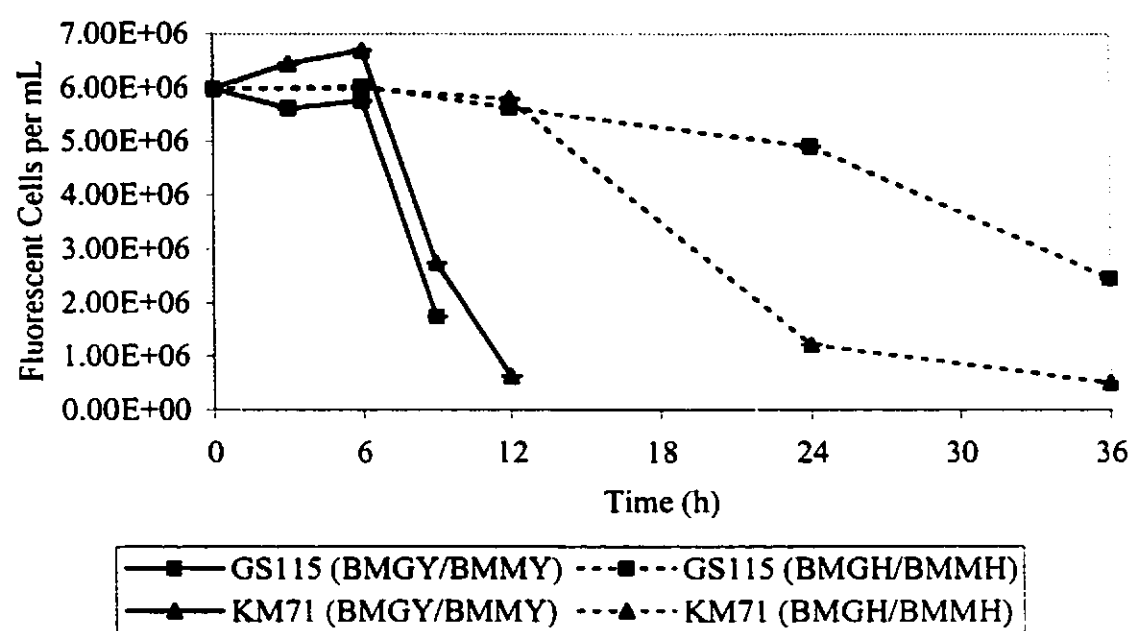
**Appendix 7.2 Determination of volume under the field of view using a 0.2 mm deep Petroff 1/5th 3900 cell counting chamber.**



**Appendix 7.3** Electromagnetic radiation spectrum. Adapted from Atlas, R. M. 1997. Principles of Microbiology. Second ed. WCB McGraw-Hill, New York, NY. 576 pp.

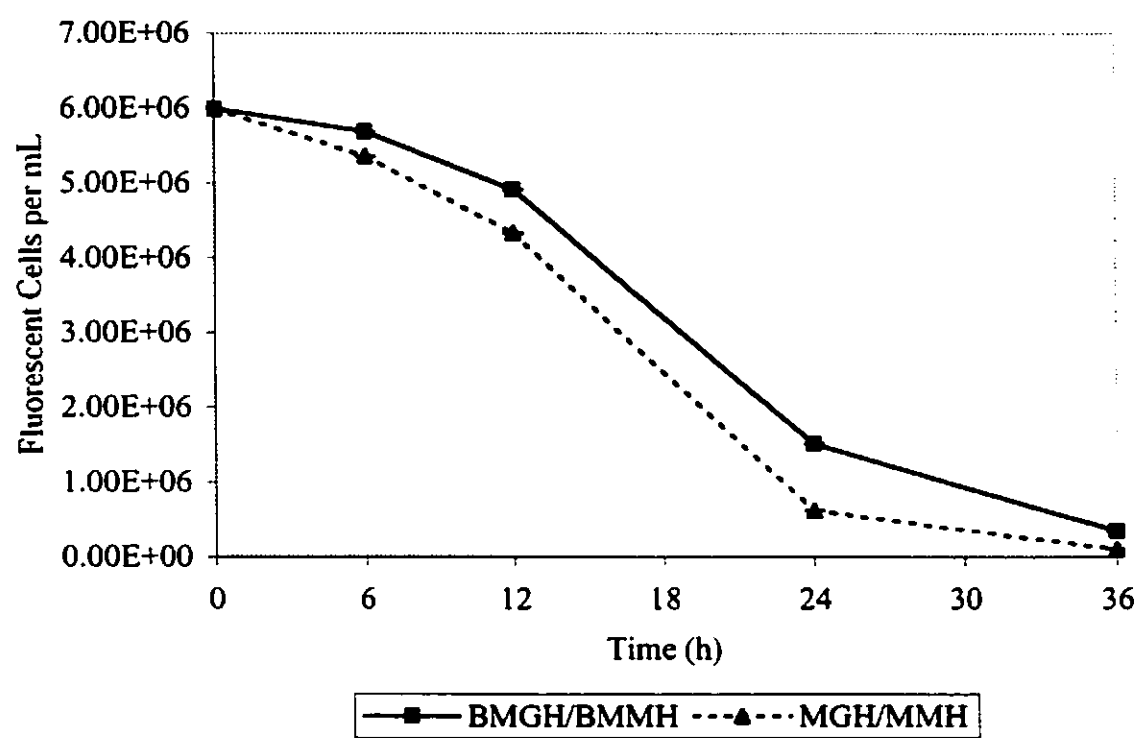


**Appendix 7.4** Evaluation of the effect of the initial inoculation rate of the induction culture on the survival of fluorescent *P. pastoris* cells in batch cultures (refer to Section 5.3.4 for methodology). Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.

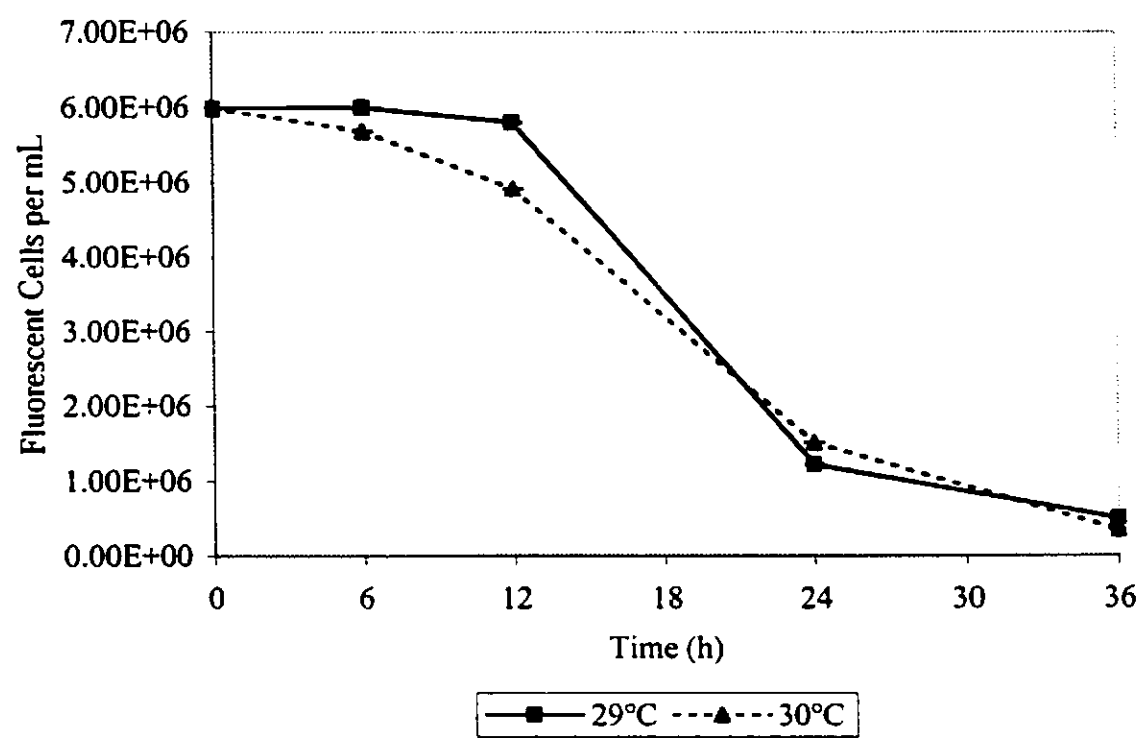


**Appendix 7.5** Evaluation of the effect of strain (KM71, GS115) and culture media (BMGY/BMMY, BMGH/BMMH) on the survival of fluorescent *P. pastoris* cells in batch cultures (refer to Section 5.3.4 for methodology). Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.





**Appendix 7.6** Evaluation of the effect of buffering agent in the induction media on the survival of fluorescent *P. pastoris* cells in batch cultures (refer to Section 5.3.4 for methodology). Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.



**Appendix 7.7** Evaluation of the effect of culture temperature on the ruminal survival of fluorescent *P. pastoris* cells in batch cultures (refer to Section 5.3.4 for methodology). Bars represent standard error of the mean, n=3. In all cases, SE bars are present and may be hidden beneath the symbol.

**Alberta Research Council production costs:**

\$44,000 for a 15,000 L three day fermentation (Mut<sup>+</sup>) or \$2.93/L

\$60,000 for a 15,000 L seven day fermentation (Mut<sup>s</sup>) or \$4.00/L

**Lethbridge Research Center production costs:**

\$240.92 for a 4.5 L four day fermentation or \$55.76/L

**Given:**

the average fermentation yield of 450 g/L (wet weight)

1 g wet weight is equivalent to 0.28 g dry weight

1 g (dry weight) is equivalent to  $4.7 \times 10^{10}$  cells

**Thus:**

3 day Mut<sup>+</sup> fermentation costs \$2.93/ $5.92 \times 10^{12}$  cells,  $2.02 \times 10^{12}$  cells/\$1

7 day Mut<sup>s</sup> fermentation costs \$4.00/ $5.92 \times 10^{12}$  cells,  $1.48 \times 10^{12}$  cells/\$1

**Given a rumen dosage rate of  $6.0 \times 10^6$  cells/mL and a rumen volume of 80 L:**

$4.8 \times 10^{11}$  cells/day cost \$0.24/day for a Mut<sup>+</sup> strain

$4.8 \times 10^{11}$  cells/day cost \$0.32 for/day a Mut<sup>s</sup> strain

Feeding  $4.8 \times 10^{11}$  cells/day is equivalent to 10.22 g/day (dry weight) of *P. pastoris* per cow per day. Given that 15.83% of the inoculum actually escapes the rumen, these  $7.6 \times 10^{10}$  cells/day must provide a production value of more than \$0.24/day for a Mut<sup>+</sup> or \$0.32/day for a Mut<sup>s</sup>.

**Appendix 7.8** Economic evaluation of the production costs associated with the use of *P. pastoris* as a ruminal escape vehicle.

Given:

the average fermentation yield of  $5.92 \times 10^{12}$  cells/L

putative expression of bovine EGF at 0.45 g/L

\$1 of a Mut<sup>+</sup> fermentation is  $2.02 \times 10^{12}$  cells

\$1 of a Mut<sup>S</sup> fermentation is  $1.48 \times 10^{12}$  cells

*P. pastoris* REV effectiveness is 15.83%

75 kg steer

Production response received from a duodenal EGF concentration of 60 µg/kg

Thus:

4.5 mg of bEGF to see a production response

bEGF is expressed at a concentration of  $7.6 \times 10^{-14}$  g per cell

4.5 mg of cells can be produced by  $5.92 \times 10^{10}$  cells

4.5 mg of cells can be duodenally delivered in  $3.74 \times 10^{11}$  cells

a Mut<sup>+</sup> bEGF strain would cost \$0.19 per day

a Mut<sup>S</sup> bEGF strain would cost \$0.25 per day

Given that 15.83% of the inoculum actually escapes the rumen,  $3.74 \times 10^{11}$  cells per day or 7.96 g per day (dry weight) of *P. pastoris* must be fed to a 75 kg steer to have a therapeutic effect on enterococcal infections at a cost of \$0.19 per day

**Appendix 7.9** Evaluation of the amount and costs associated with the use of EGF *P. pastoris* REV to have a therapeutic effect on enterococcal infections in a 75 kg steer.

## **Epilog**

The reasonable microbiologist adapts himself to the microbial world. The unreasonable one persists in trying to adapt the microbial world to himself. Therefore, all progress depends on the unreasonable microbe.

- D. Mirelman