Chapter 6
Screening for Compounds Enhancing Fibre Degradation

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Introduction

Ruminants have a unique capacity to utilize ligno-cellulosic feeds as a major component of their diet to get energy for their survival. They are also able to utilize non-protein nitrogen sources for the synthesis of microbial protein in the rumen. These tasks are accomplished in the rumen by a complex consortium of rumen microbes that live in an ecto-symbiotic relationship with the host animal. This microbial eco-system consists of bacteria, archaea, protozoa, fungi, mycoplasmas and bacteriophages. These microbes are a mixture of micro-aerophilic, facultative and obligate anaerobes that utilize a minimal amount of feed energy for their survival and conserve more than 85% of gross energy intake in the form of volatile fatty acids which are used by the host as a source of energy. The rumen contains microbes that represent a rich pool of a large number of highly active fibrolytic enzymes. The extraction of energy from the ligno-cellulosic feed is only partial and a substantial amount of dietary energy remains unutilized due to resistance of ligno-cellulose to digestion. Digestion of ligno-cellulose may be limited by the ability of enzymes to gain access to target substrates or by direct inhibition arising from the presence of anti-nutritional factors. Cereal straws, sugarcane tops, green forages and a variable amount of cereal grains are commonly used as feeds for ruminants. Enhancing the digestibility of ligno-cellulosic feeds will be beneficial to that population of ruminants that rely on high-fibre agricultural by-products as staple feed for production.

The physical and chemical characteristics of ligno-cellulosic feeds, such as degree of crystallinity of cellulose, extent of lignification and levels of other anti-nutritional factors, vary considerably. The microbial consortium of the rumen is highly sensitive to the type of diet fed to the animal and therefore the enzyme

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profile changes. Tajima et al. [18] reported a decrease in cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*), hemi-cellulolytic bacteria (*Eubacterium ruminantium)* and an increase in starch-utilizing bacteria (*Prevotella ruminicola,* 7 fold and *P. bryantii,* 263 fold) by shifting the diet from hay to grain. The variations in rumen microbial and enzyme profile with changing ratio of concentrate to roughage have been reported by Kamra et al. [9] and Agarwal et al. [2]. Their results indicated increased carboxymethylcellulase (CMCase) and xylanase activities by increasing the level of roughage in diet of buffalo. In another in vitro experiment, Agarwal et al. [1] reported that the activities of CMCase and xylanase were significantly higher with maize than with lucerne as substrate, which corresponded to the higher fibre content in maize. Similarly, Hristov et al. [7] and Martin and Michalet-Doreau [13] observed a decrease in cellulase and xylanase activities and increase in amylase activity by shifting the diet from forage to high grain. Increased lignin and nitrogen contents in the diet stimulated cellulase activity in the rumen [15]. It appears from various studies that the enzyme profile of the rumen is dependent on diet composition and that the nature of this profile changes depending on the digestibility of feed. Consequently, alteration in the activity of enzymes and their profile can be used as an indicator of the effect of various feed additives on the digestion process. In screening experiments for improving fibre degradation, enzyme activities of a group of selected enzymes (Table 6.1) responsible for degradation of ligno-cellulosic feed or those responsible for the hydrolysis of bonds between lignin and carbohydrates can be determined. Additionally, in vitro true degradability and degradation of radio-labelled substrates can be used as additional parameters for such screening experiments.

Table 6.1 Major enzymes in the degradation of ligno-cellulosic feeds in the rumen

Substrate	Enzyme	Enzyme code
Carboxymethylcellulose	Endo-β-1,4-glucanase	EC 3.2.1.4
Crystalline cellulose (avicel)	Exo-β-1,4-glucanase	EC 3.2.1.91
Cellobiose, *p*-nitrophenyl-β-D-glucopyranoside	β-1,4-glucosidase	EC 3.2.1.21
Xylan from oat spelt or birchwood	Xylanase	EC 3.2.1.8
p-Nitrophenyl-β-D-xylopyranoside	β-1,4-xylosidase	EC 3.2.1.37
Mannan	β-1,4-mannanase	EC 3.2.1.78
p-Nitrophenyl-β-D-mannopyranoside	β-1,4-mannosidase	EC 3.2.1.25
p-Nitrophenyl-α-L-arabinofuranoside	α-L-arabinofuranosidase	EC 3.2.1.55
p-Nitrophenyl-α-D-galactopyranoside	α-galactosidase	EC 3.2.1.22
2-0-(4-O-Methyl-α-D-glycopyrano-xyluronic acid)-xylobiose	α-glucuronidase	EC 3.2.1.3
Acetylated xylan	Acetyl xylan esterase	EC 3.2.1.72
Starch free wheat bran	Ferulic esterase	EC 3.1.1.73

Fibre-Degrading Enzymes

Extraction of Enzyme

The fibre-degrading enzymes are extra-cellular but mostly cell bound. Therefore, the enzymes are extracted from the microbes by cell lysis for estimation of enzyme activity [3, 7].

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
   Stock solutions
   a) 0.2 M monobasic sodium phosphate (NaH_2PO_4) 27.8 g/1000 mL
   b) 0.2 M dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) 53.65 g/1000 mL
   Mix 51 mL of solution (a) and 49 mL of solution (b) and make volume up to 200 mL to provide a 0.1 M buffer, pH 6.8.
2. Lysozyme (0.4%): Dissolve 0.4 g lysozyme in 100 mL 0.1 M phosphate buffer, pH 6.8. Make fresh before use or store in small aliquots at –20°C.
3. Carbontetrachloride.

Procedure

1. Incubate feed (200 mg) for 24 h in in vitro gas production test (method described in this laboratory manual: Chapter 7). The substrate used is either hay or a mixture of wheat straw and concentrate mixture in the ratio of 1:1. The compounds to be tested can be added as such in the substrate. To test the plants containing secondary metabolites, the plant parts are either added after drying and grinding or extracts are prepared using different solvents. A required amount of plant extract (10–500 μL/30 mL reaction mixture) is pipetted into the syringe before the buffer (containing the rumen liquor) is added. While testing of extracts, syringes containing respective solvents are to be prepared which serve as control. For each treatment, a minimum of 4–6 syringes should be used. Along with controls and treatments, 2–3 syringes should be prepared as blank (without substrate) to estimate the contribution of inoculum to the production of gas and other fermentation products, which should be subtracted from the respective observations from the treatment syringes. Every set of syringes should also contain two syringes of standard (a feed with known gas production) to check the reproducibility of the system. We use maize hay as a standard in all our experiments.
2. After the incubation, transfer the contents of each syringe to a 100 mL beaker.
3. Add 5 mL lysozyme solution and 5 mL carbon tetrachloride.
4. Incubate for 3 h at 39°C.
5. Stop reaction by placing it in ice bath.

6. Sonicate (50 mV) the sample for 6 min with 30 s pulses and intermittent cooling for 30 s/min. During sonication, the sample should be immersed in ice bath.
7. Centrifuge the sample at 27,000*g*, at 4°C for 20 min.
8. Collect supernatant and determine enzyme activity.

Cellulases

Carboxymethylcellulase (Endo-1,4-β-glucanase, EC 3.2.1.4)

Endoglucanase (endo-1,4-β-D-glucan-4-glucanohydrolase EC 3.2.1.4) hydrolyses β-1,4-glycosidic bonds of the cellulose chain at random sites, resulting in a decrease in chain length and an increase in reducing ends of the polymer. To estimate the enzyme activity, amorphous celluloses such as carboxymethylcellulose or hydroxymethylcellulose are used as substrate. With these substrates the action of exoglucanase (exo-1,4-β-D-glucan-4-cellobiohydrolase EC 3.2.1.91) is limited therefore it represents only endoglucanase activity. During incubation of substrate with the enzyme at suitable temperature for 1 h, the reducing sugars are released and then are estimated spectrophotometrically using method described by Miller [14]. The amount of reducing sugars (glucose) released per unit of time during incubation represents the enzyme activity.

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
2. Carboxymethylcellulose (1%): Place 1 g of carboxymethylcellulose in a 250 mL Erlenmeyer flask and add 100 mL distilled water. Stir on magnetic stirrer until a homogenous viscous solution is obtained. Store at 4°C.
3. Dinitrosalicylic acid (DNS): Dissolve 10 g sodium hydroxide pellets in 500 mL distilled water. Add 10 g DNS and 2 g phenol and dilute to 1 L with distilled water. Sodium sulphite (0.05%, w/v) is added just before use. Store it in an amber coloured bottle at room temperature. Without sodium sulphite, it can be stored for a period of 1 month.
4. Rochelle salt (40%): Dissolve 40 g of Rochelle salt (sodium-potassium tartrate) in distilled water and make volume up to 100 mL. Store at room temperature.
5. Standard glucose (0.1%): Dissolve 100 mg of glucose in 100 mL distilled water.

Procedure

1. Prepare tubes as follows

   - *Test*: Combine 1.0 mL phosphate buffer, 0.5 mL sample and 0.5 mL carboxymethylcellulose in a test tube and mix well. Incubate the tubes for 20 min at 39°C. Stop the reaction by adding 3 mL DNS.

Table 6.2 Plotting a calibration curve for estimation of glucose

Tube No.	Blank	1	2	3	4	5	6
Distilled water (mL)	2.00	1.75	1.50	1.25	1.00	0.75	0.50
Standard glucose (mL)	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Glucose concentration (μg)	0.00	250	500	750	1000	1250	1500
DNS reagent (mL)	3.00	3.00	3.00	3.00	3.00	3.00	3.00

- *Control:* Mix 1.0 mL phosphate buffer, 0.5 mL sample in a test tube. Add 3 mL DNS and followed by 0.5 mL carboxymethylcellulose solution (DNS to be added before the substrate so that enzyme does not get a chance to react with the substrate).

Note: Control tube is prepared to account for the reducing sugars present in the enzyme sample. It also takes care of colour imparted by the sample, if any.

- *Standard*: Prepare tubes in duplicate with the standard glucose solution and distilled water to plot a calibration curve as described in Table 6.2.

2. Keep all the tubes in boiling water bath for 10 min.
3. Add 1 mL Rochelle salt in each tube and cool to room temperature.
4. Read absorbance at 575 nm against blank (the samples may be diluted to 10 mL with distilled water, if it reads very high absorbance, but do not forget to draw a standard curve with volumes made to 10 mL).
5. Prepare calibration curve by plotting absorbance against glucose concentration.

Calculation

Change in absorbance A = Absorbance of Test – Absorbance of Control

Read ΔA on the calibration curve to determine the μg glucose released during incubation where:

$$\text{Enzyme activity (Units)} = \mu\text{mol glucose/mL/h} = (\mu\text{g glucose})/\text{T} \times \text{S} \times 180,$$

and T = Incubation time (h), S = volume of sample (mL), and 180 = molecular weight of glucose.

Avicelase (Exo-β-1,4-glucanase, EC 3.2.1.91)

Avicel (from Fluka BioChemika, catalogue number 11365) is a microcrystalline cellulose and when used as substrate it represents the activity of all the three enzymes (exoglucanase, endoglucanase and β-glucosidase) with majority of exoglucanase. The procedure for estimation of avicel-degrading activity is similar to that for endo-l,4-β-D-glucanase, but with the following differences:

1. Avicel (1%): Suspend 1 g avicel in 100 mL of 0.1 M phosphate buffer (pH 6.8) and incubate at 4°C for 48 h for proper swelling of the substrate.
2. Assay mixture: 1 mL of 1% avicel suspension (pipette with continuous shaking) and 1 mL enzyme sample.
3. Incubation time: 1 h at 39°C with continuous shaking.
4. Before measuring absorbance, filter or centrifuge the contents to remove unhydrolyzed avicel crystals.

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21)

This enzyme catalyses the hydrolysis of cellobiose and other water-soluble cellodextrins to release glucose. For the estimation of enzyme activity, commonly used substrates are cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (PNPG). With PNPG as a substrate, the enzyme activity is determined by measuring the amount of *p*-nitrophenol released during incubation of substrate with the enzyme as described by Shewale and Sadana [16].

Reagents

1. 0.1 M phosphate buffer, pH 6.8.
2. PNPG (0.1%): Dissolve 100 mg of PNPG in 100 mL of the phosphate buffer. Store in amber-coloured bottle at 4°C.
3. *p*-Nitrophenol (0.01%): Dissolve 10 mg of *p*-nitrophenol in 100 mL distilled water.
4. Sodium carbonate (2%): Dissolve 2 g sodium carbonate in 100 mL distilled water.

Procedure

1. Prepare the tubes as follows:

   - *Test:* Mix 0.1 mL enzyme and 0.9 mL PNPG. Incubate the tubes for 10 min at 39°C. Stop the reaction by adding 1 mL sodium carbonate. Read absorbance at 400 nm against blank.
   - *Control:* Mix 0.1 mL enzyme and 1 mL sodium carbonate. Add 0.9 mL PNPG solution. (Sodium carbonate is to be added first so that enzyme does not get chance to react with the substrate).

Note: Control tube will take care of colour of the sample, if any.

   - *Standard:* Prepare tubes of graded concentration of *p*-nitrophenol in duplicate as described in Table 6.3.

2. Read absorbance at 400 nm against blank.

Table 6.3 Plotting a calibration curve for estimation of *p*-nitrophenol

Tube no.	Blank	1	2	3	4	5	6	7
Distilled water (mL)	1.00	0.98	0.96	0.94	0.92	0.90	0.85	0.80
p-nitrophenol (mL)	0.00	0.02	0.04	0.06	0.08	0.10	0.15	0.20
p-nitrophenol (μg)	0.00	2.0	4.0	6.0	8.0	10.0	15.0	20.0
Sodium carbonate (mL)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

3. Prepare a calibration curve by plotting absorbance against concentration of *p*-nitrophenol.

Calculations

Change in absorbance A = Absorbance of Test – Absorbance of Control
Read ΔA on calibration curve to get the amount (μg) of *p*-nitrophenol released.

$$\text{Enzyme activity (Units)} = \mu \text{ mol } p\text{-nitrophenol/mL/min} = (\mu\text{g } p\text{-nitrophenol})/\text{T} \times \text{S} \times 139.11$$

where T = time (minutes), S = volume of sample taken (mL), and 139.11 = molecular weight of *p*-nitrophenol

Xylanases

Endoxylanase (β-1,4-xylan xylanohydrolase; Endo-β-1,4-xylanase, EC 3.2.1.8)

This enzyme breaks the backbone of xylan, producing both substituted and non-substituted shorter oligomers, xylobiose and xylose. Activity of the enzyme is determined by estimating spectrophotometrically the amount of reducing sugars released in terms of xylose during incubation of enzyme with substrate.

Procedure

The procedure for estimation of xylanase is similar to that of endoglucanase

1. 0.25% xylan: Combine 250 mg xylan (from Birchwood, catalogue number X-0502 SIGMA) and 100 mL distilled water. Warm at 70°C for 10 min with continuous shaking.
2. Prepare standard curve using graded concentration of xylose.
3. The assay mixture contains 1 mL phosphate buffer, 0.5 mL sample and 0.5 mL 0.25% xylan.

4. Incubation time 30 min.
5. Enzyme activity Units/mL = μ mol xylose/mL/min = (μg xylose)/T × S × 150,

where T = time (minutes), S = volume of sample taken (mL), and 150 = the molecular weight of xylose.

β-Xylosidase (β-1,4-D xylan xylohydrolase: Exo-β-1,4-D xylosidase, EC 3.2.1.37)

Hemicellulose is the predominant xylo-glucan polymer in plant tissues which on hydrolysis releases small chain xylose polymers and xylose. β-Xylosidase further hydrolyses these small polymers to release xylose. Using *p*-nitrophenyl-β-D-xylopyranoside as a substrate, the activity of enzyme is determined spectrophotometrically by measuring the amount of *p*-nitrophenol released during incubation of the enzyme with substrate.

Procedure

Procedure for β-xylosidase estimation is similar as that for β-glucosidase with the only difference that the substrate is 0.1% *p*-nitrophenyl-β-D-xylopyranoside.

Acetyl Esterases (EC 3.1.1.6)

These enzymes hydrolyze acetylated carbohydrates like acetyl xylose, acetyl mannose, acetyl glucose, acetyl maltose, acetyl cellobiose, etc. The enzyme activity is estimated by measuring the amount of *p*-nitrophenol released during reaction of enzyme with *p*-nitrophenyl acetate. Method of Huggins and Lapides [8] is described with some modifications.

Reagents

1. Phosphate buffer (0.1 M), pH 6.8.
2. *p*-Nitrophenyl acetate (2 μmol/mL): Dissolve 36 mg *p*-nitrophenyl acetate in minimum volume of dimethyl sulfoxide and dilute to 100 mL with the buffer. For the dilution, tip of the pipette containing the substrate is dipped in the buffer and dispensing is carried out with continuous shaking of volumetric flask otherwise precipitates are formed.

Note: The substrate is unstable; it should be used within 30 min of its preparation.

Procedure

1. Mix 1.0 mL buffer, 0.9 mL *p*-nitrophenyl acetate and 0.1 mL enzyme sample.
2. Incubate at 39°C for 10 min.
3. Read absorbance at 410 nm.
4. A reagent blank is prepared that includes all the reagents except enzyme.
5. If enzyme is coloured, prepare an enzyme blank with all the ingredients except substrate.
6. Prepare a calibration curve using graded concentration of *p*-nitrophenol.

Feruloyl and p-*coumaryl Esterases*

These esterases selectively hydrolyse the ester bond between L-arabinosyl residues of xylan and ferulic or *p*-coumeric acid. The enzyme releases ferulic acid or *p*-coumaric acid from the substrate (starch-free wheat bran) when the enzyme is incubated with substrate. The ferulic acid or *p*-coumaric acid thus released is quantified using HPLC [12].

Reagents

1. Phosphate buffer: 0.1 M, pH 6.8.
2. Starch free wheat bran: Prepare starch-free wheat bran by treating it with excess volume of potassium acetate (0.25% w/v) at 95°C for 10 min. In our laboratory, we suspend 10 g wheat bran in 200 mL potassium acetate (0.25% w/v). Wash thoroughly the treated wheat bran until the washings are neutral, which indicates removal of potassium acetate completely. Dry it in oven at 80°C and store at room temperature.

Procedure

1. Combine 100 mg starch-free wheat bran, 1.0 mL buffer and 1.0 mL enzyme in a test tube.
2. Incubate the tubes for 30 min at 39°C.
3. Stop the reaction by boiling for 3 min.
4. For control tube, add enzyme sample after denaturation by boiling for 3 min.
5. Centrifuge the tubes to remove residual substrate.
6. Filter the supernatant through a 0.2-μm membrane before loading onto the HPLC.
7. The quantification of ferulic acid and *p*-coumaric acid is done by using the following conditions:

   - Column reverse phase C18 (Octadecylsilane)
   - Column temperature 40°C

Mobile phase: Use either

(i) 10 mM NaOH solution, adjusted to pH 3.0 by adding formic acid (88%, v/v). Mix this NaOH solution and methanol (HPLC grade) in 79:21 ratio.

or

(ii) Mix 750 mL water, 250 mL acetonitrile, 40 mL of tetramethyl ammonium hydroxide (25%,v/v), and 5.7 mL of orthophasphoric acid (88%, v/v; pH 3.0).

- Flow rate 1.5 mL/min.
- Ferulic acid or *p*-coumaric acid (1μg/mL) is used as a standard.
- A Unit of enzyme activity is expressed as μg ferulic acid or *p*-coumaric acid released/mL/h.

Note: The linearity of the enzyme reaction was assessed with respect to time and enzyme concentration for all the enzymes assays. It is suggested that the workers confirm this for the enzyme preparation they obtain.

Protein Estimation

Estimating Protein Quantities

Protein estimation as described by Lowry et al. [11] is carried out by the formation of copper protein complex in alkaline medium. This complex then reduces phosphomolybdic-phosphotungstate reagent to yield intense blue colour.

Reagents

1. Standard solution of bovine serum albumin (0.06% BSA): It is prepared in distilled water to contain 0.6 mg BSA/mL.
2. Trichloroacetic acid (20% TCA): Dissolve 20 g TCA in distilled water and make the final volume to 100 mL.
3. Solution A: Dissolve 2 g sodium carbonate in 100 mL of 0.1 N NaOH.
4. Solution B: Dissolve 1 g sodium-potassium tartrate in 100 mL distilled water. Add to it 0.5 g copper sulphate and keep it overnight at room temperature. Filter to remove the precipitate, if any. Solution A and B can be stored at room temperature.
5. Solution C: Mix 50 mL solution A and 1 mL solution B just before use.
6. Solution D: Mix 1 mL of 2 N Folin and Ciocalteu's phenol reagent and 2 mL distilled water just before use.

Procedure

1. Mix 1.0 mL sample with 1.0 mL 20% TCA and leave it overnight.
2. Centrifuge at 5000 g for 5 min. Discard the supernatant.

3. Dissolve the precipitate in 1 mL of 1 N NaOH.
4. Combine 0.1 mL sample and 0.4 mL distilled water in a test tube in duplicate.
5. Prepare tubes of standard BSA in duplicate as described in Table 6.4.
6. Add 5 mL solution C in all tubes and leave for 10 min at room temperature.
7. Add 0.5 mL solution D and mix it immediately and vigorously.
8. After 10 min record absorbance (A) against blank at 600 nm.
9. Prepare calibration curve by plotting absorbance against BSA concentration.
10. Calculate protein concentration mg/mL by reading absorbance of sample on standard curve.

Table 6.4 Plotting a calibration curve for estimation of protein

Tube no.	Blank	1	2	3	4	5	6
BSA (mL)	0.0	0.05	0.10	0.20	0.30	0.40	0.50
Distilled water (mL)	0.50	0.45	0.40	0.30	0.20	0.10	0.00
BSA (μg)	000	30	60	120	180	240	300

Precautions

Folin and Ciocalteu's reagent is only stable in the acidic medium. Care should be taken while adding it to the alkaline medium. It should be well mixed immediately to reduce the solution in step 7 above before it becomes degraded in the alkaline medium.

The protein content in the sample is used to express enzyme activity in terms of specific activity as follows:

$$\text{Specific activity (units/mg protein)} = \text{Activity (in units/mL)/mg protein/mL}$$

In Vitro True Digestibility

Reagents

Neutral detergent solution

1. Sodium lauryl sulphate (SLS) 30.00 g
2. Disodium ethylene diamine tetra-acetate (EDTA) 18.61 g
3. Sodium borate decahydrate (Borax) 6.81 g
4. Disodium hydrogen phosphate anhydrous 4.56 g
5. 2-Ethoxy ethanol (purified grade) 10.00 mL

Take weighed amount of EDTA and borax in a beaker (2000 mL capacity) and add 500 mL distilled water. Heat to dissolve ingredients and add weighed amount of SLS and 2-ethoxy ethanol. In another beaker dissolve disodium hydrogen phosphate in 100 mL distilled water. Mix well the two solutions and check pH. If properly prepared, the pH should be 6.9–7.1. Adjust pH if required. Make volume to 1000 mL.

Procedure

Incubate feed (200 mg) for 24 h in in vitro gas production test (method described in this laboratory manual in Chapter 7) and transfer the of the syringe is to a spoutless beaker by repeated washing with 100 mL neutral detergent solution. Reflux the content of the beaker is refluxed for 1 h and filter through pre-weighed Gooch crucibles (Grade G1). After drying for 24 h at 80°C, weigh the crucible to obtain NDF content of the residue. In vitro true digestibility of feed is calculated as described by Van Soest and Robertson [19]:

$$\%\text{True digestibility (TD)} = \{(\text{Initial DM of feed} - \text{NDF in residue}) \times 100\}/(\text{Initial DM of feed})$$

The enhancement in fibre-degrading activity due to inclusion of a compound can be assessed by comparing the results of samples tested (enzyme activity and in vitro true digestibility) with those obtained in control reactions. If the extracts are tested, comparisons are made with the controls containing respective solvents used for the extraction of the compound. An increase in the activity of fibre-degrading enzymes and in vitro true digestibility will indicate fibre degradation-enhancing activity of the compound.

Isotopic Labelling of Plant Cell Walls

Isotopic labelling of plants has also been used as a method of defining the extent of microbial hydrolysis of plant cell walls and to assess the digestibility of forages by ruminants. Early studies almost exclusively utilized ^{14}C as a means of labelling plant tissues [6, 10], but more recent studies have used the stable isotope ^{13}C for this purpose [17]. Others have focused on specifically labelling lignin through the introduction of ^{14}C–phenylalanine into the plant [4]. Degradation of lignin is of particular focus in many studies due to its role in limiting the rate and extent of overall cell wall digestion. Increased restrictions on the use of radioactive isotopes in the laboratory and in animal feeding experiments have encouraged the use of stable isotopes to assess the rate and extent of ligno-cellulose degradation. There is also evidence that ^{13}C results in more uniform labelling of plant tissues as well. Both of these procedures require the availability of sophisticated

laboratory equipment such as solid-state oxidizers and liquid scintillation counters for radioactive isotopes and an isotope ratio mass spectrometer for stable isotopes. The sophistication of the analytical equipment required and the need for specialized training in the handling of radioactive isotopes may limit the number of laboratories that can utilize isotopes to characterize the degradation of plant cell walls.

^{14}C- and ^{13}C-Labelling of Plant Cell Walls as a Means of Assessing Ligno-Cellulose Degradation

Equipment Required

Sealed growth chamber with circulation fans
Combustion analyzer and scintillation counter; ^{14}C
Isotope mass ratio spectrometer; ^{13}C:^{12}C ratios

Reagents

1. 99% atom% $^{13}CO_2$
2. Pee Dee Belemnite standard for ^{13}C
3. $^{14}CO_2$ in the form of $Ba^{14}CO_3$ or $NaH^{14}CO_3$ (150 μCi)
4. Phosphoric or sulphuric acid for liberation of $^{14}CO_2$
5. L-[U-^{14}C] phenylalanine

^{14}C or ^{13}C Procedure for Whole Plant Labelling

1. Place plants in a transparent gas tight chamber equipped with circulation fans. Figure 6.1 illustrates a simple chamber system designed by Fallon and Pfaender [6] consisting of a Plexiglas chamber equipped with fluorescent lights and a fan to circulate the air in the chamber. Temperature of the overall system can be controlled by placement of this unit in a larger temperature-controlled chamber or by running tap water at a controlled temperature down the walls of the chamber. Include a dehumidifier in the chamber if labelling is going to be conducted over a prolonged period [10]. Fertilize plants prior to placement in the chamber and ensure adequate light.
2. For ^{14}C, directly infuse $^{14}CO_2$ into the chamber or liberate $^{14}CO_2$ via the addition of acid into an aqueous solution of $Ba^{14}CO_3$ or $NaH^{14}CO_3$.
3. Monitor levels of $^{14}CO_2$ in the chamber via collection of gas samples and their subsequent absorption into 2 N NaOH and measuring counts using a liquid scintillation counter.

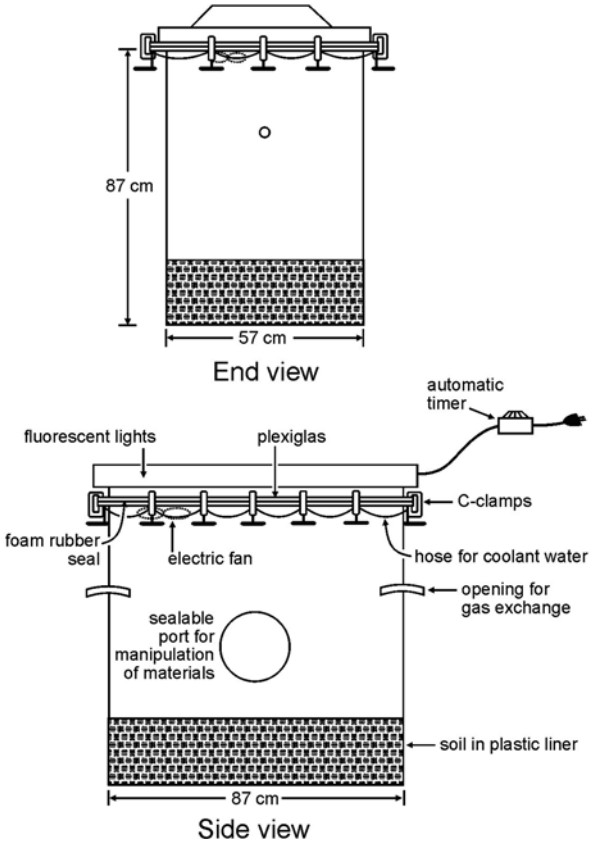

Fig. 6.1 A simplistic growth chamber for the labelling of plants with $^{14}CO_2$ or $^{13}CO_2$ for digestion studies. Adapted from [10]

4. For ^{13}C, infuse 99 atom% $^{13}CO_2$ directly into the chamber to achieve a total CO_2 concentration that is at least 640 ppm above ambient CO_2 concentration.
5. Retain plants in the presence of labelled CO_2 for a minimum of 40 min. Uniform labelling of all C fractions within the plant may require exposure periods greater than 8 h.
6. If more uniform labelling of cell wall fractions is desired, the harvest time can be extended to a week or longer. Complete uniform labelling of the cell wall may require that the plant be exposed to $^{14}CO_2$ from the seedling stage onward, as the turnover of C in the cell wall fractions occurs at a very slow rate.
7. After harvest, air-dry plants at 25–30° C for a period of 3–5 days.
8. Dissect plants into component parts (e.g., leaves, stems, roots) to examine digestion of specific components or grind the whole plant (1–4 mm screen) to assess total plant cell wall digestion.

9. Utilize labelled plant material in in vitro or in in situ digestibility procedures as described in the present manual.
10. Estimate the liberation of $^{14}CO_2$ or $^{13}CO_2$ from plant cell wall or the amount of labelled C in the residue after digestion as a means of estimating the extent and rate of degradation of the plant cell walls. Plant dry matter can be further fractionated into components as described by Alexander et al. [5] and as shown in Fig. 6.2.

Once generated ^{14}C- and ^{13}C-labelled forages can then be employed in a variety of digestive procedures that will result in the liberation of C from the labelled substrate. This could include the use of the in vitro digestibility procedure as described above. The in vitro procedure can be performed with collected digestive fluids (e.g., ruminal, intestinal, faecal), defined enzyme cocktails or pure cultures of microorganisms. Substrates are incubated with the digestive solution for a defined period (e.g., 4, 24, and 48 h).

Labelled substrates can also be employed in the in situ procedure where nylon bags of known pore size and a weighted amount of substrate are incubated within the rumen and removed at predetermined time points. Remaining residue is subsequently collected and the level of label remaining is assessed.

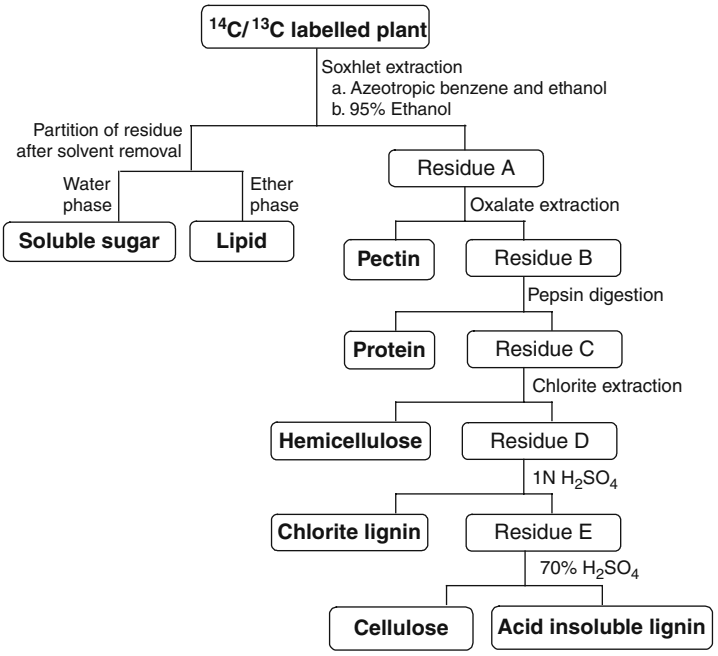

Fig. 6.2 Scheme for the chemical fractionation of labelled plant material in order to study specific degradation of soluble sugar, lipid, pectin, protein, hemicellulose, lignin and cellulose components of plants. Adapted from [5]

^{14}C-Labelled Plant Substrates

Collection of Liberated $^{14}CO_2$

Liberated $^{14}CO_2$ arising from digestion in incubation flasks is trapped by passing liberated gases through sparging tubes containing 2 N NaOH during the incubation. In prolonged incubations (>48 h), NaOH solutions are renewed every 48 h to ensure that saturation of the NaOH solution with CO_2 does not occur. Incubations can be terminated with 0.5 M H_2SO_4 at quantities to ensure that the pH of the incubation fluid is <2, with care taken to ensure that because of acidification, the released CO_2 is passed through the sparging tubes. A solution of $NaH^{14}CO_3$ of known radioactivity can be placed in the incubation vial and used to estimate the yield of $^{14}CO_2$ from acidified fermentation liquids. NaOH along with trapped $^{14}CO_2$ are introduced into counting vials. Radioactivity in the liquid is determined by liquid scintillation using a PCS solubilizer (Amersham Corp., Arlington Heights, IL) or other suitable scintillation liquid such as Hydrofluor or Picofluor 30 containing 1% Carbosorb (Packard, Stockholm, Sweden). Counting solutions should be allowed to standard for at least 1 h prior to counting in a liquid scintillation spectrophotometer. Degradation data can be expressed as $^{14}CO_2$ liberated per unit time (e.g., h, day) and expressed as a percentage of the total radioactively labelled ^{14}C substrate that was included in the incubation. Samples are corrected for background radioactivity and counting efficiency as determined by using an external standard such as ^{226}Ra.

Estimation of Retained ^{14}C in Plant Material

Retained ^{14}C in the remaining residual solid substrate is estimated by combustion of the residue in an oxidizer such as a Packard Tri Carb sample oxidizer. The liberated $^{14}CO_2$ is trapped in a mixed scintillation liquid consisting of a mixture of Carbosorb: Permafluor V at 9:13. In in vitro incubations, differential centrifugation of the culture liquid (i.e., 2500*g* for 5 min, 5°C followed by 10,000*g* for 30 min, 5°C) results in the isolation of a microbial fraction. The microbial fraction is washed/centrifuged 3 times using phosphate buffered saline and the microbial fraction dried at 60°C. The isolated microbial fraction can then be subjected to combustion in order to estimate incorporation of ^{14}C into microbial matter.

^{13}C-Labelled Plant Substrates

^{13}C-Labelled plant material can be prepared in a manner similar to that described above with the exception that $^{13}CO_2$ as opposed to $^{14}CO_2$ is provided to the plants for photosynthesis. $^{13}C/^{12}C$ ratios are determined either in trapped CO_2 or within the residual substrate using stable isotope ratio mass spectrometry. By determining the natural abundance of ^{13}C in non-labelled plants, the excess ^{13}C attributable to the labelling procedure can be determined. $^{13}C/^{12}C$ ratios are determined in the

sample and standards and reported relative to the international PeeDee Belemnite standard and expressed as parts per thousand.

Indicators of Digestive Activity

In in vitro incubations, enhanced liberation of ^{13}C- or ^{14}C-labelled CO_2 is indicative of increased digestion. Fractionation of plant material (as illustrated in Fig. 6.2) and incubation of these fractions with microbial cultures separately is a powerful approach to defining the rate and extent of degradation of various plant fractions. Conversely, conservation of labelled ^{13}C or ^{14}C in the residue remaining after digestion is indicative of those substrates that were not readily subject to enzymatic liberation by microbial enzymes. In the in situ procedure, analysis of the residue remaining after incubation is the only method of estimating the degree of degradation of cell wall fractions as trapping of any liberated $^{14}CO_2$ or $^{13}CO_2$ is not practical under these conditions. Remaining residue can still be subjected to the fractionation scheme illustrated in Fig. 6.2 as a means of assessing the extent of degradation of various chemical components within plants.

^{14}C-Labelled Lignin Using L-[U-^{14}C] Phenylalanine

1. Label lignin by administration of [L-U-^{14}C] phenylalanine through the last node using the stem infusion method. Plants are cut under water at the last node and placed in tubes containing water. Water is removed and a radioactive solutions containing L-[U-^{14}C] phenylalanine is introduced and retained with the plants until uptake of the labelled lignin precursor is complete. Once uptake is complete, a standard plant nutritive solution is added and after a period of at least 6 h in the light, plants are allowed to metabolise for a minimum of a 96 h in the dark at 25°C while being retained in the nutritive solution.
2. Grind the upper and lower halves of the apical internodes in liquid nitrogen.
3. Remove labelled proteins by incubating ground powder in pronase (2250 units/g) in 50 mL of 0.1 M phosphate buffer (pH 7) for 2 h at 35°C.
4. Wash residue in distilled water and dry at 60°C for 48 h.
5. Prepare cell wall residues by refluxing the cell wall material in 1:2 ethanol-toluene followed by 95% ethanol until the extracts become colourless.
6. Labelled phenolic residues are then used as substrates in the in vitro procedures described above.
7. Liberated $^{14}CO_2$ is trapped in sparging tubes or ^{14}C in the residue remaining residue can be determined as described above. The use of radiolabelled phenylalanine and sinapic acid results in a more targeted labelling of the phenolic elements of lignin as opposed to the use of $^{14}CO_2$, which results in the labelling of all carbon fractions within the plant.

Indicators of Digestive Activity

The level of radioactivity is examined in the solid (remaining residual) liquid (solubilised phenolic acids) and $^{14}CO_2$ fractions (liberated C) as described above. Those incubations that produce the highest fraction of $^{14}CO_2$ are indicative of high lignin degradation whereas high levels of ^{14}C in the residue reflect a low degree of lignin degradation. Label in the liquid fraction is indicative of solubilized, but undegraded phenolic acids.

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