METHODS & MATERIAL

Chapter-III

METHODS AND MATERIALS

This chapter discusses the methods and materials used to fulfill the objectives which are restated below.

OBJECTIVES

I. Restandardisation of optimum conditions for steeping and germination for the preparation of ARF from cereals, millets and legumes.

II. To study the effect of addition of ARF at graded levels, on viscosity reduction.

III. To study the effect of temperature on the activity of ARF.

IV. To study the effect of period of germination on the hydrocyanic acid (HCN) content of ARF; its distribution in the grain and the sprouts and the effect of heat treatment on the HCN content.

V. To study the microbiological quality of uncooked and cooked gruels prepared using ARF that had been stored for 1 to 6 months under ambient conditions.

VI. To study the effect of addition of jaggery and salt on viscosity and the viscosity reduction power of various ARFs on gruels prepared from cereal + pulse mixes, donated foods and commercial weaning foods.

VII. To compare the invitro carbohydrate digestibility of gruels with ARF vs gruels without ARF.

I. **Restandardisation of conditions for steeping, and germination for preparation of ARF from cereals, millets and legumes :**

The grains selected were wheat, maize, sorghum, pearlmillet, green gram, bengal gram and soya bean. The grains were purchased in bulk from the market and cleaned to remove dirt, chaff, stones and infested or broken grains. These grains were stored in air tight containers.

I.1 **Steeping :** Steeping is an important step during which the seed absorbs water and the enzymes in dormant stage are activated so that the seed can support germination. If adequate steeping time is not allowed the percentage germination is poor while on the other hand if steeping is carried out in excess of requirement the seeds develop water sensitivity and fail to germinate. Therefore, to decide the minimum steeping period required for each grain under study for maximum germination the percentage germination at the end of each hour of steeping was estimated.

Procedure : The grains were soaked in triple their volume of water. A small portion was taken at the end of each hour up to 12 hours, drained thoroughly, wrapped in a piece of wet muslin cloth and kept covered in a petridish. At the end of 6 hours the total number of grains and the total number of germinated grains were counted. From this, percentage germination was calculated using the following formula:

$$\text{Percent Germination} = \frac{\text{Total number of germinated grains}}{\text{Total number of grains}} \times 100$$

The steeping period at the end of which maximum percentage germination was obtained for a particular grain was finally utilised for the preparation of ARF from that grain.

I.2 **Germination :** Germination is the process where a whole array of enzymes are mobilised to break the stored food in the grain into simpler substances to be utilised by the growing seedling. In our study we were interested in obtaining α-amylase in adequate amount to bring about maximum viscosity reduction. Therefore, each grain subjected to various periods of germination was tested for its liquifying power.

Procedure : Six hundred g of each grain was soaked in a plastic bowl in triple volume of water for the standardised period of time. The steeped grains were drained thoroughly, wrapped in a wet muslin cloth and kept covered. Approximately 100 g portion was drawn at the end of 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of germination and dried at 50 ± 1 ° C in a hot air oven. The dried grains were devegetated and ground to a fine powder of +60 BSS. This ARF was tested for its liquifying power by viscosity reduction test.

Materials : Distilled water, purified corn starch (purchased in bulk from Anil Starch, Ahmedabad) and ARF from various grains.

I.3 **Standardisation of viscometer :** The Brookfield synchrolectric viscometer RVT model was used for the entire study. The viscometer does not need any standardisation as per the instructions manual. However, it is necessary to standardise the speed and rotation time for the particular food system under study. Allowing too long a rotation time at a high speed may lead to faulty readings due to thixotrophy while a short time interval and reading at low shear rate may produce faulty results due to failure of the instrument to stabilise. After preliminary trials a speed of 50 rpm and a time period of 1 minute was found to give accurate results. The instrument was operated under these standardised conditions during the entire study.

Procedure : Preliminary trials were carried out to establish the concentration of starch slurry and the ratio of starch to ARF in order to obtain reproducible results. A 10% starch slurry and 96 : 4 ratio of starch to ARF was found to give reproducible results. Table 11 gives the composition of the control and experimental slurry.

Table 11 : Composition of Control and Experimental slurries from starch

Slurry	Starch (g)	Wate (ml)	ARF (g)
Control	40.0	360	Nil
Experimental	38.4	360	1.6

The starch was mixed with 360 ml of water (350 ml in case of experimental sample) in a 500 ml beaker and cooked over a boiling water bath with continuous stirring. The slurry was held for 5 minutes once a temperature of 90°C was reached, to allow complete gelatinisation of starch. This is an important step since a-amylase does not act on raw or ungelatinised starch. The slurries were then removed from the water bath. The ARF was dissolved in the remaining 10 ml of water and added to the experimental slurry and stirred thoroughly. The slurries were cooled to 40°C (or for 20 minutes approximately) with continuous stirring. The viscosity of control and experimental slurry was measured using appropriate spindles and under standardised conditions. The reading was converted into viscosity in centipoise units using the factor finder (Appendix 1). The percent reduction in viscosity was calculated as under :

$$\text{Percentage reduction in viscosity} = \frac{\text{Viscosity in Cp of control - Viscosity in Cp of Experimental}}{\text{Viscosity in Cp of control}} \times 100$$

The germination period at the end of which maximum viscosity reduction was obtained or the one beyond which insignificant increase was obtained was utilised for the preparation of ARF. Thus the conditions for steeping and germination were standardised and utilised for the entire study. An attempt was made to quantitate the α-amylase activity of the ARFs prepared under standardised conditions. However, this was not possible for reasons given below :

1. The colorimetric procedures tested namely Sanstedt, Kneen and Blish (1939), Wohlgemuth (1908) and Bernfield (1955) which are based on estimation of free sugars liberated by the action of α-amylase on starch under standard conditions yielded results which failed to correlate with those obtained by viscosity reduction test. This was especially the case with soya bean where

the estimated amylase activity was very high while the viscosity reduction was low. Germinated soyabean is reported to contain both a and β amylase. Thus the free sugars produced are by the combined action of α and β-amylases. Hence, these methods are inappropriate for systems where both the amylases are present.

2. Attempts were made to inactivate α-amylase by heating the extract at 70°C for 15 minutes (Beleia and Marston, 1981). However, α-amylase too was destroyed in the process as indicated by lowered viscosity reduction power.

3. Since α-amylase acts from the non-reducing end only, substituted starch namely hydroxy ethyl starch (with >90% degree of substitution) was used as the substrate (McHale 1988). The results again failed to correlate with the viscosity reduction test. The α-amylase randomly cleaves a C-C bond in the starch thus exposing an unsubstituted non-reducing end for α- amylase to act.

Thus the α-amylase activity could be expressed in comparative terms of viscosity reduction only.

II. **To study the effect of concentration of ARF on viscosity reduction:**

There is a definite quantitative relationship between the enzyme and the substrate. Increasing the enzyme concentration beyond a certain level does not lead to an increased conversion of substrate to product. Hence it is essential to determine the minimum level of enzyme required for optimum viscosity reduction.

Materials : Distilled water, purified corn starch and ARF from wheat, sorghum, pearl millet and maize.

Procedure : The procedure described in I.2. was followed for the preparation of slurries. ARF was added at different concentrations namely 1% to 7% of solids (Table 12). Percentage reduction in viscosity at each level of ARF concentration was calculated.

Table 12 : Composition of Control and Experimental starch slurries used for viscosity reduction studies

Slurry	Starch (g)	Water (ml)	ARF (g)
Control	40.0	360	Nil
Experimental 1	39.6	360	0.4
Experimental 2	39.2	360	0.8
Experimental 3	38.8	360	1.2
Experimental 4	38.4	360	1.6
Experimental 5	38.0	360	2.0
Experimental 6	37.6	360	2.4
Experimental 7	37.2	360	2.8

The concentration at which the ARF yielded optimum viscosity reduction was utilised in further studies.

III. **To study the effect of temperature on the activity of ARF.**

The enzymes are heat labile and at the same time are more active within a certain temperature range. Therefore it is essential to establish the favourable temperature for enzyme activity in order to get optimum viscosity reduction.

Materials : Distilled water, purified corn starch and ARF from wheat, sorghum, pearmillet and maize.

Procedure : Five sets of slurries were prepared for each ARF using previously described procedure. ARF was added at 4% of solid contents. To the first set ARF dissolved in 10 ml of water was added at a slurry temperature of 90°C. Both the control and the experimental slurries were held at 90°C for 10 minutes with continuous stirring and viscosity measured at the end of the 10th minute. The next four sets were cooled to 80°C, 70°C, 60°C and 50°C respectively and the ARF dissolved in 10 ml of water was added. The slurries were held at respective temperatures for 10 minutes with continuous stirring. The viscosity was measured at the end of 10th minute and viscosity reduction was calculated.

IV. **To study the effect of period of germination on hydrocyanic acid (HCN) content of ARF; its distribution in the grain and the sprouts and the effect of heat treatment on the HCN content :**

ARF was prepared from three varieties of sorghum viz. GJ 35 (red grain), GJ 36 (white grain) and market variety of wheat, pearl millet and maize by steeping using previously standardised method and germination for various periods of time viz. 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. The vegetative portion was separated carefully after drying the grains at 50°C. The grains and the sprouts were milled separately to yield a fine powder of +60 BSS. Hydrocyanic acid was estimated in the raw ARF and sprouts and in the heat treated sample by the method of Indira and Sinha (1969) with suitable

modifications. The modification involved was, reading of samples at 480 nm instead of 540 mm as in the original method. This was based on our observation on scanning of the sample with a series of dilutions against the blank. The estimations were carried out on the day of preparation of samples to avoid loss of HCN due to action of endogenous enzymes on the glycoside.

Accurate methods for the extraction and determination of cyanogenic glycosides have been developed by Butler and Conn (1964) and Butler (1965). These methods make use of purified linamarase as a reagent for the release of HCN. Though these methods are sensitive and accurate they can not be used for the determination of cyano-glucosides like HCN in a large number of samples. In sorghum, endogenous enzyme was used by Akazawa et al (1960) for the release of HCN from dhurrin. The released HCN was trapped in NaOH and determined by the method of Aldridge (1944). Gilchrist (1967) quantitated the qualitative method of alkaline picrate and Sinha and Nair (1968) used the same for estimating HCN in cassava. To date this is the most simple and sensitive method and hence was used with suitable modifications mentioned previously.

Materials : (a) Alkaline picrate reagent : 25 g of sodium carbonate (AR) and 5 g of picric acid (AR) dissolved in glass distilled water and volume made up to 1 litre.

(b) Standard cyanide solution : A cyanide solution of 0.01 g/100 ml concentration was prepared by dissolving 0.0188 of sodium cyanide in glass distilled water and making up the volume to 100 ml.

Procedure :

Standardisation : One blank and five sample tubes were set up and treated as given in the table:

	ARF Extract (ml)	CN Std. Soln. (ml)	Water (ml)	Alkaline picrate (ml)	Incubation
Blank	-	-	2	2	}
Standard 1	-	0.2	1.8	2	} For 45
Standard 2	-	0.4	1.6	2	} at 60°C
Standard 3	-	0.6	1.4	2	}
Standard 4	-	0.8	1.2	2	}
Standard 5	-	1.0	1.0	2	}
Sample	2	-	-	-	}
Sample					} No
blank	2	-	-	2	} incubation

The final volume was made up to 10 ml. Extract from various ARFs and sprouts was prepared by suspending 5 g of the ARF and 1 g of powdered sprouts in 50 ml of water for 30 minutes. The suspension was centrifuged at 2000 rpm for 10 minutes and filtered through whatman No. 1 to obtain a clear extract. The extract was divided in to five portions. One portion was left aside. The remaining four portions were heated in a water bath at the following temperatures respectively, i.e. 90°C, 80°C, 70°C, and 60°C. Each sample was treated as shown in

the table. The samples were read at 480 nm. The HCN concentration per g of sample was calculated from the standard curve (Appendix 2) using the following formula:

$$\text{mcg HCN/g of sample} = \frac{\text{OD of sample} - \text{OD of blank}}{\text{OD of standard}} \times \text{Conc of std.} \times \text{Dilution factor}$$

V. **To study the microbiological quality of uncooked and cooked gruels prepared using ARF stored for 1 to 6 months under ambient conditions :**

The ARFs prepared from wheat, sorghum, pearl-millet and maize as per the standardised methods were stored in 200 gauge polythene bags, heat sealed, kept in air tight glass jars and stored at room temperature. Six such sets were prepared and samples were analysed at the end of 1st, 2nd, 3rd, 4th, 5th and 6th month for the total plate count, E.Coli and yeast and mould using the Indian standard methods (1969).

Since there are Indian standards specifications for the total plate count of weaning foods it was thought essential to analyse the gruels for the same (IS 1973).

Water is utilised in the preparation of ARF and if it is not potable, as in the case in slums or low income households, it can be the source of E.coli. Therefore the gruels were tested for the presence of E.coli.

Further, ARF being high in free sugars can serve as a good medium for proliferation of yeast and mould. Hence, the yeast and mould count in gruels was assessed.

Preparation of sample : Gruels were prepared from wheat, sorghum, pearlmillet and maize flour and ARF from the same source was added respectively to each gruel. For the control gruel 20 g of cereal or millet flour was mixed with 80 ml of water and stirred well. The experimental gruel was prepared by mixing 19.2 g of cereal or millet flour and 0.8 g of ARF with 80 ml of water. Each mixture was divided into two halves. One half was left uncooked and the other half was cooked on an open flame. The gruel was boiled for 5 minutes and 1 ml sample was drawn from each at the end of 0, 1, 2, 3, 4, 5 and 6 hours and analysed for the parameters mentioned previously.

V.1 **Total Plate Count :**

Materials : (a) Diluent - stock phosphate buffer - 34 g of potassium dihydrogen phosphate (KH_2PO_4) was dissolved in 500 ml of distilled water. The pH was adjusted to 7.2 with 1N sodium hydroxide solution and the volume made up to 1 litre with distilled water. For use 1.25 ml of stock was made up to one litre with distilled water.

(b) Plating medium - The media had composition as follows:

Tryptone	5.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Sodium chloride	6.5 g
Agar, bacteriological grade	15.0 g
Distilled water	1000.0 ml
Final pH	7.0 ± 0.1

Procedure : (a) Preparation of glassware : The pipettes and petriplates were sterilised in their containers with lid placed loosely in a hot air oven at 160°C for 15 minutes Precaution was taken not to overload the oven.

(b) Prepation of media : The materials weighed in appropriate amounts were soaked for about 3 to 5 minutes and then the mixture was boiled to bring the mixture into solution with minimum delay. The mixture was stirred continuously to prevent charring. Approximately 15 ml of media was poured into tubes and the tubes were plugged loosely and autoclaved at 121°C for 15 minutes. Aseptically a small portion of media was removed before use and the pH was checked and adjusted to 7.0 ± 0.1.

(c) Preparation of dilution blanks : The dilution bottles were filled with phosphate buffer up to the 99 ml mark. The bottles were stoppered loosely and autoclaved at 121°C for 15 minutes. Blanks with a variation of ± 2 were discarded. One ml of the sample was taken and four serial dilutions were made under aseptic conditions. The dilution bottles were capped after transferring the sample and shaken thoroughly. The sample was pipetted into sterile petriplates under aseptic conditions. The last drop from the pipette was drained by touching on a dry spot.

(d) Plating the medium : The media was poured by first sterilizing the tip of media container over a flame. The cover of the petridish was lifted gently and 12 to 15 ml of the medium was poured. The media container was sterilised over flame and the next plate was poured. The medium was thoroughly mixed with the test portion by rotating and tilting the dish without splashing the mixture over the edge. The samples were run in triplicate.

The sterility of containers was checked by pouring one plate and the sterility of dilution blank checked by pouring control plates for each lot of dilution blank.

The plates were inverted once the media solidified and incubated at 37°C for 48 hours.

Spreader free plates were selected with 30-300 colonies. Colonies were counted, the dilution was recorded and the total count was calculated.

V.2 Detection and estimation of E.Coli :

Materials : (a) Diluent - same as mentioned in V.1

(b) Plating medium - violet red bile agar with the following composition:

Yeast extract	0.90 g
Peptone	12.10 g
Bile salts	5.45 g
Lactose	13.00 g
Sodium chloride	1.50 g
Agar	14.50 g
Neutral red	0.09 g
Crystal violet	0.06 g
Distilled water	300.00 ml
Final pH	7.4 ± 0.1

Procedure : (a) Preparation of glassware : Same as described in V.1

(b) Preparation of media : The ingredients were weighed out accurately and mixed with water and rehydrated. The mixture

was brought to boil quickly taking precautious to avoid charring. The pH was checked and adjusted to 7.4 ± 0.1. The media was prepared shortly before use. This media should not be sterilised.

(c) Preparation of dilution blanks : Same as mentioned in V.1

(d) Plating the medium : Same as mentioned in V.1 After the mixture solidified an additional 3 to 4 ml of medium was poured to cover the surface to prevent surface growth. The petriplates were inverted and incubated at 37°C for 18 to 24 hours. Presence of dark red colonies measuring at least 0.5 mm in diameter constituted a positive test.

V.3 Yeast and mould count :

Materials : (a) Diluent : Same as mentioned in V.1

(b) Plating medium : Potato glucose agar with the following composition was prepared:

Infusion from 200 g of potatoes	100 ml	(Boil 200 g of white potatoes, peeled and sliced in about 500 ml of water, filter through fine cotton, and make upto 1000 ml)
Glucose	20 g	-
Agar	15 g	-
Final pH	3.5 ± 0.1	-

Procedure : (a) Preparation of glassware : Same as in V.1

(b) Preparation of media : The acccurately weighed and measured ingredients were soaked for 3-5 minutes and then boiled quickly. The reaction of the autoclaved medium was adjusted to 3.5 ± 0.1 with sterile 10% tartaric acid. The amount of acid required for adjustment in one tube/flask was established and the adjustment was made for other tubes/flasks accordingly.

(c) Preparation of dilution blanks : Same as in V.1

(d) Plating the medium : Same as in V.1

The petriplates were inverted and incubated at 30°C for 5 days. Yeast and mould colonies were counted at the end of the incubation period. Yeast colonies are characterised as growth, moist, elevated or surface colonies. Mould colonies are easily recognised by their profuse growth of hyphae. The yeast and mould count per gram of sample was reported as the total yeast and mould count.

VI. **To study the effect of addition of jaggery vs salt on viscosity and the viscosity reduction power of various ARFs in gruels prepared from cereal+pulse mix, donated foods and commercial weaning foods :**

A cereal and pulse in the ratio of 4:1 makes a nutritionally well balanced mix. Therefore, the following cereal pulse mixes were selected namely, wheat, sorghum, pearlmillet and maize in combination with red gram and bengal gram in 4:1 ratio. The grains were cleaned and the cereal and pulse ground separately into a fine powder of +60 BSS. The cereal and pulse flour was then mixed in the required ratio.

The donated foods studied were corn soya milk (CSM) corn soya blend (CSB) and Energy Food.

The commercial weaning foods used were Farex (A), Farex veg (B), Nestum rice (C) and Cerelac wheat (D).

VI.1. The effect on viscosity of jaggery vs salt was studied in all the cereal + pulse mixes. The gruels were prepared from the cereal + pulse mixes following the traditional method of preparation as described below :

Table 13 Composition of gruels prepared from cereal+pulse mix

Cereal + pulse mix	100.0 g
Jaggery or	75.0 g
Salt	2.5 g
Oil	10.0 g
Water	300.0 ml

Jaggery or salt was mixed in water. Cereal + pulse mix was roasted (for 2 minutes) in heated oil and taken off the fire. Jaggery or salt water was added slowly with stirring and the mixture was cooked thoroughly. The gruel was then cooled to 40°C and the viscosity was measured as described in I.2. A gruel without jaggery or salt served as the control. The effect of addition of jaggery or salt on viscosity was compared against this control.

VI.2. The effect of addition of ARFs on the viscosity of gruel from cereal + pulse mix and donated foods was studied. The gruels were prepared from cereal + pulse mix and CSM and CSB as described below. To cereal + pulse mix CSM, CSB and Energy Food all the ARFs were added at graded levels (1- 7%). The ARF which yielded maximum viscosity reduction at comparable solid contents level was chosen for final viscosity reduction trials.

Table 14 Composition of gruels prepared from cereal + pulse mix and donated foods

Control	Gruel	Experimental	Gruel
Mix	100 g	Mix	96 g
Jaggery	75 g	Jaggery	75 g
Oil	10 g	Oil	10 g
ARF	Nil	ARF	4 g
Water	300 ml	Water	300 ml

Jaggery was dissolved in water. The oil was heated in a pan and the mix, was roasted (for 2 minutes). The pan was taken off the fire and the jaggery water was added to it. This mixture was warm to which ARF was added in case of the experimental gruel and the mixture was stirred for 10 minutes. The pan was then kept on the fire (for both control and experimental gruel) and the gruel was cooked thoroughly by boiling. The gruel was cooled to 40°C and the viscosity was measured as described in I.2. The viscosity reduction was calculated using previously described formula.

VI.3 Since the commercial weaning foods are ready to reconstitute mixes, ARF was added to the dry mix and viscosity reduction on reconstitution studied. Similarly if ARF has to be utilised in mass feeding programs like the Integrated Child Development Services (ICDS) it needs to be added at the point of manufacture to the mix to simplify the logistics of delivery. Therefore, ARF at graded levels

namely 4-7% of solid contents level was added to CSM, CSB and Energy Food and the commercial weaning foods. ARF was mixed thoroughly by seiving several times and the mixes were stored in air tight containers. This served as the experimental sample. The mix without ARF served as the control sample. The gruels could not be prepared by roasting the mix as described previously in VI.2. because roasting inactiviated the enzyme. Therefore gruels were prepared from CSM, CSB and Energy Food as follows:

Table 15 Composition of control and experimental gruels prepared from plain mix and ARF added mix

Control gruel		Experimental gruel	
Plain Mix	100 g	Mix with ARF	100 g
Jaggery	75 g	Jaggery	75 g
Oil	10 g	Oil	75 g
Water	300 ml	Water	300 ml

Jaggery was dissolved in water. Oil was added to the dry mix and stirred well. The jaggery water was then added with stirring to the mix. The mixture was cooked initially over a low flame (for first 10 minutes) to avoid inactivation of amylase. It was then cooked thoroughly by boiling. The gruels were cooled to 40°C and the viscosity in centipoise and percent viscosity reduction was calculated.

The commercial weaning foods were reconstituted as per the instructions of the manufacturers. The control feeds had a viscosity suitable for young child

feeding. The experimental samples had a very low viscosity. Therefore, the solid contents were increased in the experimental sample to obtain a feed of viscosity equivalent to its control counterpart. Table 16 gives the composition of the control and experimental feeds.

Table 16 Composition of gruels prepared from commercial baby foods

Baby Food	% Solid Control	Concentration Experimental
Farex (A)	21 7	37.7
Farex Veg. (B)	17.3	30.8
Nestum Rice (C)	9.0	20.0
Cerelac Wheat (D)	20.0	35.0

VII. **To compare the in vitro carbohydrate digestibility of gruels with ARF vs gruels without ARF :**

The invitro carbohydrate digestibility was studied using the method of Kon et al (1971). The method utilises pepsin and pancreatin digestion of the sample followed by estimation of free sugars by the modified dinitrosalicylic acid method (Bernfield 1955). Recently Robyt and Whelan (1972) have reported that equimolar quantities of maltodextrius (maltose through maltoheptaose) did not give equal reducing value with increase in the size of dextrin. Similar studies with alkaline copper reagent showed that equimolar quantities of maltodextrines gave equal reducing values. This has been further confirmed by Dias et al (personal communications). Therefore, Nelson's alkaline copper reagent method

(1944) was used for the determination of free sugars.

Preparation of slurry : Slurries from wheat, sorghum, pearlmillet and maize in combination with green gram and red gram in the ratio of 4 : 1 were used. ARF from respective cereal/millet source was utilised. For the control slurry 20 g of mix was taken and for the experimental slurry 19.2 g of mix was taken and mixed with 80 ml of water. The slurry was heated in a water bath to 90°C and held at that temperature for 5 minutes. To the experimental slurry 0.8 g of ARF was added and slurries were cooled to 40°C. A 5 ml aliquot was taken and homogenised in a blender with 45 ml of water. The pH was adjusted to 2.0 with 0.1 N HCl and a 2 ml aliquot was drawn for the analysis.

Materials : (a) Phosphate buffer PH 7.0:

A O.1 M solution of di sodium hydrogen phosphate (Na2HPO4) and sodium hydrogen phosphate was prepared and 39 ml and 61 ml respectively of each was mixed. The pH was checked and adjusted if required.

(b) Glycine HCl buffer pH 2.0:

A O.1M solution of HCl and glycine was prepared and 49.1 ml and 59.1 respectively of each was mixed. The pH was checked and adjusted if required.

(c) Pepsin : (Loba Chemie 1 : 10000)

A solution containing 2 mg pepsin/ ml of glycine HCl buffer was prepared.

(d) Pancreatin (Loba Chemie)

A solution containing 5 mg pancreatin/ml of phosphate buffer was prepared.

(e) Reagent A

Anhydrous sodium carbonate 25 mg, sodium potassium tartrate 25 mg, sodium bi carbonate 20 g and anhydrous sodium sulphate 200 g was dissolved in 800 ml of water diluted to 1 litre and filtered if necessary.

(f) Reagent B

Thirty grams of cupric sulphate pentahydrate mixed in 200 ml of water containing four drops of sulphuric acid.

(g) Reagent C

Ammonium molybdate 25 g + was dissolved in 450 ml of water, 21 ml of sulphuric acid was added to it and 3g of sodium arsenate heptahydrate added slowely to it with stirring. The solution was diluted to 500 ml and warmed overnight at 37°C.

(h) Reagent D

One ml of reagent B was added to 25 ml of reagent A.

(i) Standard maltose solution:

A solution of 0.5 mg/ml was prepared.

Procedure :

Standardisation: To 1 ml of reagent D 0.2 ml to 1 ml of maltose solution was added and the volume made up to 2 ml. The blank contained 1 ml of water instead of the maltose solution. The tubes were capped and placed in a boiling water bath for 20 minutes and then cooled under running water. One ml of reagent C was added and the tubes were allowed to stand for 5 minutes. The volume was then made upto 25 ml. The obsorbance was measured in a spectrophotometer at 520 nm. A standard curve was obtained (Appendix 3).

The sample was prepared as just described and 2 ml of sample (PH 2.0) was drawn. To the sample blank 3 ml of glycine HCl buffer was added. The contents were neutralised to pH 7.0 with 0.1 N NaOH. To the sample blank 1 ml of phosphate buffer was added and 1 ml of pancreatin solution was added to the sample. The tubes were incubated at 37°C and 1 ml samples drawn at the end of 5,10,15 and 20 minutes intervals and analysed for free sugars as mentioned previously. The free sugars as maltose were calculated using the following formula:

$$\text{Maltose/g of sample} = \frac{\text{OD of sample - OD of sample blank}}{\text{OD of standard}} \times \text{Conc. of standard} \times \text{Dilution factor}$$

Thus carbohydrate digestibilty in terms of mg maltose released/g of sample was calculated for the control and experimental slurry and the starch equivalent was calculated using the conversion factor of 0.9.