# *Chapter 3*

# Materials and methods

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## 3.1 Introduction

This chapter discusses the materials and methods used in the research work. Three locally grown yams from Assam, India were used for the preparation of flours and the isolation of starches. The physicochemical properties of the yam flour were determined and then mixed with almond protein isolate (API) for the preparation of cookies. Proximate composition, texture analysis (hardness), and antioxidant properties of the cookies were determined; and sensory analysis of the cookies was conducted. Physical (HMT and annealing) and chemical (hydroxypropylation and cross-linking) methods were used for the modification of the native starches. The physicochemical, functional, thermal, morphological, pasting, and rheological properties of the native and physically modified starches were investigated. The functional, morphological, and rheological properties of the chemically modified starches were also investigated. Finally, the physically modified starches along with walnut oil were used for the preparation of edible films, and the film-forming solution was applied as coatings on grapes for shelf life studies. The results of the study were analyzed using various analytical and instrumental methods. Instruments used for the investigations in this study include rheometer, Attenuated total reflectance - Fourier transform infrared (ATR-FTIR) spectroscopy, X-ray diffraction (XRD), scanning electron microscope (SEM), texture analyzer, differential scanning calorimetry (DSC), UV-spectrophotometer, atomic absorption spectrophotometer, etc.

## 3.2 Materials

### 3.2.1 Collection of yam species

Three locally grown yams (**Fig. 3.1**) were collected from Kuthori Bagisha, Morigaon, Assam, India (26°04'46.6"N 92°16'10.6"E) and were identified to be species belonging to *Dioscorea esculenta* (Lour.) Burkill and *Dioscorea alata* (purple yam and

yellow yam). The harvesting period of the yams was in December before the Bihu festival of Assam.

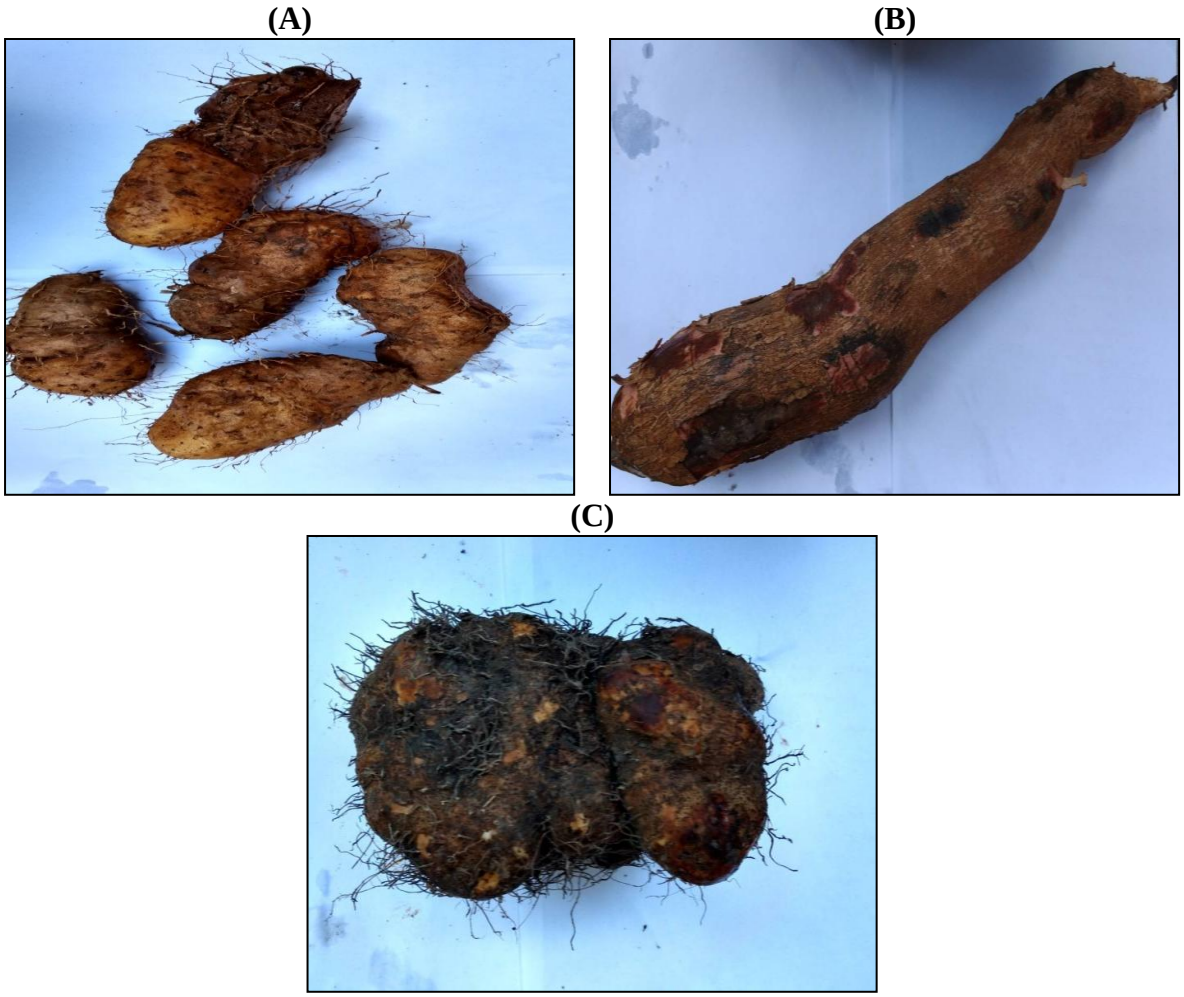


**Fig. 3.1:** Yam samples collected. (A) *Dioscorea esculenta* (Lour.) Burkill; (B) *Dioscorea alata* (purple yam); and (C) *Dioscorea alata* (yellow yam).

### 3.2.2 Chemicals and reagents

Amylose (potato), amylopectin (potato), α-amylase, DPPH (2,2-diphenylpicrylhydrazyl) and amyloglucosidase were purchased from Sigma-Aldrich Co. LLC., India. Cold-pressed walnut oil manufactured by Essentia Extracts was purchased from Amazon, India. All other reagents used in the study were of analytical grade.

## 3.3 Preparation of yam flours

Preparation of flours (**Fig. 3.2**) from the yam tubers was carried out as described by **Olapade and Akinyanju [20]**.

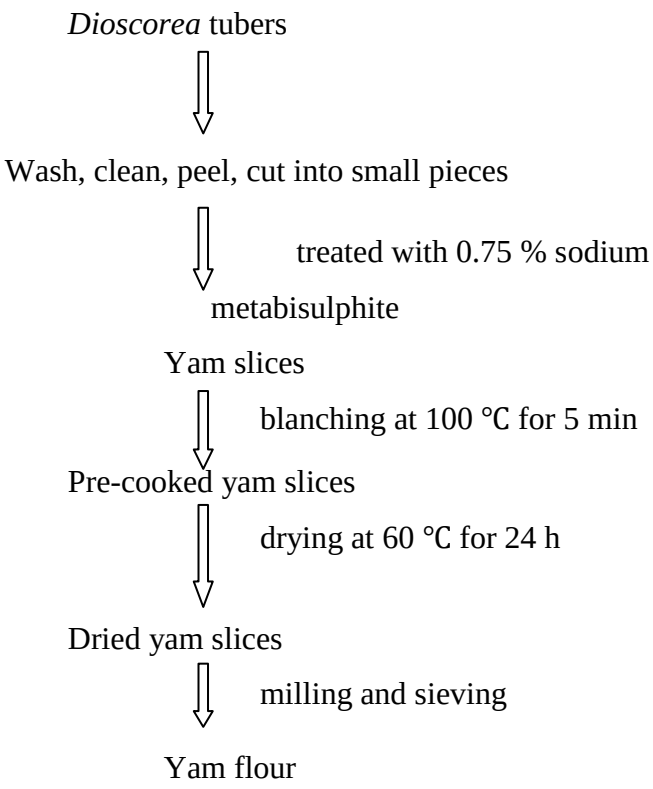


**Fig. 3.2:** Flowchart followed for the preparation of yam flours.

The flours obtained from the yam species *Dioscorea esculenta* (Lour.) Burkill, *Dioscorea alata* (purple yam), and *Dioscorea alata* (yellow yam) were named as YF1, YF2 and YF3, respectively.

### 3.4 Extraction of almond protein isolate

Almond protein isolate (API) was extracted from defatted almond presscake flour using the method of **Shi et al. [27]** with slight modification. Briefly, proteins were isolated using isoelectric precipitation, in which defatted almond press cake flour was used to precipitate proteins under alkaline and acidic conditions. A slurry was prepared by adding the defatted flour to distilled water at a ratio of 1:10 (w/w). Proteins were solubilized under alkaline conditions with 2M NaOH and the pH was adjusted to 8.5 with constant stirring for 2 h. After solubilization, the slurry was centrifuged at 4000 rpm using a laboratory centrifuge (Hermle Labortechnik, Germany) for 20 min (25 °C), followed by removal of pellet and collection of supernatant in a beaker. Here, the pH of the supernatant was adjusted to 4.5 with 2M HCl to aggregate proteins. The solution was left for 2 h (without stirring), and then centrifuged at 4000 rpm for 20 min at 25 °C. The precipitates were collected from the centrifuge tubes after discarding the supernatant.

The resulting protein precipitate was neutralized by washing (twice) with distilled water, and then lyophilized. The resulting freeze-dried almond protein isolate (API) powder was stored at -20 °C for further use.

### 3.5 Preparation of blends of yam flour and API

The yam flours YF1, YF2 and YF3 were mixed with 10 % API to obtain the blends of yam flour and API, and were named as PY1, PY2 and PY3, respectively.

### 3.6 Characterization of yam flours, and blends of yam flour and API

#### 3.6.1 Proximate analysis of flours and API

The standard methods of the Association of Official Analytical Chemists (AOAC) 2005 **[2]** was used for the determination of moisture (Method No. 935.29), protein (Method No. 978.04), crude fat (Method No. 930.09), crude fiber (Method No. 978.10) and ash (Method No. 930.05) content of the flours and API. The carbohydrate content of the flours and API were calculated by difference.

#### 3.6.2 Antioxidant properties of flours

The total phenolic content (TPC) in flours was measured according to **Azeem et al. [4]** using the Folin–Ciocalteu (FC) method with slight modification. Briefly, 1 g of sample was mixed with 20 ml of 70 % ethanol, kept in shaker incubator (Metrex Scientific Instruments Pvt. Ltd., New Delhi) set at 50 °C for 25 min, and then centrifuged at 5000×g for 15 min. After centrifugation, 0.5 ml of the supernatant (10-fold diluted) were mixed with 1 ml of FC reagent and kept in the dark for 30 min. To the mixture, 2 ml of $Na_2CO_3$ was added and kept again for 30 min. The absorbance was measured at 736 nm in a UV-VIS spectrophotometer (Inkarp Pvt Ltd), and the TPC values were expressed as mg gallic acid equivalents (GAE) per g or 100 g.

DPPH radical scavenging activity of the samples was determined using a modified method of **Brand-Williams et al. [7]** reported by **Shukla et al. [28]**. Briefly, 100 mg of sample was mixed with 2 ml of methanol and kept for 2 h on a laboratory shaker. After the extraction, the mixture was centrifuged at 3000×g for 10 min and 100 µl of supernatant was reacted with 3.9 ml of 6×10-5 mol/l of DPPH methanolic solution in a test tube. Then, the mixture was kept in the dark for 30 min before the measurement

of absorbance in a UV-VIS spectrophotometer (Inkarp Pvt Ltd) at 515 nm using methanol as blank.

$$\text{DPPH radical scavenging activity (\% inhibition)} = (1-\frac{A_s}{A_c})\times 100$$

where,

$A_s$ = absorbance of the sample

$A_c$ = absorbance of the control

### 3.6.3 Functional properties of flours

Bulk density was determined using the method reported by **Suriya et al. [31]**. Briefly, flour samples (3 g) were gradually placed in graduated cylinders of 10 ml capacity, respectively. The bottoms of the cylinders were gently tapped on the laboratory bench several times until no further attenuation of the sample level was observed. The weight of the flour per unit volume of the flour (g/ml) represents the bulk density.

$$\text{Bulk density (g/ml)} = \frac{\text{Weight of the flour (g)}}{\text{Volume taken by the flour (ml)}}$$

Water absorption capacity (WAC) and oil absorption capacity (OAC) of the samples were determined following the method of **Ashwar et al. [3]**. 2.5 g of sample on a dry weight basis (db) was mixed with 20 ml of distilled water or mustard oil, and was stirred for 30 min at 25 °C. The suspension was then centrifuged at 3000xg for 10 min and the supernatant was decanted. Weight gain was expressed as percentage water/oil absorption capacity.

The swelling power of flours was determined using the method described by **Gani et al. [9]**, with slight modification. In short, sample (3g) was mixed with 30 ml of distilled water in a centrifuge tube, and then the tubes were placed in a water bath at 70 °C for 30 min. After heating, the tubes were centrifuged at 1000 rpm for 15 min, and the supernatant was decanted to take the weight of the paste was taken.

$$\text{Swelling power (g/g)} = \frac{\text{Weight of the paste (g)}}{\text{Weight of the dry flour (g)}}$$

Foaming capacity (FC) and foaming stability (FS) was determined using the method described by **Onwuka [21]**. Briefly, flour sample (2 g) was added to 50 ml of distilled water in a 100 ml graduated cylinder. The suspension was mixed and shaken vigorously until foamy, and the total volume was recorded after 30 s and 30 min.

$$\text{Foaming capacity (\%)} = \frac{\text{Volume after whipping (30 s)} - \text{Volume before whipping}}{\text{Volume before whipping}} \times 100$$

$$\text{Foaming stability (\%)} = \frac{\text{Volume after whipping (30 min)} - \text{Volume before whipping}}{\text{Volume before whipping}} \times 100$$

### 3.6.4 Pasting properties of flours

The pasting properties of the starch samples (12 % w/v) were evaluated in a Rapid Visco Analyzer (StarcMaster2, Newport Scientific, Australia). The programmed cycle was set at 50 to 95 ºC in 5 min (heating), 95 ºC for 2 min (holding), 95 to 50 ºC in 4 min (cooling), and 50 ºC for 4.5 min (holding). Pasting temperature (PT), Peak viscosity (PV), Hot-paste viscosity (HPV), Final viscosity (FV), Breakdown (BD), and Setback (SB) were recorded.

### 3.6.5 ATR-FTIR spectra of flours

The ATR-FTIR spectra of the samples were recorded using Spectrum-2 spectrometer (Perkin-Elmer, USA). The IR spectra were obtained with the help of Universal Attenuator Total Reflectance (UATR) and spectra in the range of 4000 - 400 $cm^{-1}$ were recorded with a resolution of 4 $cm^{-1}$ at ambient temperature.

## 3.7 Preparation of cookie dough and cookies

The cookie dough was prepared by following the formula reported by **Tyagi et al. [33]**. The cookie dough formula based on the method was: flour (100 g), shortening (26.5 g), sugar (53 g), sodium chloride (0.89 g), sodium bicarbonate (1.1 g), and 12 ml water. The prepared cookie dough was sheeted to a thickness of 0.5 cm using a roller, followed by cutting of the sheet using a 4 cm circular die. The round cut sheet were transferred to a tray lined with aluminum foil, and were kept for baking in an

electric oven at 160 ºC for 10 min. The baked cookies were stored in airtight glass containers after cooling to room temperature.

### 3.7.1 Rheological characteristics of cookie dough

The dynamic rheological properties of the cookie dough samples were studied by following the procedures described by **Inglett et al. [13]** using a rheometer (MCR 101, Anton Paar, Germany), which was equipped with parallel-plate geometry (50 mm diameter) with 1.5 mm gap at 30 ºC. After loading the sample, the outer edge of the plates was sealed with petroleum jelly to avoid dehydration of sample during the test. The dynamic frequency sweep (frequency range of 0.1 – 10 rad/s) test was performed at a strain value of 1.5 % (within the linear viscoelastic region) to record the loss modulus (G´´), storage modulus (G´), and loss tangent (tan δ= G´´/ G´) as a function of frequency (ω).

## 3.8 Characteristics of cookies

### 3.8.1 Proximate analysis of cookies

Same as Section **3.6.1**

### 3.8.2 Antioxidant properties of cookies

Same as Section **3.6.2**

### 3.8.3 Physical characteristics of cookies

The total weights of each batch of cookie was measured by placing the cookies together in an electronic weighing balance, and then the weight of each cookie was calculated by dividing total weight of each batch by the number of cookies in each batch. The thickness (T) of the baked cookies was measured with a Vernier Caliper, and the diameter (D) of the cookies was determined using a scale by placing them side by side. After the measurements of T and D were taken, the spread ratio (D/T) was calculated, where D is the diameter and T is the thickness of baked cookies.

### 3.8.4 Texture analysis (hardness) of cookies

Hardness of the cookies were measured by Texture Analyzer (Model-TA-XD, Stable Micro System, UK) using biscuit cutting probe with 30 kg load cell and a test

speed of 2 mm/sec. The hardness of cookies was expressed as maximum peak force required to snap the cookies.

### 3.8.5 Color analysis of cookies

A Lovibond LC 100 spectrocolorimeter (The Tintometer Ltd., Amesbury, UK) was used to determine the color profile (L*, a* and b* values) of the samples according to the method described by **Shukla et al. [28]**. The colorimeter was first calibrated, then the instrument optics were placed over the sample and the L*, a*, and b* values were measured.

## 3.9 Sensory evaluation of cookies

Baked cookies made from yam flour and API blends were subjected to sensory evaluation based on color, flavor, texture, aroma, and overall acceptability in comparison to control (wheat flour cookies). A team of semi-trained panelists (aged 21-40 years) were selected after a test to exclude sensory blind panelists. Then, the panelists rated the sample cookies after a brief on how to assign score on a 9-point hedonic scale, where 1 = extremely dislike and 9 = extremely like.

## 3.10 Isolation of native starch from yam species

Isolation of starches from the yams was carried out by the method described by **Nindjin et al. [18]** with modifications. The washed and cleaned tubers were cut into small pieces and immediately dipped in distilled water containing 0.2 % (w/v) sodium metabisulphite. The pieces were crushed in a blender and suspended in excess distilled water containing 4 % (w/v) NaCl to maintain a solid to liquid ratio of 1:5. The slurry was filtered through cheesecloth and the filtrate was centrifuged at 3000 rpm for 10 min using a laboratory centrifuge (Hermle Labortechnik, Germany). The obtained starch cake was re-suspended in distilled water and centrifuged. The process was repeated till no brownish layer on the surface was seen. The purified starch cake was dried in a tray drier at 40 ºC for overnight, followed by pulverization, sieving, and storage for further analysis. The native starches obtained from *D. esculenta, D. alata* (purple yam) and *D. alata* (yellow yam) were named as 1YNS, 2YNS and 3YNS, respectively.

### 3.11 Hydrothermal modifications of yam starch

For heat moisture treatment (HMT) of the isolated starches, the method described by **Piecyk et al. [24]** was followed with slight modifications. Samples were adjusted to 20 % and 30 % (w/w) moisture levels and kept at 4 ºC for 24 h in a sealed container. The sealed sample containers were then placed in a hot air oven at 110 ºC for 3 h with occasional shaking of the containers. The treated samples were cooled down to ambient temperature and dried at 40 ºC overnight. Accordingly, the treated starches were named 1HMT-20, 1HMT-30, 2HMT-20, 2HMT-30, 3HMT-20, and 3HMT-30, where the prefix '1', '2', and '3' indicate the yam species *D. esculenta*, *D. alata* (purple yam), and *D. alata* (yellow yam), respectively, and the suffix -20 and -30 indicate moisture levels.

Annealing of the starches was carried out using the method reported by **Yeh and Lai [35],** with minor modifications. Samples were suspended in distilled water (1:2 and 1: 4, w/v), mixed well in a sealed container for equilibration, and incubated at 50 ºC in a water bath for 24 h. After incubation, the samples were cooled to ambient temperature and dried at 40 ºC for 24 h. Accordingly, the treated starches were named 1ANN-12, 1ANN-14, 2ANN-12, 2ANN-14, 3ANN-12, and 3ANN-14, where the prefix '1', '2', and '3' indicate the yam species *D. esculenta*, *D. alata* (purple yam), and *D. alata* (yellow yam), respectively, and the suffix -12 and -14 indicate starch to water ratio of 1:2 and 1:4, respectively.

### 3.12 Properties of native and hydrothermally treated starch

#### 3.12.1 Proximate analysis of native starch

Same as Section **3.6.1**

#### 3.12.2 Total amylose content, amylose leaching, water and oil absorption capacity

Total amylose content (AM) was determined using a standard curve prepared by mixtures of amylose and amylopectin standards, according to the method described by **Hoover and Ratnayake [10]**. Briefly, 20 mg of defatted starch sample was mixed with 8 ml of DMSO in a round bottom screw cap tube. The mixture was vigorously mixed using a vortex for 2 min, and then kept in a water bath at 85°C for 15 min with intermittent shaking. After cooling, the dispersed solution of the tube was diluted to 25 ml. 1 ml of the diluted solution, 40 ml of distilled water and 5 ml of iodine solution was mixed in a

50 ml volumetric flask, and the volume was adjusted to 50 ml with distilled water. The solution was mixed vigorously and allowed to develop color. After 15 min, the absorbance was taken at 600 nm using an UV-VIS spectrophotometer (Inkarp Pvt Ltd) against a blank (all reagents except the sample). The amylose content was determined using a calibration curve constructed by mixtures of standard potato amylose/amylopectin.

Amylose leaching (AML) of the native and modified starches was determined at 80 ºC according to the method of **Jayakody et al. [14]** using the amylose content determination method of **Hoover and Ratnayake [10]**. Briefly, starch sample (20 mg) was mixed in distilled water (10 ml) and were heated at 90 °C in sealed tubes for 30 min. The tubes were allowed to cool at room temperature and was centrifuged at 2000 rpm for 10 min. The supernatant liquid (2 ml) was collected and its amylose content was determined. Percentage AML was expressed as mg amylose leached per 100 g dry starch.

Water absorption capacity (WAC) and oil absorption capacity (OAC): Same as Section **3.6.3**

### 3.12.3 Scanning electron microscopy

The native and modified starch powdered samples were dried initially at 40 ± 2 $^{o}$C in a hot air oven for 4 h. The samples were first gently placed on a double-sided carbon tape attached to a metal stub. The stub along with the sample was later coated with a gold layer of 20 nm using a magnetron sputtering machine (Q150R ES, Quorum, England). The samples thus prepared, were morphologically examined under a scanning electron microscope (FESEM, Sigma 300, Zeiss, Germany) at 5 kV.

### 3.12.4 ATR-FTIR spectroscopy

The ATR-FTIR spectra of the samples were recorded using Spectrum-2 spectrometer (Perkin-Elmer, USA). The IR spectra were obtained with the help of Universal Attenuator Total Reflectance (UATR) and spectra in the range of 4000 - 400 $cm^{-1}$ were recorded with a resolution of 4 $cm^{-1}$ at ambient temperature.

### 3.12.5 Swelling power and solubility

Swelling power and solubility of the starches were measured by following the method reported by **Bernardo et al. [6]**, with minor modifications. Briefly, 50 mg of starch was mixed with 10 ml of distilled water and heated at 55, 65, 75, 85 and 95 ºC for 1 h. After cooling to room temperature, the mixture was centrifuged at 4000xg for 15 min. The supernatant was dried in a hot air oven at 105 ºC for 4 h and the solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{dried supernatant (g)}}{\text{mass of starch (g)}} \times 100$$

For determination of swelling power, the sediment was weighed and the swelling power was calculated as follows:

$$\text{Swelling power } \left({}^{\text{g}}/_{\text{g}}\right) = \frac{\text{sediment weight (g)}}{\text{mass of starch (g)} \times (100\% - \%\ \text{dried supernatant})} \times 100$$

### 3.12.6 Thermal properties

Differential scanning calorimeter (Micro DSC III, Setaram, France) was used to analyze the starch gelatinization using the method reported by **Liu et al. [17]**. Starch samples (3 mg) in distilled water at a ratio of (1:3.5) were scanned from 30 to 150 ºC at a heating rate of 10 ºC/min in a sealed aluminum pan. An empty sealed DSC pan was used as reference. The onset temperature ($T_o$), peak temperature ($P_o$), conclusion temperature ($T_c$), and endothermic enthalpy ($\Delta H$) were determined.

### 3.12.7 Pasting properties

The pasting properties of the starch samples (12 % w/v) were evaluated in a Rapid Visco Analyzer (StarcMaster2, Newport Scientific, Australia). The programmed cycle was set at 50 to 95 ºC in 5 min (heating), 95 ºC for 2 min (holding), 95 to 50 ºC in 4 min (cooling), and 50 ºC for 2 min (holding). Pasting temperature (PT), Peak viscosity (PV), Hot-paste viscosity (HPV), Final viscosity (FV), Breakdown (BD), and Setback (SB) were recorded.

### 3.12.8 Static and dynamic rheological parameters

The static and dynamic rheological properties of the starch gelatinized paste (12 % w/v) obtained from RVA were studied by following the procedures described by **Qin**

**et al. [25]** using a rheometer (MCR 101, Anton Paar, Germany), which was equipped with parallel-plate geometry (50 mm diameter) with 0.5 mm gap at 30 ºC. The steady shear (flow behavior) test was performed by varying the shear rate from 0 – 400 $s^{-1}$ to measure the impact of shear rate on apparent viscosity and shear stress. The dynamic frequency sweep (frequency range of 0.1 – 100 rad/s) test was performed at a strain value of 1 % (within the linear viscoelastic region) to record the loss modulus (G´´), storage modulus (G´), and loss tangent (tan δ= G´´/ G´) as a function of frequency (ω). To further investigate the rheological behavior, Power-law as mentioned in Eq.(1) and (2 and 3) were fitted to the experimental data of steady shear and oscillatory testing conducted on the starch pastes, respectively.

$$\sigma = k(\gamma)^n \quad (1)$$

where, $\sigma$ = shear stress (Pa); $\gamma$ = shear rate (1/s); $k$ = consistency coefficient ($Pa.s^n$); $n$ = flow behaviour index (dimensionless).

$$\text{Storage modulus } (G') = k'(\omega)^{n'} \quad (2)$$

$$\text{Loss modulus } (G'') = k''(\omega)^{n''} \quad (3)$$

where, k´, k´´, n´ and n´´ are the corresponding fitting parameters.

### 3.12.9 *In vitro* starch digestibility

The percentage of rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) of the native and modified yam starches were determined following the method of **Huang et al. [12]** with slight modification. Briefly, 200 mg starch was weighed in a centrifuge tube and 15 ml of phosphate buffer (pH 5.2 ± 0.2) was added to it. The mixture was allowed to boil in a water bath for 20 min and then equilibrated at 37 ºC for 5 min. After equilibration, 5 ml of enzyme solution (1200 U/ml α-amylase and 15 U/ml amyloglucosidase) was added and incubated in a shaking (180 rpm) water bath at 37 ºC. After 20 min and 120 min, 0.5 ml aliquots of hydrolyzed solution were mixed with 4 ml of ethanol (80 %) in a centrifuge tube to stop the activity of the enzymes. Then, the tubes were centrifuged at 3000 g for 10 min and the supernatant was collected to measure the content of glucose using the Anthrone method. The percentage of hydrolyzed starch was determined by multiplying the glucose content

with a factor of 0.9. The RDS (%), SDS (%), and RS (%) in each starch sample were determined using Eq. (4), (5) and (6), respectively.

$$\text{RDS (\%)} = \frac{\text{G20}-\text{FG}}{\text{TS}} \times 0.9 \times 100 \quad (4)$$

$$\text{SDS (\%)} = \frac{\text{G120}-\text{G20}}{\text{TS}} \times 0.9 \times 100 \quad (5)$$

$$\text{RS (\%)} = \frac{\text{TS}-\text{RDS}-\text{SDS}}{\text{TS}} \times 100 \quad (6)$$

where, G20 and G120 are the content of glucose determined after 20 min and 120 min of hydrolysis, respectively; FG is the free glucose content in starch; and TS is the total starch content.

## 3.13 Chemical modifications of yam starch

### 3.13.1 Hydroxypropylation, hydroxypropyl group and molar substitution (MS)

Hydroxypropylated starch samples were prepared by reacting the isolated starch with propylene oxide according to the procedure of **Perera and Hoover [22]**. The procedure that was followed for hydroxypropylation is presented in **Fig. 3.3**.

Isolated starch (200 g, dry basis)

⇩

Dissolved in a solution of NaOH (2.6 g) and $Na_2CO_3$ (30 g) in distilled water (240 ml)

⇩

Placed in water bath at 40$^{o}$C and propylene oxide (5 or 10 ml) to be added

⇩

Reaction at 40$^{o}$C for 24 h with shaking

⇩

Suspensions neutralized to pH 5.5 with dilute $H_2SO_4$ (1 M)

⇩

Starch cakes were washed with distilled water until negative to sulfate ions when tested with $BaCl_2$

⇩

Freeze dried until the moisture content was reduced to 10–12%

**Fig. 3.3:** Flow sheet of starch modification by hydroxypropylation **[22]**.

Hydroxypropyl group percentage and degree of substitution of the modified starches were determined according to the method of **Wang et al. [34]**. Briefly, starch sample (100 mg) was mixed with 25 ml of 0.5 M $H_2SO_4$ in a 100 ml volumetric flask and heated in a water bath at 100 ºC until the solution become clear. After heating, the solution was allowed to cool at room temperature and the volume was adjusted to 100 ml with distilled water. 1 ml of this solution was placed in a 25 ml glass stoppered test tube, immersed in an ice bath, and 8 ml of concentrated $H_2SO_4$ was added. After vigorous shaking, the tube was placed in a water bath at 100 ºC for 3 min and immediately chilled using an ice bath. 2 percent ninhydrin reagent (0.6 ml) was added and gently swept down the walls of the test tube. The solution was immediately mixed well and left at room temperature for 100 min. The volume was made up to 25 m with concentrated $H_2SO_4$ and the tube was inverted several times to mix. Absorbance of the solution was immediately measured at 590 nm using a starch blank as reference and a calibration curve was established using standard aqueous solutions of propylene glycol (mg/ml). Hydroxypropyl groups (% HP) were calculated using the following formula:

$$\%\,\mathrm{HP} = \frac{\mathrm{C} \times 0.7763 \times 10\mathrm{F}}{\mathrm{W}} \times 100$$

where,

C = amount of propylene glycol in the sample solution read from the calibration curve (mg/mL); F = dilution factor; and W = weight of the sample (mg)

The molar substitution (MS) of the starches of MYS and DMYS was calculated using the following formula [11]:

$$\mathrm{MS} = \frac{\%\mathrm{HP} \times 162}{59{:}08 \times (100 - \%\mathrm{HP})} \times 100$$

### 3.13.2 Cross-linking modification, phosphorus content and degree of substitution (DS)

Crosslinking starch modification with sodium trimetaphosphate (STMP) will be done according to the procedure of **Perez et al. [23]**. The procedure that was followed for crosslinking modification is presented in **Fig. 3.4**.

Phosphorus content was determined using the method described by **Wang et al. [34]**. A sample (1 g) was heated in a muffle furnace at 600 °C for 4 h and cooled to room temperature. 10 ml of 5M HCl was then added to the ash. This solution was diluted with

distilled water to 100 ml, 10 ml of which was placed in a 25 ml test tube, and 1 ml of 2 % vitamin C solution and 2 ml of 4 % vanadate molybdate reagent were sequentially added to prepare a solution. The solution was immediately mixed and the absorbance was measured at 470 nm. A calibration curve was generated from standard solutions of $KH_2PO_4$ (mg/ml) using deionized water as reference. Phosphorus content (% P) was determined from the prepared calibration curve using the following formula:

$$\% \, P = \frac{P \times V \times 100}{10 \times W \times 1000}$$

where,

P = phosphorous content (mg/100 ml) from the calibration curve; V = dilution volume (ml); and W = weight of the sample (mg)

The degree of substitution (DS) was determined according to Morrison using the following formula **[11]**:

$$DS = \frac{162 \times \%P}{3100 - (124 \times \%P)}$$

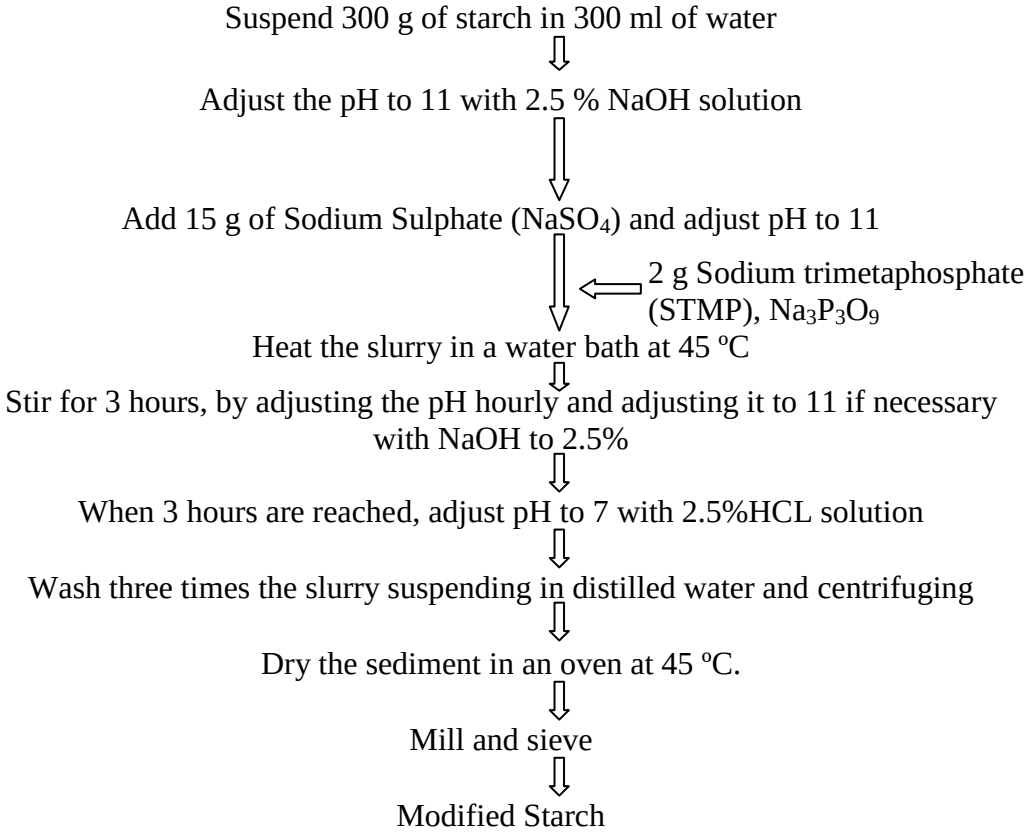


**Fig. 3.4:** Flow sheet of starch modification with with sodium trimetaphosphate (STMP) **[23]**.

## 3.14 Properties of chemically modified starch

### 3.14.1 Scanning electron microscopy

Same as Section **3.12.3**

### 3.14.2 ATR-FTIR spectroscopy

Same as Section **3.12.4**

### 3.14.3 Swelling power and solubility

Same as Section **3.12.5**

### 3.14.4 Paste clarity and freeze thaw stability

The paste clarity values of native and chemically modified starch pastes were measured by preparing a hot paste from starch dispersions (1 g/100 g) in a hot water bath. After heating, the hot starch paste was allowed to cool at room temperature for 1 h, and then its paste clarity was measured at 640 nm with a spectrophotometer using percent transmittance (%*T*) against a water blank.

Freeze thaw stability of the native and modified starches was conducted according to method described by **Lee and Yoo [15]**. Starch suspension (5 %, w/w) was heated in a water bath at 95ºC with constant mild agitation for 30 min, and was allowed to cool at room temperature.15 g of the gelatinized paste was taken in a centrifuge tube, and weighed. Successive freeze-thaw cycles (FTC) was conducted till 3$^{rd}$ FTC by freezing at 18 ºC for 24 h, and then thawing at 30 ºC for 1.5 h, followed by centrifugation at 2300× g for 30 min. After each FTC, the supernatant was eliminated from the tubes, and the tubes were weighed.

$$\text{Syneresis (\%)} = \frac{\text{liquid seperated (g)}}{\text{total weigth of sample (g)}} \times 100$$

### 3.14.5 Static and dynamic rheological parameters

Same as Section **3.12.8**

### 3.15 Preparation of films

The native or modified starch (4%) was mixed in distilled water and the solution was kept in a water bath at 90$^{o}$C for 1 h with continuous shaking. The solution was transferred to a beaker and magnetically stirred at 1500- 1800 rpm for 2 h. Then, glycerol (25 % of starch mass) was gently added to the solution. After 1 h, walnut oil (0.5 % of starch mass) was added at temperature ~50$^{o}$C and kept under stirring for 2 h. The final solution was poured in teflon coated plates, then cooled to room temperature and oven dried at 40 $^{o}$C for overnight to obtain films. The obtained films were named as NS0F: native starch film without walnut oil; NSWF: native starch film with walnut oil; H1WF and H2WF: films prepared from HMT starch with 20% moisture level and 30% moisture level, respectively, and walnut oil; A1WF and A2WF: films prepared from annealed starch with 1:2 and 1:4 starch to moisture ratio, respectively, and walnut oil.

### 3.16 Properties of films

#### 3.16.1 Thickness, moisture content, swelling index and solubility of films

The film thickness to the nearest 0.001 mm at 10 random positions around the film was determined using a Micrometer by the method described by **Rodríguez et al. [26]**. The average value of measurements was used in the calculations.

Film specimens (2 cm × 2 cm) were cut from the films, and then dried at 105 ± 1 °C in a hot air oven for 24 h and weighed. The moisture content of the specimens was calculated using the following equation:

$$\text{Moisture content (\%)} = \frac{\text{W1} - \text{W2}}{\text{W2}} \times 100$$

where,

W1 = weight of pre-dried specimens

W2 = weight of dried specimens

The swelling index of the films was measured according to the method of **Nisar et al. [19]**. Film specimens (2 cm × 2 cm) were cut from the films, and then dried at 105 ± 1 °C in a hot air oven for 24 h and weighed. Dried specimens were immersed in 50 ml distilled water for 24 h. The specimens were then removed from distilled water and

the excess amount of water from the swelled specimens was removed by placing them between two layers of filter paper, and weighed.

$$\text{Swelling index} = \frac{W2 - W1}{W1}$$

where,

W1 = weight of swelled specimens after removal of excess water

W2 = weight of dried specimens

The solubility (%) of the films was measured according to the method of **Nisar et al. [19]**. Film specimens (2 cm × 2 cm) were cut from the films, and then dried at 105 ± 1 °C in a hot air oven for 24 h and weighed. Dried film specimens were immersed in 50 ml distilled water with constant agitation at 25ºC for 6 h. The residual film specimens were then removed from distilled water and the remaining mass was dried at 105 ± 1 °C in a hot air oven for 24 h and weighed.

$$\text{Solubility (\%)} = \frac{W1 - W2}{W1} \times 100$$

where,

W1 = weight of dried specimens before immersion in water

W2 = weight of dried specimens before immersion in water

### 3.16.2 Transparency of films

Film specimens (2 cm × 2 cm) were cut from the films and attached to one side of the cuvette. The absorbance of each film specimen was measured at 600 nm using an UV-VIS spectrophotometer (Inkarp Pvt Ltd) against a blank cuvette. The transparency ($mm^{-1}$) of the film specimen was calculated as follows:

$$\text{Transparency (mm} - 1) = \frac{\text{Absorbance}_{600\text{nm}}}{\text{Film thickness}_{\text{mm}}}$$

### 3.16.3 Color of films

Same as Section **3.8.5**

### 3.16.4 SEM morphology of films

The film samples were morphologically examined using a scanning electron microscope (JEOL JSM-6390LV, Singapore). Film samples were conditioned for 1 week in a desiccator to ensure the absence of moisture in the film samples. The film pieces were mounted on a double-sided carbon tape attached to an aluminum stub, platinum coated and observed using an accelerating voltage of 20 kV at 1500x magnification.

### 3.16.5 FTIR spectra of films

Same as Section **3.6.5**

### 3.16.6 XRD spectra of films

XRD analysis of the film samples was conducted using an X-ray diffractometer (Miniflex, Japan) by adopting the methodology of **de Souza et al. [8]**. Equipment conditions were 15 mA and 30 kV with X-ray beam. Measurements were taken at a diffraction angle (2θ) in the range of 10° to 40° with a step width of 0.05°.The crystallinity percentage was calculated as follows:

$$\text{Crystallinity}\ (\%) = \frac{\text{Ic}}{\text{Ic} + \text{Ia}} \times 100$$

where,

$I_c$ = crystalline peak

$I_a$ = amorphous halo

Identification and area analysis of XRD peaks were performed using Peak Analyzer of Origin software (OriginPro 8.5, OriginLab Corporation).

### 3.16.7 Tensile strength of films

The tensile strength (TS) of films was determined following the method described by **Souza et al. [29]**. TS ($N/mm^2$) measurement was performed on a texture analyzer (Model-TA-XD, Stable Micro System, UK) with a load cell of 5 kg according to ASTM D882-09 [30]. Three strips of film specimens (130 mm × 25 mm) were cut from each preconditioned films, and each one was mounted between the grips of texture analyzer for testing. Testing conditions were as follows: Initial grip separation (50 mm); and test speed (0.8 mm $s^{-1}$). The tensile strength of the film strips was calculated dividing the

maximum load by the original minimum cross-sectional area (related to minimum thickness) of the film specimen.

### 3.16.8 Water vapor permeability (WVP) of films

WVP was calculated according to the gravimetric method described by **Aitboulahsen et al. [1]**, with minor modifications. Briefly, film specimens (50 mm diameter) were sealed on the mouth of weighing tubes (25 × 150 mm) containing 15 g of silica gel (0% RH). Then, the tubes were conditioned with a saturated NaCl solution (75% RH) in desiccators. The change in weight of the tubes were recorded at an interval of 12 h for 7 days. The slopes of weight gained (Δm, g) versus time interval (Δt, day) were obtained and divided by the exposed film area (area A, $m^2$). WVP (g $day^{-1}m^{-1}$ $Pa^{-1}$) was determined using the following equation:

$$\mathrm{WVP} = \frac{\Delta \mathrm{m}}{\Delta \mathrm{t} \times \mathrm{A}} \times \mathrm{T}/\Delta \mathrm{p}$$

where,

T (m) = average thickness of films

Δp (Pa) = difference in water vapor pressure between the two sides of the film

## 3.17 Shelf life study of grapes

Fresh table grapes (*Vitis vinifera*) were brought from Okhla Fruit Market, New Delhi. After discarding the diseased or damaged grapes, the grapes were washed with distilled water and divided into 7 different groups (approximately 200 g each group). Each group of grapes were dipped in to the coating solutions prepared from the film forming solutions, and one is left as uncoated (UC). The coating solutions used for grapes were NS0F: native starch film-forming solution without walnut oil; NSWF: native film-forming solution with walnut oil; H1WF and H2WF: film-forming solution prepared from HMT starch with 20% moisture level and 30% moisture level, respectively, and walnut oil; A1WF and A2WF: film-forming solution prepared from annealed starch with 1:2 and 1:4 starch to moisture ratio, respectively, and walnut oil.

Each group of grapes were coated with respective coating were stored at 4$^{o}$C for a storage period of 15 days. Various parameters associated with the quality of grapes such as weight loss, total soluble solids (TSS), pH, titratable acidity, total phenolic content

(TPC), DPPH scavenging activity, and monomeric anthocyanin content were determined on every third day of storage, and the results were compared to uncoated grapes sample.

### 3.17.1 Weight loss, pH and TSS

Weight loss (WL) of uncoated and coated grapes during storage was measured by weighting fruits in the trays before storage and at every third day of storage period, following the standard method of AOAC (1994) **[5]**. The pH of grapes was analyzed by using a digital pH meter. 10 ml of the grape juice sample was taken in a beaker, and the pH was measured at 25 ± 0.5 $^{o}$C after continuously magnetic stirring for 5 min. TSS ($^{o}$Brix) of grapes was determined by placing few drops of grape juice in the prism of hand refractometer (True Sense Technologies) at 25 ± 0.5 $^{o}$C.

### 3.17.2 Titratable acidity

Titratable acidity (TA) was determined by the method reported by **Takma et al. [32]**, with slight modifications. Briefly, 1 ml of the grape juice was diluted (10-fold) with distilled water, and then titrated with 0.1 N NaOH using phenolphthalein (0.1%) as an indicator. The results of TA were expressed as a percentage of tartaric acid per 100 g.

### 3.17.3 Total phenolic content and antioxidant activity

TPC: Same as Section **3.6.2**

DPPH scavenging activity: Same as Section **3.6.2**

### 3.17.4 Total monomeric anthocyanin content

Total monomeric anthocyanin content (TAC) of the grapes was determined by pH differential method reported by **Lee et al. [16]**, with slight modification. All the pH buffers (pH 1 and pH 4.5) made with standard procedures were mixed with grape juice, and 10 ml of the mixed solution was transferred into test tubes. Then, the test tubes were covered with aluminium foil to prevent evaporation, and heated to 40 $^{o}$C for 15 min. After heat treatment, the samples were cooled rapidly by diluting with pH 1 and pH 4.5 buffers and then were placed in the dark for 30 min. The absorbance was measured at 520 and 700 nm using UV-Vis spectrophotometer. The difference of absorbance (A) of the diluted samples are calculated using the following equation:

$$A = (A_{1\text{vis-max}} - A_{700})pH1.0 - (A_{1\text{vis-max}} - A_{700})pH_{4.5}$$

The total monomeric anthocyanin content (TAC) was determined by using the following equation in cyanidin-3-glucoside equivalents.

$$TAC(mg/L) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

where, MW is the molecular weight of cyanidin-3-gluoside (MW = 449.2 g/mol), DF is the dilution factor, ϵ is the molar absorptivity (26900 L/mol cm) and l is the path length of the cuvette.

### 3.18 Statistical analysis

All the tests of this study were conducted in triplicate. The results were expressed as mean ± standard deviation and evaluated for significance using one-way ANOVA and Turkey post hoc test at a significance level of $p<0.5$ using IBM SPSS Statistic Version 20 software (IBM Corporation, Armonk, N.Y., USA). For the graphs and plots, OriginPro 8.5 software (OriginLab Corporation, Northampton, MA, USA) was used.

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