INVESIGATION ON EXTRACTION OF STARCH FROM CASSAVA (*Manihot esculenta* Crantz) STEM

By

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(2014-09-102)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Investigation on extraction of starch from cassava (*Manihot esculenta* Crantz) stem"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Hasmi Sulain K K

DEDICATED TO MY PARENTS

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LIST OF ABBREVIATIONS

%	Percentage
A_{260}	Absorbance at 260nm wavelength
A_{280}	Absorbance at 280nm wavelength
bp	Base pair
cm	Centi metre
DNA	Deoxyribo Nucleic Acid
et al.	et alia
EtBr	Ethidium bromide
F	Forward primer
FAO	Food and Agricultural Organization
g	Gram
Hz	Hertz
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute
kb	Kilobases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometer
^{o}C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
R	Reverse primer

RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
s	Second
T	Time
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tg	Tera gram
TM	Trademark
Tm	Melting temperature
Tris HCl	Tris (Hydroxymethyl) amino methane hydrochloride
UV	Ultraviolet
V	Volt
w/v	weight/volume
µg	Microgram
µl	Microlitre
µM	Micromolar

INTRODUCTION

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub, currently is the sixth world food crop. It is an important food source for over one billion people in developing countries of tropical and sub-tropical Africa, Asia and Latin America (FAOSTAT, 2016). It is mainly cultivated by resource-limited small farmers and is cultivated over an area of 18 million ha. It gives reasonable yield in dry and poor soils and does not require high level management and cost compared to other major crops. Cassava has inherent tolerance to stressful environments and produce more energy due to its high yield potential under optimal environmental conditions when compared other staple food crops, hence it is considered as a food security source against famine.

Today more than one in seven people in the world are suffering from malnutrition (Glover *et al.*, 2010) and between now and 2050; the world's population is predicted to increase more than 2 billion people (Granham-Rowe, 2011). Moreover, during last few decades, the excessive consumption of fossil fuels has led to an increasing demand for alternative sources of energy (Zaldivar *et al.*, 2001). So, food and energy are an indispensable thing for this rocketing global population and cassava can meet the global challenges such as food security and sustainable energy.

Cassava is a staple food crop mainly cultivated for its tuber starch. The other parts include leaf, stem and peels. Cassava leaves and peels have some applications in daily life. Cassava leaf is widely consumed in many parts of central Africa. They are good source of protein (lysine), vitamin C and vitamin B. It is a powerful anti-oxidant to prevent cardio vascular diseases, strokes and cancer. Cassava peels can be used as roughage and as an energy feed in ruminant diets. Cassava is primarily grown for its root starch, in which 48% is used as food, 34% is used as feed and 18% is used as feedstock for bio fuels and bio chemicals (FAO, 2008). The global cassava root production for food is 256 billion tonnes per year and there are about 34 billion tons of stem residues (dry mass) also formed, in which only 10–20% is used for propagation and the rest is often considered as waste (Howeler, 2012).

Starch is an important carbohydrate stored in the plants which is a main factor for quality determination in food products and it is one of the most abundant polymers that is daily used in both food and non-food application (Yazid *et al.*, 2018). It is an easily available polymer from natural sources with high yield and low cost and usually edible and usually the natural sources include barley, corn, potato, wheat, tapioca and rice (Alcazar-Alay, 2015).

Recently the global trend is producing more bio-based products or green chemical products which are mainly from starch and starch derivatives. This increases pressure on existing starch production and crops grown for human and animal starch consumption. Hence globally the interest is growing for finding new starch resources and cassava (*Manihot esculenta* Crantz) starch from root and stem is an important research area for a long time which is used for both food and non-food applications (Zhu *et al.*, 2015).Cassava stem is a crop residue having a worldwide production of 40 Tg (Teragram) of dry weight in 2014 in which 80% is discarded as waste and the fact is it contains 40% starch (Zhu *et al.*, 2015). So, when compared to other plant stems and types of agricultural waste, the starch content in cassava stems is much higher and this starch can be easily extracted using water. Cassava stem starch is an ideal source to increase the availability of starch without using additional land, water and fertilizers. Hence understanding the structural and functional properties of stem starch is an important aspect before substituting with root starch because there is a lack of knowledge of starch properties when compared to root starch.

The starches from different parts of a plant such as roots, stems, leaves and fruits vary considerably in morphological and physicochemical characteristics in which the sizes and size distributions of starch granules are highly variable because they are the major parameters in determining the time of sedimentation, and settings for centrifuging during starch extraction processes and starch refining. More over starch granule size has important effect on starch physicochemical properties. For example, the amylopectin chain length of potato starch varied with the size of the granules and large granules tend to have a higher amylose content and lower swelling power than smaller ones and the amylose /amylopectin ratio

and pasting characteristics of a starch are also important parameters in determining their potential uses (Zhu *et al.*, 2015).

Cassava stem contain more than 30% of starch (dry mass) and the biomass of cassava stem is higher than 50% of the root mass (Wei *et al.*, 2015). Even though the role of cassava stems in both starch and energy production is neglected and the discarded cassava stems are currently removed and considered as waste.

If the cassava stem starch could substitute root starch in some applications, the scientists anticipate that a further 100 million individuals could be fed by 2030 and to unlock the full potential of cassava stem starch, more study is required (Zhu *et al.*, 2015). The wasted cassava stem starch can be utilized for both food and non-food applications. So, cassava can increase both food and fuel resources where cassava roots are for food and stems for fuel and even reduce poverty without using additional land.

Hence, keeping above points in mind, the present research entitled "Investigation on extraction of starch from cassava stem (*Manihot esculenta* Crantz)" were carried out with the following objectives:

1. To identify the cassava (*Manihot esculenta* Crantz) genotypes suitable for extraction of starch from stem.
2. To identify suitable method of extraction of starch from cassava stem.
3. To characterize the starch obtained from stem tissue.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 CASSAVA: A CROP FOR SUSTAINABLE FOOD SECURITY AND ENERGY

Cassava (*Manihot esculenta* Crantz) also known as manioc coming under the family euphorbiaceae is originally a perennial shrub. It has a chromosome number of 36 (2n = 36) and is an amphi diploid out breeding species. It is a staple food crop for humans and animals widely growing in the developing countries of tropical and sub-tropical Africa, Asia and Latin America with a total cultivated area of over 18 million hectares and utilized by over one billion people (FAOSTAT, 2016). It gives reasonable yield in dry and poor soils and does not require high level management and cost compared to other major crops.

Cassava has inherent tolerance to stressful environments and produce more energy due to its high yield potential under optimal environmental conditions when compared other staple food crops hence it is considered as a food security source against famine. It is mainly cultivated for its root starch with dry matter containing more than 80% starch while in some areas like central Africa the young leaves are also used for human consumption as a vegetable. They are good source of protein (lysine), vitamin C and vitamin B. It is a powerful anti-oxidant to prevent cardio vascular diseases, strokes and cancer. Cassava peels can be used as a roughage and as an energy feed in ruminant diets.

About 70% of world cassava root production is used for human consumption and the remaining 30% is used for animal feed and other industrial applications. Cultivars use varieties with low in cyanogen (sweet) content to avoid health problems when cassava is directly used for human consumption. When there are varieties with high cyanogen (bitter) content, the hydrocyanic acid is removed from cassava roots and leaves by using a mix of complex traditional methods and modern technologies during food processing and preparation (Essers, 1995).

For optimum growth and production of cassava, a temperature of more than 20◦C (mean day temperature) with a warm climate and for maximum leaf

photosynthesis, an optimum leaf temperature of 25–35 ◦C is required (EL-Sharkawy *et al.*, 1992). Cassava has vegetative propagation and mature woody stem cuttings of 15–30 cm long with 5000 to 20000 cuttings per hectare is planted depending on the cropping system and purpose of cultivation (Alves *et al.*, 2002). The cassava roots are harvested after 7–24 months of planting, depending on the variety, application and the growth conditions. The harvested tuber can be used as food source either freshly or cooked or marketed for consumption and can be processed for starch extraction, dried for flour production, and used for animal feed.

2.2 STARCH

Starch is a major carbohydrate reserve in plants which is an important polysaccharide in human diet and is the most abundant biomolecule on earth. It is the main source of energy for sustainable life on earth and has many other food and non-food applications in everyday life. It is a polymer of glucose joined together by glycosidic linkages.

The word starch is derived from German word "starke". Naturally starch is produced by plant leaves by using light energy and stored in amyloplasts. Starch is insoluble in water and alcohol and pure starch is solid, white in colour, odorless and tasteless powder. Native starch granules are semi crystalline forms with a density about 1.5 g/cm3.

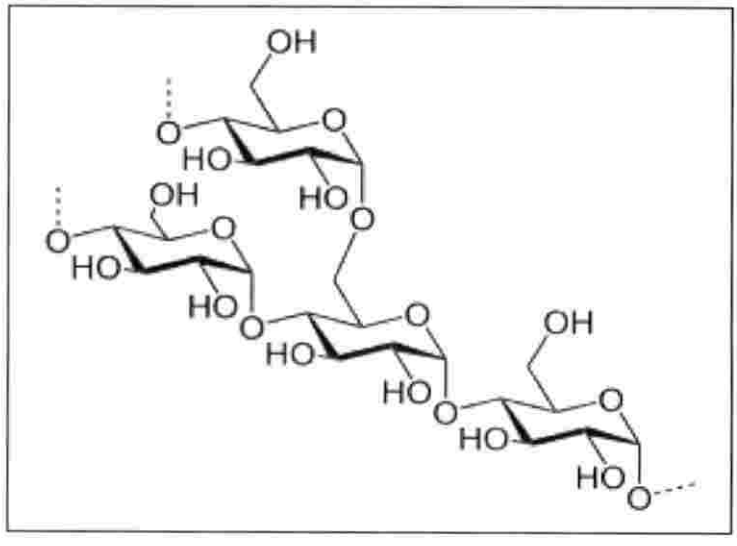


Figure 1: Structure of starch molecule

2.2.1 Structural aspect of starch

Starch has two different polysaccharide units such as amylose and amylopectin in which amylose is a linear polymer and amylopectin is branched polymer. It generally contains 20-30% amylose and 70-80% amylopectin by weight and both of these are polymer of α-D-glucose. Amylose is linked by alpha (1⟶4) glycosidic bonds and amylopectin is linked by alpha (1⟶6) bonds. The functional characteristics of starch such as viscosity, gelatinization, solubility, texture, gel stability, retrogradation, shear resistance etc. are directly related to the amylose/amylopectin ratio.

2.2.1.1 Amylose

Amylose is a linear polysaccharide made up of 500-20,000 α-(1⟶4)-D-glucose units. During the iodine test used for starch conformation the blue-violet color appears due to the formation of the amylose-iodine complex. This test is very sensitive to detect minute amounts of starch in solution.

Amylose contain long linear chains which readily crystallize when compared to amylopectin and amylose is insoluble in cold water. So, starch containing high-amylose content is more resistant to digestion (Birt *et al.*, 2013). When amylose content is higher, then the expansion potential and gel strength become low for the same starch concentration and this can be countered partially by increasing the granule size (Pycia *et al., 2014*).

α-amylase can digest the starch molecule into maltose, which can be used as energy source. Amylose has important applications in both food and non-food contexts such as thickener, water binder, emulsion stabilizer, and gelling agent but when the amylose concentration is increases, then the gel stickiness decreases but gel firmness increases. Amylose also have good film forming properties; hence it could be used for food packaging and it is better for both barrier and mechanical properties when compared to the amylopectin (Myllarinen, 2002).

2.2.1.2 Amylopectin

Amylopectin is water soluble structural unit of starch found as a branch in the amylose chain by forming α-1⟶6 glycosidic bonds that occur in every 24 to 30 glucose units of amylose chain. Amylopectin is highly branched and made up of 2,000 to 200,000 glucose units. It is larger molecule than amylose.

An amylopectin molecule consists of three types of chains such as A, B and C chain. A chains are unbranched and connected to another chain via a (1 → 6) linkage, B chains are connected to the C chain or another B chain via a (1 → 6) linkage and C chain carries the one reducing end group and numerous branches termed B chains and each of these chains have right-handed α-helix conformation (BeMiller, 2018).

Starch is formed generally by 70-80% amylopectin but the amount varies with different starches (Amylopectin content in waxy varieties are almost 100%). Due to the branching amylopectin shows a lesser tendency to gelation, retrogradation, and syneresis (Gunaratne, 2004).

2.2.3 Starch Morphology

Starches from different sources differ in their chemical and morphological structures. Starch molecules are stored in the plant as semi-crystalline granules. Different plant varieties have unique starch granular size and different morphology. The starch granule size ranges from about 2 – 100 μm such as rice starch is relatively small and is about 2 μm while potato starches have larger granules and the size is up to 100 μm (Kaur, 2009).

Table 1. Granule Size of Various Starches

Starch Species	Granule Size Range (μm) (Coulter Counter)	Average size (μm)
Waxy Rice	2-13	5.5
High Amylose Corn	4-22	9.8
Corn	5-25	14.3
Cassava	3-28	14
Sorghum	3-27	16
Wheat	3-34	6.5, 19.5
Sweet Potato	4-40	18.5
Arrowroot	9-40	23
Sago	15-50	33
Potato	10-70	36
Canna (Aust. Arrowroot)	22-85	53

The shape of starch granules also varies depending up on the source such as potato and canna, have oval shape, maize, oats, and rice starches are of polygonal and round shapes; wheat and barley starches are disk shaped (Cauvain, 2017).

2.2.4 Main sources of starch

Plants are the natural reservoirs of starch and many plants are used for the commercial production of starch. Cereals and tubers are the main sources of starch and it provides almost 80% of the calories globally. The main plant sources are maize, rice, wheat, potatoes, cassava, bananas, yams, sorghum etc. in which maize is grown in temperate and subtropical zones, cassava and banana in tropical environments and potatoes in cold climates. Banana is a new source of starch which gives excellent starch yield (Carvalho, 2008.).

Globally the demand is increasing for food materials based on renewable resources and therefore the demand is certainly increasing for new starch sources.

2.2.5 Starch properties

Starch properties include viscosity, gelatinization, solubility, texture, gel stability, retrogradation, shear resistance etc. which could affect the amylose/amylopectin ratio of starch there by functional properties.

2.2.5.1 Starch Gelatinization

Starch gelatinization is a process in which the intermolecular bonds of starch are breaking by the usage of water and heat, and thus the starch become water soluble. Three main events are happening during the processes which are granule swelling, double helical melting, and amylose leaching and finally the granule structure disintegrates (Jane, 1995). Water can act as a plasticizer during gelatinization process.

2.2.5.2 Starch Retrogradation

When cooled for a long time the gelatinized starch become thick like a gel and again become a more crystalline structure and this process is called retrogradation. There are three main molecular associations occur during the process which are amylose - amylose, amylose - amylopectin, and amylopectin - amylopectin associations. Due to strong hydrogen bonding starch which has higher amylose content will form a stiff gel but starches with high amylopectin content will have a stable gel and is softer than high amylose gels (Hegenbart, 1996). Retrogradation is enhanced by a low temperature (0-5^{o}C) and high starch concentrations and it reduces the digestibility of the starch.

2.2.5.3 Crystalline Structures

The crystalline structure of starch is a double helical structure with a 10.5 A repeat distance and six glucose units per pitch (21A°). The helix is stabilized by hydrogen bonds between the hydroxyl groups and hydrophobic interactions between the hydrocarbon moieties of the two strands. The crystalline starch is resistant to acidic and enzymatic hydrolyses.

2.2.5.4 Dextrinization

When the starch is subjected to dry heat it breaks down to form dextrins and this process is known as dextrinization. These dextrins are also called pyro dextrins and are yellow to brown in color.

2.2.5.5 Hydrolysis

Alpha-amylases are responsible for the hydrolysis of starch. Human saliva is rich in amylase and cuts starch into maltose. This process is important in the digestion of starch.

2.2.6 Functionality

Starch is an easily available, versatile and cheap material has many uses in everyday life such as thickener, water binder, emulsion stabilizer, and gelling agent. The size of starch granules determines its swelling property. When the amylose content is higher, then its swelling power decreases and the gel strength become low. But when the granule size is higher, its swelling power also increases and high amylose content can be counteracted by a larger granule size.

By considering amylose and amylopectin, amylose has the most useful functions as a hydrocolloid with high viscosity and forms useful gels and films. Its association and retrogradation on cooling and storage decreases stability and causes shrinkage and the release of water (syneresis). When amylose concentration increase, it decreases gel stickiness but increases gel firmness. Lipid content, amylose / amylopectin ratio, amylose chain length and amylopectin, and strong concentration affect retrogradation (Hegenbart, 1996). Functional derivatives of starches include cross-linked, oxidized, acetylated, hydroxyl propylated and partially hydrolyzed starch.

2.3 CASSAVA STARCH BIOSYNTHESIS GENES AND ENZYMES

The synthesis and storage of starch takes place in the plastids which consist of chloroplasts in leaves and amyloplasts in storage tissues (Pfister and Zeeman, 2016). The synthesis of starch happens through three main pathways which are the Calvin cycle, sucrose synthesis and storage starch bio-synthesis (Saithong *et al.*, 2013). Sakurai *et al.* (2007) reported that starch biosynthesis can occur through sucrose metabolism.

There are a lot of starch biosynthesis pathways and almost 45 genes such as ADPG pyrophosphorylase (AGPase), granule bound starch synthase (GBSS), starch synthase (SS), starch branching enzyme (SBE), de-branching enzyme (DBE) etc. are participating in this starch biosynthesis (Tappiban, 2018). Starch is synthesized from sucrose and is converted to hexose phosphates. It is then transported to the amyloplast and where it forms glucose-6-phosphate. The enzyme phosphor gluco mutase converts glucose-6-phosphate into glucose-1-phosphate (Viola *et al.*, 1991). The key step in starch biosynthesis is the synthesis of ADP-glucose from glucose-1-amylose is formed from ADPG by the enzyme phosphate by using ATP and the enzyme adenosine diphosphate glucose pyrophosphorylase (AGPase) (Preiss, 1982). Then granule bound starch synthase (GBSS) enzyme convert ADP-glucose into amylose. Amylopectin is formed by the catalysis of branching enzyme and soluble starch synthase (SSS) (Smith and Martin, 1995). So, the starch biosynthesis pathway mainly involves only four enzymes such as AGPase, GBSS, SSS and BE. But the enzymes involved in starch biosynthesis have several isoforms and which differ in their tissue specificity, timing of expression, kinetic properties and products.

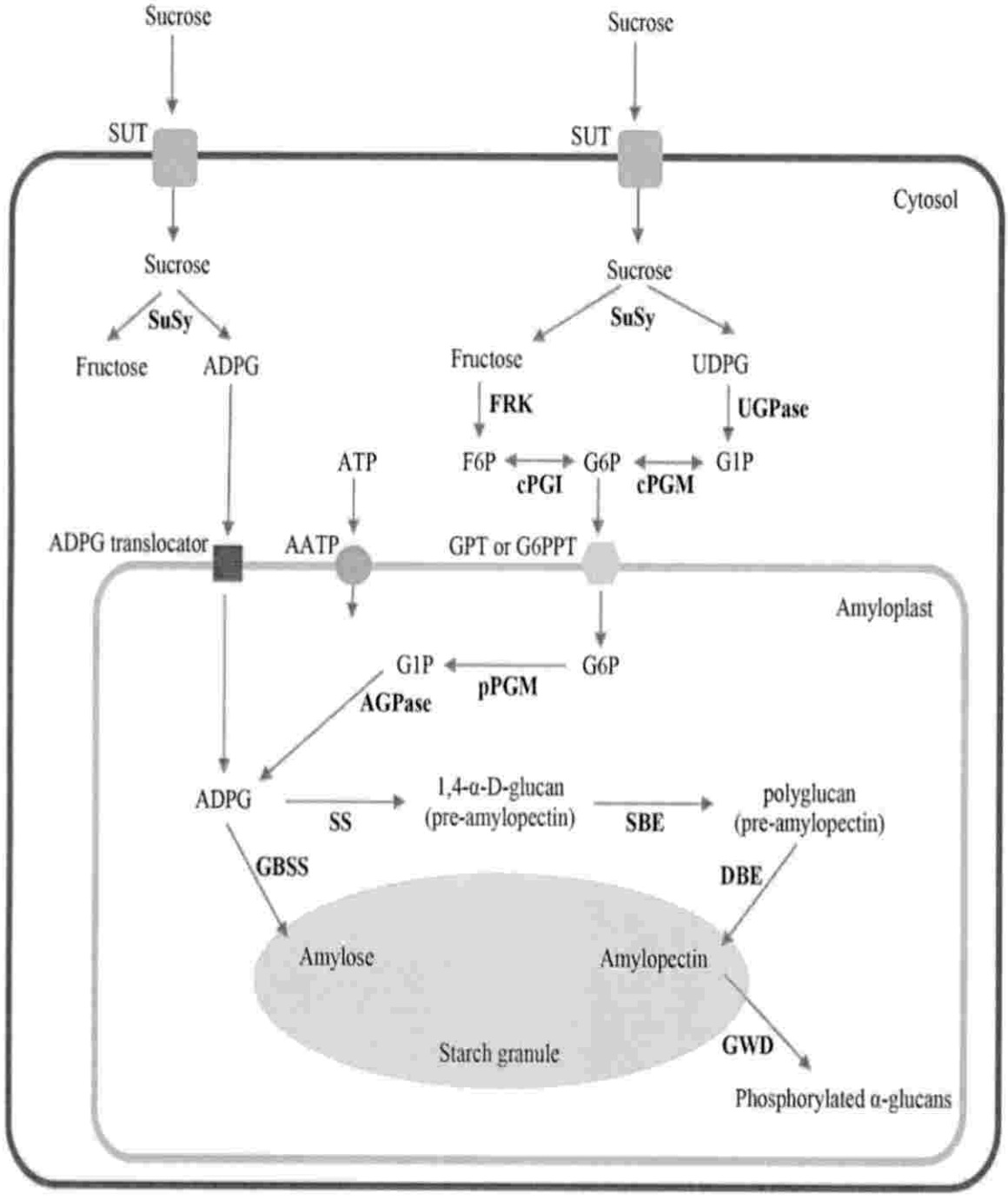


Figure 2: Pathway for biosynthesis of starch in cassava

2.3.1 ADP Glucose Pyro phosphorylase

AGPases are hetero tetrameric proteins belonging to the family of glycosyl-transferases (210-240 kDa) and composed of four subunits (2 small and 2 large called AGPase B and S respectively) (Kleczkowski *et al.*, 1991). It catalyzes the production of ADP Glucose from Glucose-1-Phosphate and a rate-limiting enzyme in starch biosynthesis which is required for accumulation of starch in cassava roots (Raemakers *et al.*, 2001). An abundance of evidence demonstrates the prominent role of AGPase in the biosynthesis of starch in plants.

Both the AGPase B and S can be expressed in all cassava tissues, but AGPase B is more expressed in leaves and tuberous root tissue than AGPaseS and there is no or a little activity in AGPase activity in petioles, stems and non-tuberous roots (Raemakers *et al.*, 2001).

2.3.2 Starch Synthases

Amylopectin is produced by the enzyme starch synthases which can be divided into two groups such as granule bound starch synthase (GBSS) and soluble starch synthase (SSS) (Keeling and Myers, 2010). GBSS is found tightly bound to the starch granule and SBSS is found in the stroma of the amyloplast or chloroplast (MacDonald and Preiss, 1985). Amylose is mainly produced by the activity of GBSS (Zhao *et al.*, 2011). GBSS can be subdivided into two groups GBSS1 located on chromosome 1 and GBSS2 located on chromosome 2(Gu *et al.*, 2015).

GBSSI synthesis amylose in storage tissue such as tubers, endosperm, embryos and pollen while GBSSII is responsible for synthesis of amylose in for non-storage tissues such as leaves, stems and roots (Karlstrom *et al.*, 2016).

The GBSSI was highly expressed in tuberous roots and their expression was low in leaves, stems and roots (Koehorst-van Putten *et al.*, 2012). The genomic DNA sequence of GBSSI has 3366 bp and contains 13 exons and 12introns (Aiemnaka *et al.*, 2012). Cassava GBSSI is also called the waxy (wx) locus (Karlstrom *et al.*, 2016)

2.3.3 Branching Enzyme

Branching enzyme catalyses the formation of the α-1,6 glycosidic linkage of amylopectin synthesis (Smith *et al.*, 1997).

The branching enzymes have multiple isoforms and plays important role in the structure and physical properties of the starch granules (Tetlow and Emes, 2014). This enzyme shows different expressions in different cassava genotypes.

2.4 CASSAVA STEM STARCH – A NEW STARCH RESOURCE

Nonfood residues of certain crops contain rarely a high content of starch in various xylem cells and usually starch is accumulated in the phloem cells (Kozlowski, 1992). So, planting stakes contain a high content of starch (Lopez Molina and Sharkawy, 1995) and starch accumulation in the stem is an important factor for vegetative propagation of plants using stem cuttings.

It is reported that cassava stems have a high content of starch (Wei *et al.*, 2018) and that starch can be easily extracted could open new way to replace the roots that are used as feedstock for producing biofuel and biochemicals which indicates the possibility of using the stem starch as a new resource and it can save the roots for increasing demands of food for growing population. So if the cassava stem starch can replace the root starch, the saved cassava roots can feed an additional 30 million people today by increasing dietary energy requirements (FAO, 2016) and these findings are very important for those one billion people who are dependent on cassava starch in terms of food, fuel, the environment, and social development.

During next two decades the cassava stem and root production is going to increase based on the fact that the world wide cassava root production increased by 30% over the last decade (FAO, 2010) and increase in yield per hectare had the largest contribution. Potassium (K) can also help to increase both root and stem yields (Lopez Molina and Sharkawy, 1995) without expansion of the cultivation areas. Based on some preliminary analysis, due to social and technological development it is optimistically say that in coming 20–30 years the starch extraction from stems can provide food for additional 100 million people globally.

Wei *et al.* (2018) reported that the cassava stem starch is oval shaped and granule size ranges from 2-15μm. The granule size of root starch is greater than stem starch but the granule morphology is similar. More over the X-ray diffraction pattern,

crystallinity and amylase content are similar to root starch but higher pasting temperature (72.1°C).

Zhu *et al.* (2015) reported that cassava can increase both food and fuel production where cassava roots are for food and stems for fuel because xylem tissues rather than phloem contain about 30% starch (dry mass) in the stem and these starches can be extracted using simple water based techniques which can increase an 87% cassava starch production worldwide. About 15% of stem starch can be extracted similar to starch production from cassava roots or potatoes by means of simple processes – milling, washing, sedimentation and no need for a large investment but some small adjustments like change in the design of the feeding devices are needed. If the particle size is reduced and improved techniques are used, a higher rate of starch can be extracted using the same extraction method (Guo, 2004).

According to Wei *et al.* (2015) the cassava stem contains about 42% starch (dry mass) and yield up to 2.0 Mg DM ha^{-1}.The location, variety and harvest time can significantly affect the stem starch content in which the yield is mostly affected by the location followed by variety and harvest time. More over the starch and soluble sugar content can be affected by soil properties such as soil pH and organic carbon, S and P contents.

Starch from cassava can be used as a potential source for the commercial bioethanol production. Due to its easy availability and cheap price, itis considered as a basic source for large-scale bioethanol production using microbial amylases. So, cassava stem starch could be used as an alternative source instead of tuber starch and complex starch polymer can be converted into various valuable metabolites by using starch liquefying and saccharifying enzymes which can selectively cleave the internal linkages of starch molecule to produce free glucose which can be utilized to produce bioethanol by microbial fermentation. Cassava stem is a potential crop residue and future food security crop. So, cassava stem starch can increase both food and fuel resources without using additional land and even reduce poverty.

2.5 STARCH EXTRACTION FROM TUBER CROPS

2.5.1 Chemical methods

Moorthy (1991) reported that 0.03 M ammonia solution can be used to extract starch from different tuber crops such as colocasia, *Dioscorea esculenta* and sweet potato by the conventional settling method. It increased the starch yield in Colocasia (6-16%) but decreased in sweet potato while remained almost the same for the other starches. Extraction using ammonia increased the peak viscosity in colocasia and *Dioscorea esculenta* but lowered in sweet potato.

Rani and Chawhaan (2012) studied the extraction of starch using 1% ammonium oxalate and 0.03 M ammonia solution in tikhur and they reported that extraction using 0.03M ammonia solution is best and yields significant quantity of starch. They got 38.46 and 37.64% yield of starch in 0.03 M ammonia solution and 1% ammonium oxalate respectively.

Witono *et al.* (2013) studied the extraction of starch using sodium meta bisulphite and NaOH from *Canna edulis* and it gave high starch yield, low ash, low fiber, and high carbohydrate content. The extraction is highly affected by NaOH concentration by the interaction with $Na_2S_2O_5$and physical treatment.

Fagbohun *et al.* (2013) extracted the starch using 1 % w/v sodium metabisulphite solution from *Moringa oleifera* and the starch obtained was a pure white, crystalline, non- hygroscopic powder and the physicochemical characterization showed that it has high potential for industrial application such as in the food, textile and pharmaceutical industries.

Afolayan *et al.* (2014) extracted starch using 1% w/v sodium metabisulphite solution from ginger (*Zingiber officinale*) roots. The starch obtained was a white, crystalline, non-hygroscopic powder with yield of about 18%.

2.5.2 Extraction of starch using simple water techniques

Daiuto *et al.* (2014) studied the starch extraction of starch from roots and tubers using water, and starch is recovered by decantation. But the starch extraction from yam is difficult due to non-starch polysaccharides which causes high viscosity

of the slurry. If the tubers were digested with an aqueous oxalic acid/ammonium oxalate, it is easy to separate because viscosity was reduced. For all the others methods tested, the viscosity remained almost the same.

2.5.3 Microwave and ultra sound assisted extraction

Kaufmann and Christen (2002) reported that microwave assisted extraction and ultra sound assisted extraction is used to extract natural products and both of the techniques allow reduced solvent consumption and shorter extraction times but the extraction yields is same or even higher than those obtained with conventional methods.

Many researchers are used ultrasound assisted extraction technique for increased yield of starch from cereals like maize or to modify the starch (Benmoussa and Hamaker, 2011).

Kamalini (2018) reported the extraction of starch from cassava stem using microwave assisted extraction with alkaline pretreatment. They used four different variables such as reaction time (60–120 s), NaOH concentration (2–4% w/v), solid to liquid ratio (1:25–1:75 g/ml), and microwave frequency (360–720 Hz) to study the effect and increase the sugar recovery. The results show that reaction time of 116.4 s, NaOH concentration of 3.21% (w/v), substrate to liquid ratio of 1:62.07 g/ml and microwave frequency of 719.86 Hz is optimum and obtained a maximum yield of 43.60 μg/ml of reducing sugar.

2.6 APPLICATIONS OF STARCH

Humans use starch as a main source of energy and it has many roles in everyday life. It plays a major role in food, pharmaceutical and packaging industries like filler, emulsion stabilizer, coating, etc.

Globally starch is a major food source which is utilized in the food industry in a number of forms such as frozen food, dairy products, puddings, flavor encapsulation, canned foods etc. More over now a days modified starches also used to enhance paste consistency, thickening, smoothness and clarity and also to impart cold storage stability and freeze thaw stability (Wurzburg, 1970).

Recently pharmaceutical companies use modified starches for various applications which plays major role in dosage formulation by providing mechanical strength, stability and tablet disintegration. Native starches were used as binder and disintegrant in solid dosage form. But its flowability is poor and now a days pregelatinized starch is used under the name of starch1500. Recent multifunctional excipients in pharmaceutical industry are modified rice starch, starch acetate and acid hydrolyzed diascorea etc. (Saboktakin, 2007).

Starch also used as tablet super disintegrant which are used for immediate release tablet formulations and sodium carboxy methylated starch is employed and marketed as sodium starch glycolate (Nattapulawat, 2009). Modified starch is used as sustained release polymer in different forms such as acetylated and phosphate ester derivative for sustaining the release of drug for better patient compliances (Pohja, 2004). The hydroxyethyl starch is used as plasma volume expander for the patients suffering from trauma, heavy blood loss and cancer treatment.

Starch used as a warp size to increase yarn strength and abrasion resistance during the weaving process. It provides a protective coating, good affinity to the yarn, good film flexibility, and stable viscosity during application.

Starch has applications in plastic industry due to its renewable, biodegradable property and it can be obtained from a variety of plant sources with low-cost. Thermoplastic starch can be obtained by the addition of polyvinyl and biodegradable starch is obtain by addition of Polystyrene also granular starch can be used as a filler to enhance biodegradation of plastics (Maddever,1989).

The petroleum industry uses modified starch to impart desirable changes to the mud used in drilling oil wells, and thousands of wells have been drilled using mixtures of sand, water and pre-gelatinized starch. It can enhance oil recovery (EOR), helps in drag reduction, and water shutoff (Lesile, 2005).

2.7 CASSAVA STEM FOR BIOFUEL PRODUCTION

Cassava stems contain high amount of starch and that starch can be extracted easily indicates the cassava stems as a new starch resource which can replace the roots that are used as feedstock for producing starch, biofuel and

biochemicals, and thus save the roots for increasing demands of food for humans. The studies show that up to 15% of stem dry mass can be currently extracted as starch by means of simple processes – milling, washing, sedimentation – similar to those for producing starch from cassava roots or potatoes.

Cassava can used for food and fuel (CFF concept) in a harmonized way without using additional land and it has a great importance for the world's growing population as it may increase both food and fuel resources and even reduce poverty. The CFF concept means the case where cassava roots can used for food and stems for fuel. Moreover, solid fuel and biogas can be produced from cassava stem residues and wastewater after starch extraction (Nuwamanya *et al.*, 2012) but the technological processes, economic feasibility and environmental risks will need to be analyzed in detail.

Usually the cassava tuberous roots are harvested and the remaining stems are left on the fields or sometimes burned considered as a waste and also the fiber residue in the wastewater after starch manufacture is discharged into water bodies. With the CFF concept, resources can be more efficiently used and both starch and fuel productions are increased and, in addition, pollution is minimized when the stems are integrated into the combined starch and fuel model. Moreover, sludge from biogas production and the ash remaining after the combustion of solid fuel from cassava stems can be recycled as fertilizers. In addition, filtrated water after the fermentation for biogas can be reused for some processes in the starch production. Exploration of and research into these left-over crop materials for fuel as well as for food and feed production is essential.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Investigation on extraction of starch from cassava (*Manihot esculenta* Crantz) stem" was carried out at the Division of Crop Utilization, ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the year 2018-2019. Details regarding the experimental materials used and procedures followed in the study are detailed in this chapter.

3.1 SOURCE OF GERMPLASM

15 high yielding and high stem biomass genotypes of cassava were selected from already screened genotypes from ICAR-CTCRI for the present study. The genotypes include H - 226, H - 165, Sree Athulya, Me - 833, Black Thailand, Quintal Kappa, M - 4, Sree Jaya, Sree Vijaya, Sree Pavithra, H - 1687 (SreeVisakam), Sree Swarna, Kunguma Rose, Ci - 848 and Kalpaka. These were raised in fields using stem cuttings as planting material.

3.2 SAMPLING AND SAMPLE PREPARATION

One plant each from fifteen genotypes was randomly chosen and harvested at the 8th month. The stems excluding the leaf and tuber were collected. The samples were preliminarily handled at a processing laboratory of CTCRI, where they were cleaned; chopped using a chopping machine; air dried in sun drier; milled and sealed in plastic bags for further use.

3.3 PHYSIOLOGICAL STUDIES OF CASSAVA STEM

After harvesting the stem, it was taken into a laboratory at CTCRI where it is cleaned, removed all leaves. Then it was weighed using a weighing balance for determining the stem fresh weight and measured its length and girth. After sun drying for one week, it again weighed for determining stem dry weight and moisture content.

3.4 RAW STARCH EXTRACTION

The milled samples (100g for each genotype) were washed in 1 L tap water at room temperature and stirred manually for about 1 minute. It was again grinded using a mixer before it was filtered through a sieve with a mesh size of 25. The material left in the container was rinsed and the remaining water was pressed out. The filtrate was left to settle for overnight. A drop of toluene was added to avoid microbial activity. The supernatant was decanted and the sediment was collected, air dried for one day, pooled, grinded, weighed and sealed in plastic bottles.

3.5 ESTIMATION OF TOTAL STARCH

Rapid titrimetric method (Moorthy and Padmaja, 2002) using alkaline potassium ferricyanide solution was used for the estimation of starch. One gram of extracted stem starch from each sample was weighed into 100ml Erlenmeyer flasks. To each flask, 20ml 80% ethanol was added and left overnight to extract the sugars.

The extracted sugars were separated from the residue by filtration through Whatman No.1 filter paper. The filtrates were collected separately for sugar estimation. The residue was transferred back into the conical flask using 20 ml of 2N HCl and 1ml concentrated HCl was added to the alcoholic sugar filtrate. The starch in the residue and sugar filtrate was then hydrolyzed by leaving the flasks on hot plate at 100°C for 30 minutes. The hydrolysates were cooled to room temperature and the volume of starch residue was increased to 100 ml and the sugar extract was raised to 50ml using distilled water for further titration. Both the extracts were titrated against 10ml of boiling potassium ferricyanide solution (1%) containing 5ml of 2.5N NaOH solution over the flame.

$$\text{Starch (\%, Dry wt. basis)} = \frac{10\times100\times0.9\times100}{T\times2\times1000} \quad \text{.... Equation (3.1)}$$

$$\text{Sugar (\%, Dry wt. basis)} = \frac{10\times50\times100}{T\times2\times1000} \quad \text{...Equation (3.2)}$$

3.6 FUNCTIONAL ANALYSIS OF STEM STARCH

3.6.1 Determination of moisture content

The moisture content in various samples was analyzed according to the AOAC method. Two grams of each sample was accurately weighed into previously weighed Petri dishes. The Petri dishes containing the samples were placed in an electric oven maintained at 105 °C for 3h. From the weights of the initial and dried samples, moisture content was determined. The dry matter content was also determined from this weight difference.

3.6.2 Swelling volume and Solubility

The swelling volume and solubility of the starch complexes were determined according to the standard procedures (Schoch, 1964). The sample (400mg) was weighed out into a conical flask (100mL), and distilled water (40 mL) was added. The samples were kept in a boiling water bath for 20 minutes with continuous swirling of the flask. After cooling the samples were transferred to graduated centrifuge tubes and centrifuged at 2000 rpm for 20 minutes. The height of the gel was noted and recorded as swelling volume in mL. From the supernatant, 10 mL was pipetted out carefully into a pre-weighed Petri dish and evaporated in an air oven at 65°Cover night. The weight of the residue was measured for calculating solubility.

$$\text{Solubility (\%)} = \frac{\text{Weight of residue (g)} \times (10 - \text{volume of swollen part})}{\text{Weight of starch taken (g)} \times 5} \quad \text{-Equation (3.3)}$$

3.6.3 Water binding capacity (WBC)

The water binding capacity of the native starch and starch complexes was determined using the standard procedure of Nuwamanya *et al.* (2011). A suspension of starch (1g) in distilled water (15 mL) was agitated using a mechanical stirrer for 1 h and centrifuged (3000 x g) for 10 minutes. The water layer was decanted from the wet starch and the residue was weighed. The water binding capacity was calculated as follows:

WBC (%) = Gel Weight-Weight of starch (g) .....Equation (3.4)
Weight of starch taken

3.6.4 *In vitro* enzyme digestibility

In vitro enzyme digestibility of the starch samples was estimated by using pancreatic α - amylase [pancreatin 3X (100units/mg/min) (SRL, Mumbai, India)]. The sample (100 mg) was weighed, sodium phosphate buffer (10 mL, 0.02 M, pH-6.9) was added and the solution was heated in a boiling water bath. After cooling, the volume was made up to 20 mL using the buffer; pancreatic amylase solution (0.5 mL of a solution of 25 mg of enzyme dissolved in 25 mL phosphate buffer) was added and incubated at 30oC for 1 h. After the incubation period, the samples were heated in a boiling water bath to deactivate the enzyme. The reducing sugar formed was estimated by the Nelson's method (1944). The digestibility was calculated as percentage.

3.6.5 Pasting Properties (Viscometry)

The viscosity parameters (peak viscosity, breakdown, setback viscosity, and pasting temperature) of the complexes and the native starch were obtained using a Rapid Visco Analyzer (RVA Tec Master, Perten Instruments, Australia) controlled by thermo cline for Windows software. A fixed starch concentration (10%) was used for the study. Standard 1 measurement profile was chosen. The temperature programme was as follows: heating from 50°C to 95°C at 12°C/min, holding at 95°C for 2 minutes, cooling to 50°C at 12°C/min and holding at 50°C for 2 minutes. The sample + water was stirred in an RVA canister at 960 rpm for 10s, then at 160 rpm for the remainder of the test. Determination was done in triplicate. The viscosity was recorded in centipoises (cP) (1cP= 1mPas). The viscosity profile recorded by the RVA reflects the peak viscosity (PV), breakdown (BD), final viscosity (FV), setback (SB) and pasting temperature.

3.7 STARCH LOCALISATION USING STAINING

The cassava stem sections were taken using a sterile blade and sections were kept in IKI (Iodine-Potassium iodide) solution for fifteen minutes and the sections were visualized using Nikon ECLIPSE E200 microscope. (To make this solution,

dissolve 2g of KI in 100ml of water and then dissolve 0.2g of iodine in the KI solution. Starch will appear as a blue black in a few minutes. Newly formed starch will appear red to purple).

3.8 ENZYMOLOGICAL STUDIES

3.8.1 Estimation of peroxidase content

The extract of peroxidase (POD) was prepared using 2.0 g stem starch in 15 ml extraction buffer (0.05M Sodium phosphate buffer pH 6.0 containing 0.05% ascorbic acid and 1% polyvinyl pyrrolidine). The tissue was homogenized for 3-5 minutes and the homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and used for the estimation of peroxidase content.

Two types of assay system were used: a control system and a test system. Test system consist of 0.5ml 0.05M pyrrogallol (the substrate), 0.1ml of 12.3mM hydrogen peroxide (the hydrogen donor), 2.9ml of citrate phosphate buffer (0.1M: pH 7.0) and 0.5ml of enzyme extract. In the control system, 3.5 ml of citrate phosphate buffer (0.1M: pH 7.0) and 0.5ml of enzyme extract alone were taken. The test and control systems were incubated for 20 min at room temperature and then kept in boiling water bath for 2 min. The absorbance was measured at 436nm using UV/Vis spectrophotometer.

3.8.2 Estimation of poly phenol oxidase content

The enzyme extract was prepared by homogenizing 2.0g stem starch powder in 15ml extraction buffer (0.1M citrate phosphate buffer, pH 7.0) containing 1% PVP. It was kept in a refrigerator for 20 min and then centrifuged at 10,000 rpm for 10 min in a cooling centrifuge. The clear supernatant was used as the enzyme source. Two types of assay systems were used for the estimation of polyphenol oxidase: a control system and a test system both consisting of 3.0ml 0.5% catechol (the substrate), 1.0ml of citrate phosphate buffer (0.1 M: pH 7.0) and 1 ml of enzyme extract. In the control system, immediately after the addition of enzyme extract, the tubes were kept in boiling water bath for 5 min to inactivate the enzyme. The test system was incubated for 30 min at RT and then kept in a boiling water bath for 2 min. The absorbance was measured at 400 nm using UV-Vis

spectrophotometer.

3.9 EXPRESSION PROFILING OF STARCH BIOSYNTHESIS GENE

3.9.1 Glass wares and other materials

1.5 ml and 2 ml Eppendorf tubes, PCR tubes, mortar and pestle, micropipette tips for 10 µl, 200 µl and 1000 µl were autoclaved and used. 1.5 ml tube stand, PCR tube holders, micropipettes, ice bags, measuring cylinder, bottles, spatula, polythene covers, labels, wipes, weighing pot and the other materials needed for molecular work.

3.9.2 Instruments

The equipments viz., water bath, weighing balance, microwave oven, ice machine, vortex mixer, spinner, cooling centrifuge, Nano Drop spectrophotometer, pH meter, deep freezer (-20 °C, -80 °C), refrigerator, electrophoresis apparatus, gel documentation system, PCR machine, hot air oven, autoclave and distilled water unit were used for the study.

3.9.3 RNA Extraction

RNA was extracted from fresh tender stems of two different cassava plants having high starch content in stem (H-1687) and low starch content in stem (Quintal) using manual TRIzol method. TRIzol reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. TRIzol reagent is a monophasic solution of phenol, guanidine isothiocyanate, and other proprietary components which maintains the integrity of the RNA due to its highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. It is a single-step RNA isolation method developed by Chomczynski and Sacchi in 1987.

A 0.1g of plant tissue was weighed and pulverised in pre chilled mortar and pestle using liquid nitrogen and 1ml TRIzol reagent and was transferred to a 2 ml sterile centrifuge tube. It was kept in room temperature for 5 min and centrifuged at 13,000rpm for 5 min at 4^0C. Then the supernatant was transferred to a fresh centrifuge tube and 200µl chloroform was added and mixed by vortexing. Again, incubated in RT for 5 min and centrifuged at 13000 rpm for 5 min. Upper phase

was transferred to a new tube and 0.5ml ice cold isopropanol was added, mixed well and incubated for 10 minutes at RT. RNA was precipitated by centrifugation at 12000 rpm for 10 minutes at 4 °C. Pellet was dissolved in 1ml 75% ice cold ethanol and is suspended completely and centrifuged at 8000 rpm for 5 minutes at 4 °C. Discarded the supernatant and pellet was air dried for 20-30 minutes and was dissolved in 30µl of nuclease free DEPC water. This was stored at -80 °C for further usage. All samples were checked for RNA in 1.5% agarose gel and confirmed.

3.9.4 Agarose gel electrophoresis

A1.5% of agarose gel was used to check the quality and integrity of the extracted RNA. 1.5% agarose solution was prepared by weighing out 1.5 g agarose in a conical flask and dissolving it using 100 ml 1X TBE buffer. Every reagent was prepared in DEPC treated water. Agarose was dissolved by heating and after that the flask was allowed to cool and when the temperature of the flask decreases, about 0.9 µl (10 mg/ml) of EtBr was added directly to the gel and gentle mixing was done. Casting tray was prepared with combs to which gel was poured and allowed to solidify. 2µl of isolated RNA sample mixed with 3 µl of 1X loading dye was loaded into the wells of prepared gel. Horizontal gel electrophoresis unit was used to run the gel. The gel was run for about 30 minutes at 55V. The run was terminated after the dye front reached 3/4th of the gel. Then it was visualized in UV light using a gel documentation system.

3.9.5 Quantification of RNA

RNA was quantified using UV spectrophotometer (Denovix DS - 11+ spectrophotometer). Nuclease free water in which RNA was dissolved was used to calibrate the machine to blank i.e. zero absorbance. The advantage of Nano Drop is that it requires only 1 µl sample to measure its quantity and quality. The quantity of RNA was determined at OD_{260} and the purity was determined by OD_{260}/OD_{280} ratio.

3.9.6 cDNA synthesis

RNA is inherently susceptible to RNase degradation and it is a chemically

unstable molecule. For the RT-PCR, the mRNA is converted to cDNA by the reverse transcriptase enzyme as it is more stable. This enzyme catalyzes cDNA synthesis starting with the poly A tails on the individual mRNA molecules.

Mix-up the RNA sample and primer oligo d(T) in a sterile tube. Make the volume of 10 µl using DEPC treated water or sterile distilled water. Then hold the mixture for denaturing, at 65°C for 5 minutes. Spin briefly and hold on ice.

COMPONENT	VOLUME
RNA	1 µg
Oligo d(T)	1 µl

Nuclease-free H_2O to a total volume of 10 µl

Next step is the preparation of master mix (Table 2).

Table 2: Master mix preparation for cDNA synthesis

COMPONENT	VOLUME
100 mM dTT	1µl
5X Assay Buffer	4 µl
25 mM dNTP	2 µl
RT Enzyme	1 µl
DEPC water	2 µl

Nuclease-free H_2O to a total volume of 10 µl

Incubate the 10 µl cDNA synthesis reaction at 37°C for one hour using ProFlex PCR machine. Inactivate the enzyme at 94°C for 2 minutes. The cDNA product should be stored at -20°C. In general, the volume of cDNA product should not exceed 1/10 of the PCR reaction volume.

3.9.7 Primer Designing

Primer was designed for Starch Synthase gene (SS1) using Primer 3+ software. The composition of the primer pair designed and used for the study were as follows:

CASSAVA SS1

Forward Primer- 5' TGGTGACCACGACACAAACA 3'

Reverse Primer- 5'ATTGTCCCCAGCCAAAGGAG 3'

Synthesis of the primers was done by Integrated DNA Technologies (Cochin).

3.9.8 RT-PCR

The components used in a PCR reaction are Taq polymerase, primers, template DNA, and dNTPs nucleotides (DNA building blocks). The components are added sequentially in a tube, along with the cofactors required by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.

Table 3- PCR mix for PCR amplification

REACTION INGRADIENTS	REQUIRED VOLUME
Emarald Master Mix	7.5 µl
Primer (F and R)	2 µl
DNA sample	2 µl
Sterile distilled water	3.5 µl

PCR is done using the ProFlex PCR machine.

3.9.9 Thermal profile

1. Initial denaturation : 95 °C 3min
2. Denaturation : 95 °C 30s
3. Annealing : 57.5 °C 30s
4. Extension : 72 °C 30s

   72 °C 7 min

Number of cycles: 35 cycles, step 2-4

After completion, the expression was analyzed using agarose gel.

3.9.10 Agarose gel electrophoresis to visualize the results of PCR

Agarose gel electrophoresis was used to visualize the SS1 gene expression. For this, 1.5% of Agarose gel was prepared in the 1X TBE buffer. The PCR products were loaded and also an I Kb Plus ladder was added in the gel to estimate the size of the gene. The run was at 60 volts. After run, the gel was documented using Gel-documentation system.

3.9 IDENTIFICATION OF SUITABLE STEM STARCH EXTRACTION METHOD

3.9.1 Extraction of starch using simple water technique

A 100g of cassava fresh stem and dried stem powder (H-1687) were used for the extraction of starch. The milled samples (100g for each variety) were washed in 1 L tap water at room temperature and stirred manually for about 1 minute. It is again grinded using a mixer before it was filtered through a sieve with a mesh size of 25. The material left in the container was rinsed and the remaining water was pressed out. The filtrate was left to settle for 6h. A drop of toluene was added to avoid microbial activity. The supernatant was decanted and the sediment was collected, air dried for one day, pooled, grinded, weighed and sealed in plastic bottles.

3.9.2 Extraction of starch with the application of chemicals

0.2% NaOH and 0.5% $Na_2S_2O_5$ (sodium meta bisulphite) are used for the extraction of starch from fresh cassava stem and dried stem powder (H-1687). 100g samples were weighed and suspended in 1L 0.2% NaOH and 0.5% $Na_2S_2O_5$ and kept at room temperature and stirred manually for about 1 minute. It is again grinded using a mixer before it was filtered through a sieve with a mesh size of 25. The material left in the container was rinsed and the remaining water was pressed out. The filtrate was left to settle for 6h. The supernatant was decanted and the sediment was collected, air dried for one day, pooled, grinded, weighed and sealed in plastic bottles.

3.9.3 Microwave assisted extraction

Microwave assisted extraction was used for the extraction of starch from cassava stem. Three different variables were used such as reaction time (60–120 s), Different solvents (0.2% NaOH, 0.5% $Na_2S_2O_5$, water) and microwave frequency (360–720 Hz) to study the starch recovery. The sample was passed through a 25 µm sieve; the filtrate was left to settle for 6h. The supernatant was decanted and the sediment was collected, air dried for one day, pooled, grinded, weighed and sealed in plastic bottles.

3.9.4 Ultra sound assisted extraction

Sample was sonicated using Rivotek™ ULTRASONIC SONICATOR/20 kHz. Different levels of time were tested. The sample was passed through a 25 µm sieve, the filtrate was left to settle for 6h. The supernatant was decanted and the sediment was collected, air dried for one day, pooled, grinded, weighed and sealed in plastic bottles.

RESULTS

4. RESULTS

The present study entitled the "Investigation on extraction of starch from cassava (*Manihot esculenta* Crantz) stem" was conducted at the Division of Crop Utilization, Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram, Kerala. The results obtained from the study are summarized below.

4.1 CASSAVA GENOTYPES FOR THE STEM STARCH EXTRACTABILITY

Fifteen high yielding and high stem biomass genotypes of cassava were selected from ICAR-CTCRI collection. The genotypes include H - 226, H - 165, Sree Athulya, Me - 833, Black Thailand, Quintal Kappa, M - 4, Sree Jaya, Sree Vijaya, Sree Pavithra, H - 1687 (Sree Visakam), Sree Swarna, Kunguma Rose, Ci - 848 and Kalpaka (Plate 1).

4.2 STARCH EXTRACTION USING SIMPLE WATER TECHNIQUE

The stem starch for the functional analysis was extracted using simple water technique. Using the routine water extraction method as described earlier, starch was extracted from the selected cassava genotypes. The extracted starch powder of each variety is depicted in Plate-2. Significant differences were observed starch yields from stems of different cassava genotypes. The starch content was in the range of 16-30% by dry weight and it was found that H-1687 genotype had the highest starch yield (0.297 g/g) and the cassava genotype Sree Vijaya had lowest starch yield (0.1687g/g). The data on starch yield from different genotypes is represented in the Figure - 3.

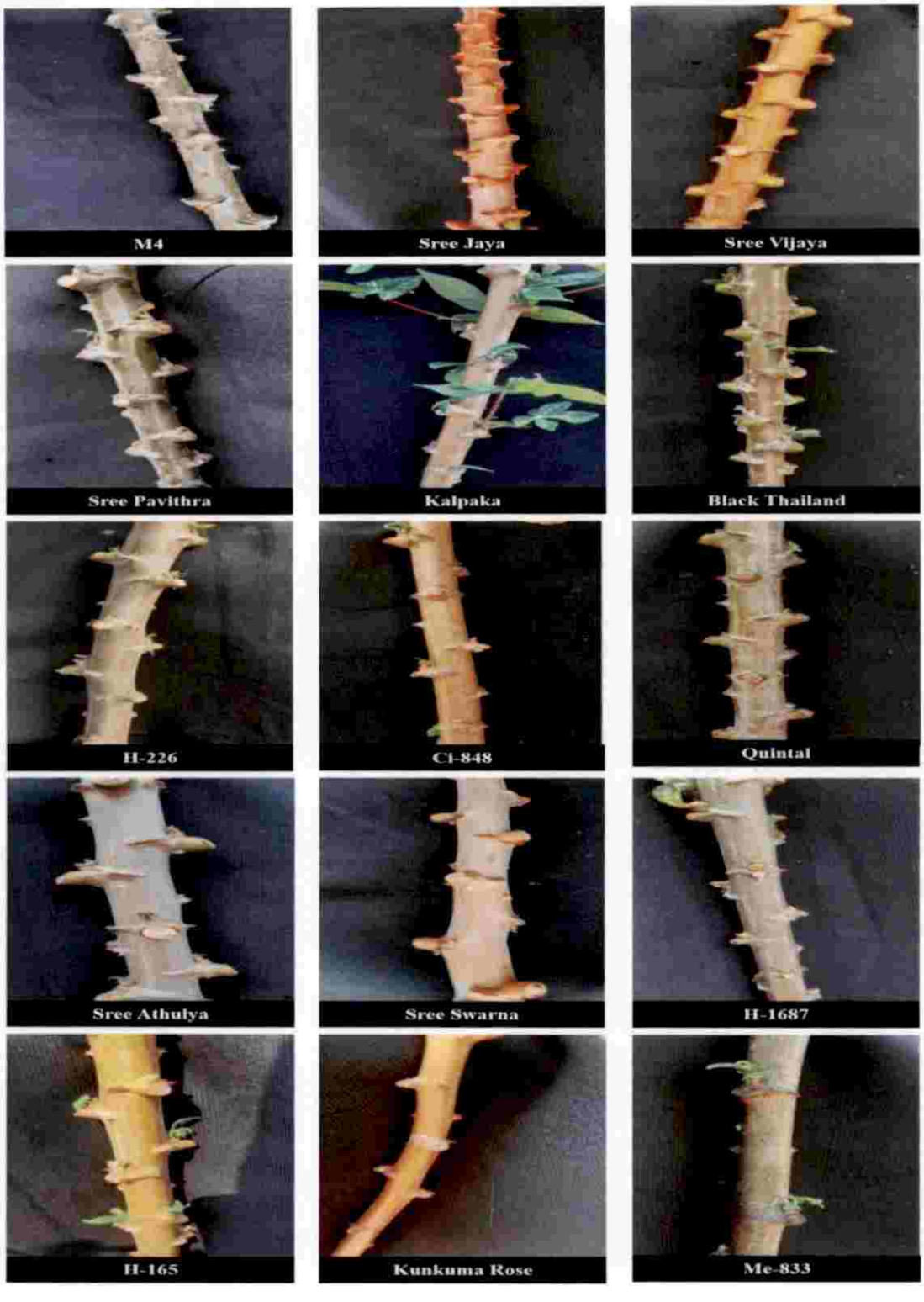


Plate 1: Cassava stem samples from different cassava genotypes for the extraction of starch

Plate 2: Starch extracted from stem samples of different cassava genotypes

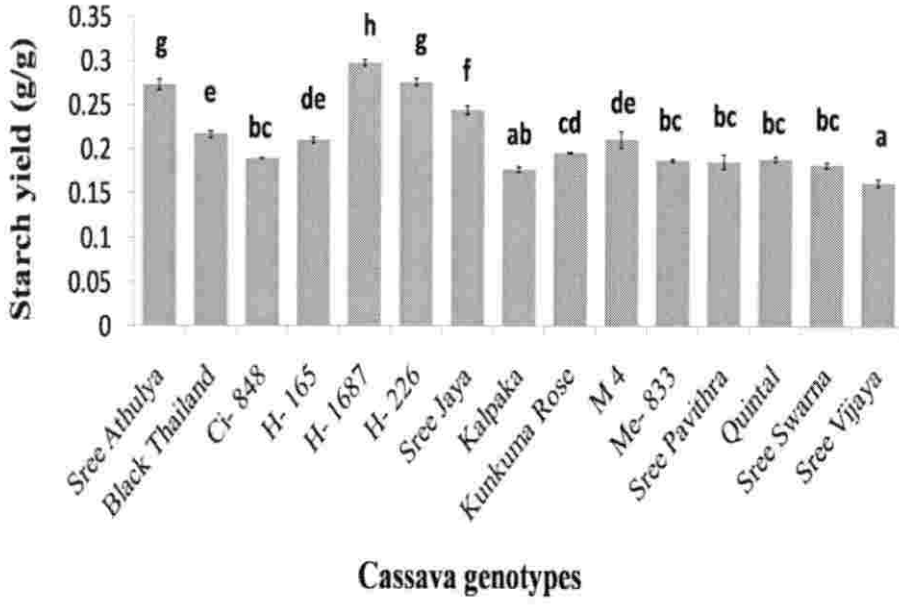


Figure-3: Starch yield from stems of different cassava genotypes using simple water extraction technique

4.3 PHYSIOLOGICAL STUDIES OF CASSAVA STEM

Stem average length, girth, fresh weight, dry weight and moisture content of each variety were determined.

4.3.1 Cassava stem height

Significant difference was observed in the stem height of cassava. The height of cassava stem ranged from 86.12 to 262.0 cm. Sree Athulya had the longest stem (262 cm), then Sree Pavithra (200cm) and the shortest stem was found for Kalpaka (86.5cm) (Figure-4).

4.3.2 Cassava stem girth

Significant difference was observed in the stem girth of different genotypes of cassava. The stem girth of cassava ranged from 3.3 to 7.2cm. The genotypes Quintal, Sree Pavithra and H-165 had more than 8.0 cm stem girth at the bottom. Kalpaka and Ci-848 had lowest stem girth (Table-4).

4.3.3 Stem fresh weight and dry weight of cassava stem

Significant difference was observed in the fresh weight and dry weight of cassava stem. The fresh weight and dry weight of cassava stem ranged between 0.5

and 2.5 kg and from 0.2 to 0.8 kg respectively. Sree Swarna and Sree Pavithra had maximum stem fresh weight and dry weight whereas Kalpaka and Me-833 has lowest fresh weight and dry weight (Figure-5).

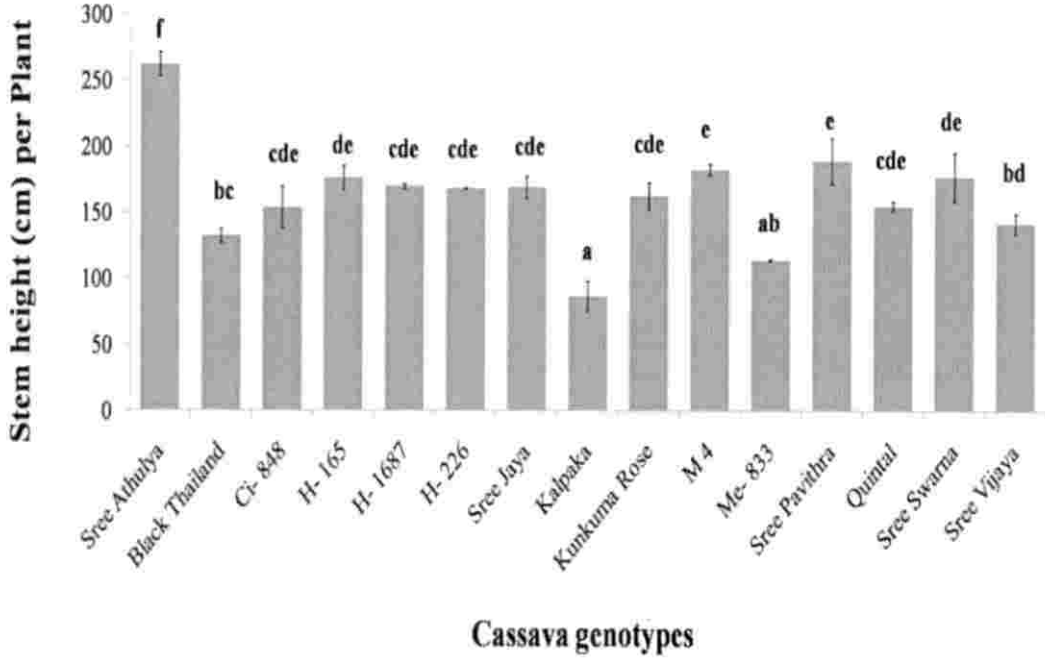


Figure 4: Characteristics of stem height in selected genotypes of cassava

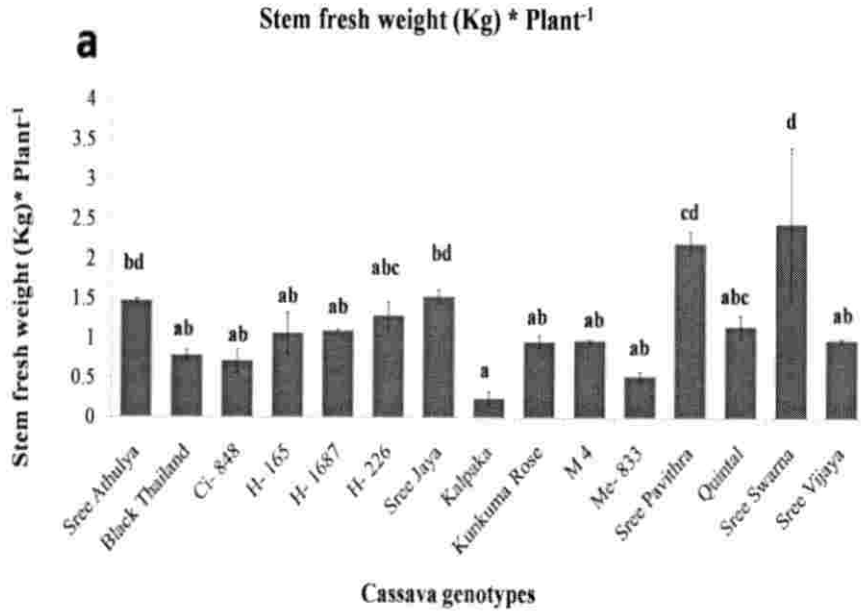
4.3.4 Moisture content of cassava stem

Significant difference was observed in the case of moisture content of cassava stem. The moisture content of cassava stem varied from 62-72%. The cassava varieties Me-833, Kalpaka and Kunkuma Rose had more than 70% moisture content and Sree Athulya, Sree Jaya, Sree Pavithra and Quintal had lowest moisture content(less than 66%) (Figure-6).

Table 4: Stem girth of different genotypes of cassava

Sl. No.	**Cassava genotypes**	Stem Girth Bottom (cm)	Stem Girth Middle (cm)	Stem Girth Top (cm)
1	Sree Athulya	6.97 ± 0.24^{ac}	5.15 ± 0.49^{ac}	2.35 ± 0.42^{ab}
2	Black Thailand	6.5 ± 0.91^{ac}	5.5 ± 0.07^{ac}	3.65 ± 0.14^{cdf}
3	Ci- 848	5.5 ± 1.34^{ab}	3.7 ± 0.49^{ab}	1.77 ± 0.10^{a}
4	H- 165	8.07 ± 1.02^{bc}	6.02 ± 0.77^{ac}	4.67 ± 0.10^{fg}
5	H- 1687	7.12 ± 0.81^{ac}	5.32 ± 0.84^{ac}	2.65 ± 0.35^{ad}
6	H- 226	6.22 ± 0.32^{ac}	4.95 ± 0.68^{ac}	3.12 ± 0.17^{bde}
7	Sree Jaya	7.3 ± 0.14^{ac}	5.8 ± 1.07^{ac}	3.82 ± 0.24^{df}
8	Kalpaka	4.57 ± 1.09^{a}	3.3 ± 0.85^{a}	2.02 ± 0.17^{ab}
9	Kunkuma Rose	5.77 ± 1.09^{ab}	4.65 ± 0.24^{ac}	2.47 ± 0.45^{abc}
10	M 4	6.9 ± 0.92^{ac}	5.8 ± 0.38^{ac}	4 ± 0.35^{ef}
11	Me- 833	6 ± 0.49^{ac}	5.6 ± 0.247^{ac}	4.3 ± 0.07^{efg}
12	Sree Pavithra	8.47 ± 0.39^{bc}	6.37 ± 0.35^{bc}	4.6 ± 0.28^{fg}
13	Quintal	8.57 ± 1.23^{bc}	7.2 ± 0.77^{c}	5.32 ± 0.45^{g}
14	Sree Swarna	9.32 ± 0.17^{c}	6.8 ± 0.74^{c}	4.2 ± 0.07^{efg}
15	Sree Vijaya	5.87 ± 0.95^{ab}	5.22 ± 0.23^{ac}	3.77 ± 0.67^{df}

*Means with same alphabets in the same column is not significantly different.



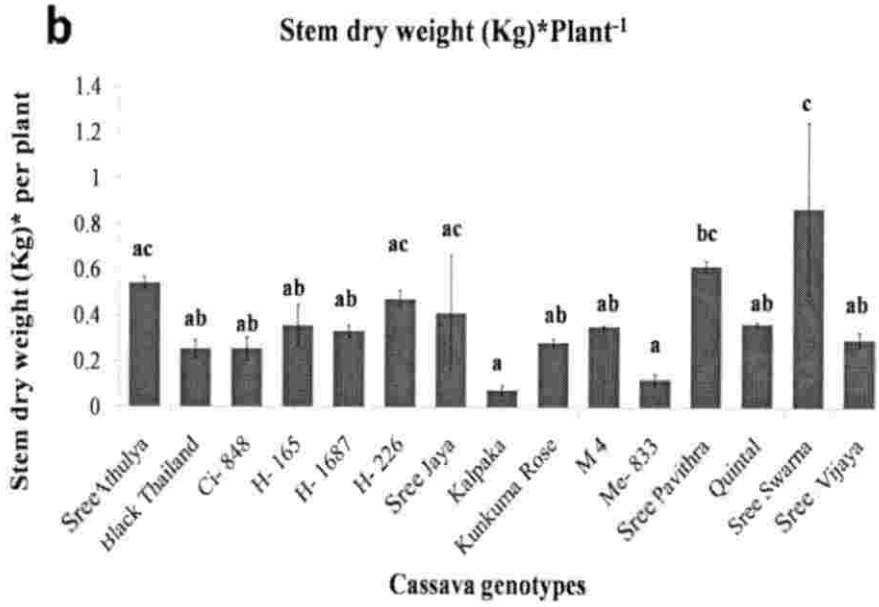


Figure 5: Characteristics of stem fresh and dry weight of cassava genotypes

a. Stem fresh weight b. Stem dry weight

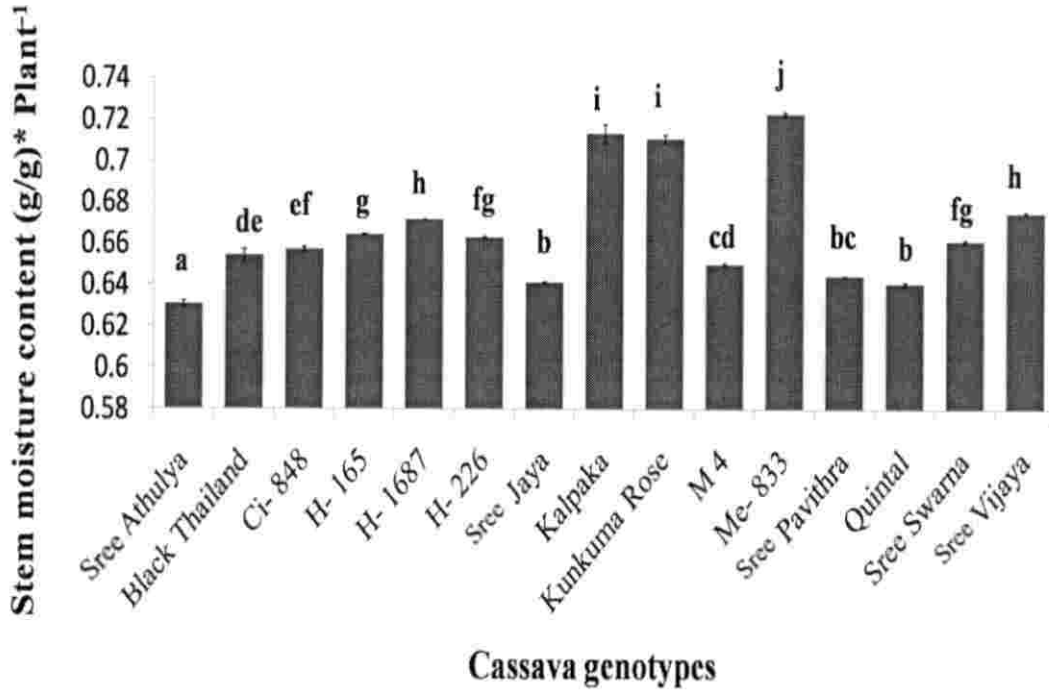


Figure 6: Characteristics of stem moisture content of cassava genotypes

4.4 Stem starch localization

Starch localization in cassava stem was done by KI staining and observed using Nikon ECLIPSE E200 microscope. Starch was found localized mostly near the primary and secondary xylem (Plate -3).

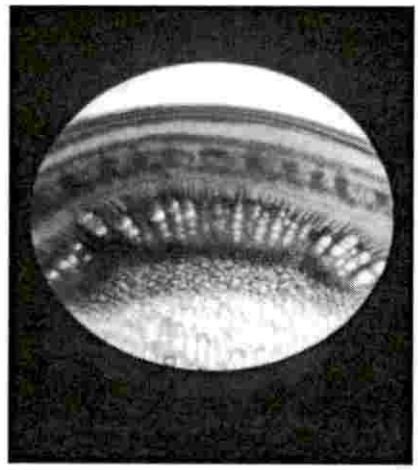


Plate 3: Starch localization near the primary and secondary xylem in the cassava stem

4.5 Estimation of starch content

Total starch content in cassava stem was estimated by standard tritrimetric assay. The starch content in cassava stem varied from 37 to 55% on dry weight basis. The highest starch content in selected genotypes was observed in H-1687 (55%) and lowest was observed for Quintal (37%) (Figure-7).

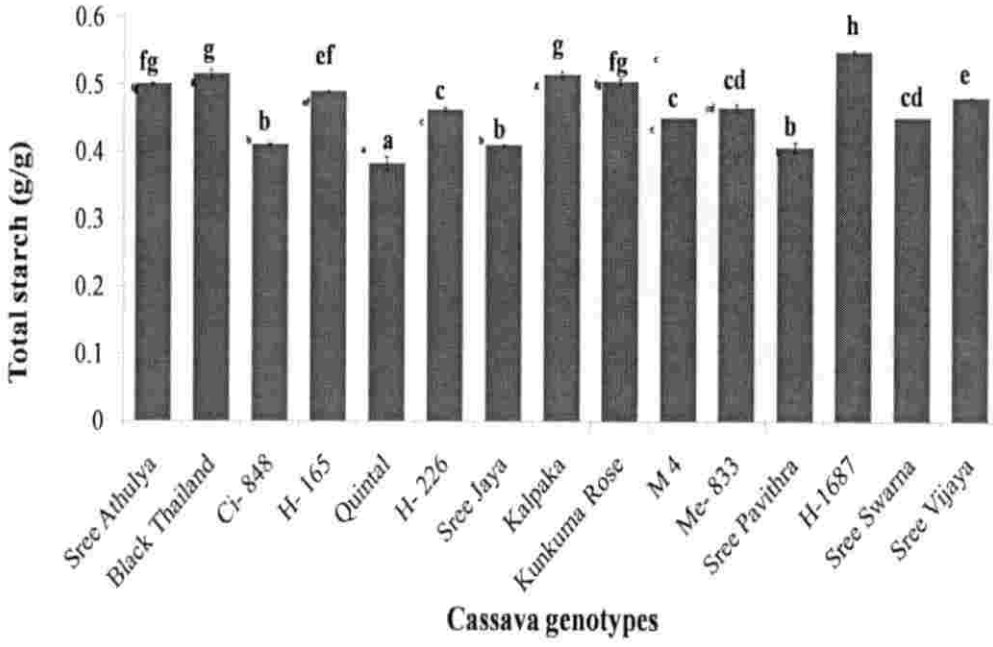


Figure-7: Characteristics of stem starch in different cassava genotypes

4.6 Functional analysis of stem starches

For native starches, the functional properties like moisture content, swelling volume, solubility, water binding capacity, *in vitro* digestibility and pasting properties are significantly related to the average granule size of the starches separated from various source.

4.6.1 Starch moisture content

The moisture content was less than 2% in all stem starches. Moisture content was highest in Kalpaka (1.5%) and lowest in Sree Athulya (1%). There was not much difference in moisture content among the different genotypes of cassava stem starches (Figure-8).

4.6.2 Swelling volume and solubility

The strong swelling power of starch granules makes it easy for them to reach their maximum viscosity and they are likely to breakdown easily because of their weak intermolecular forces, thus becoming more sensitive to shear force as the temperature increases. The swelling volume was same for all the stem starches (15ml) (Figure-9a) and the solubility was less than 10% (Figure-9b). The solubility was higher for M4 and H-226(7.5%) and lowest for Ci-848(3.75%).

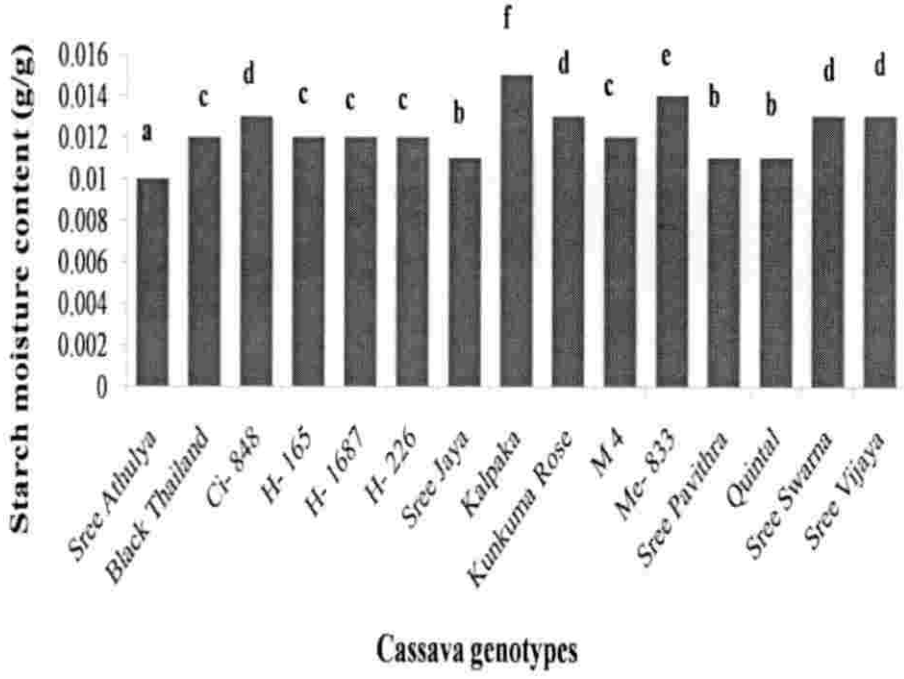
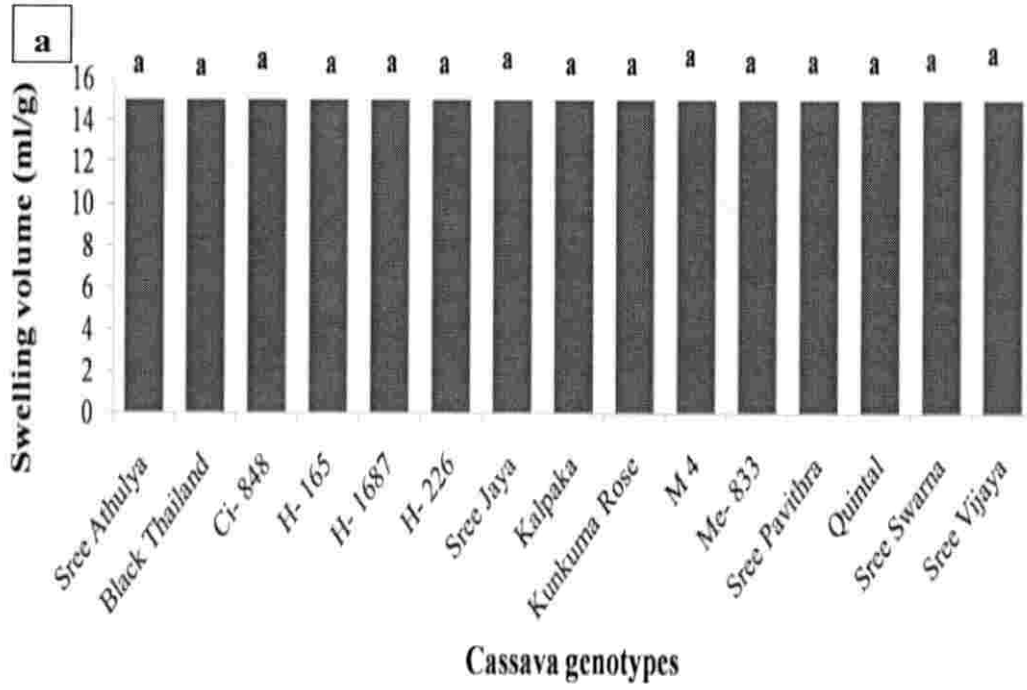


Figure-8: Moisture content of starch extracted from stems of different cassava genotypes



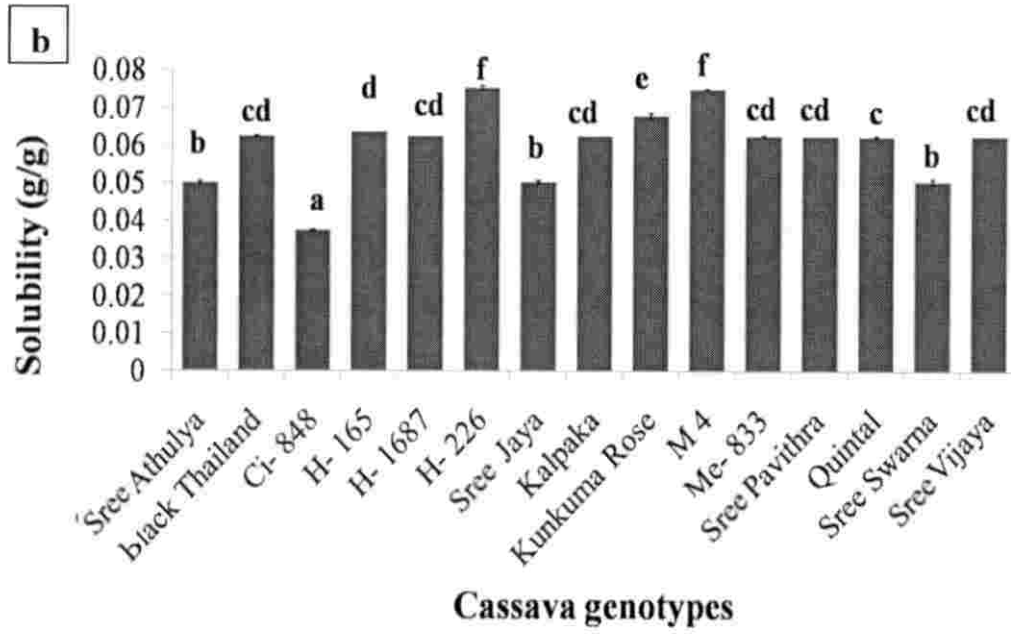


Figure-9: a) Swelling volume of cassava stem starch b) Solubility of stem starch

4.6.3 Water binding capacity

The water binding capacity varied from 46.09% to 77.50% for stem starches from the selected genotypes of cassava (Figure-10).

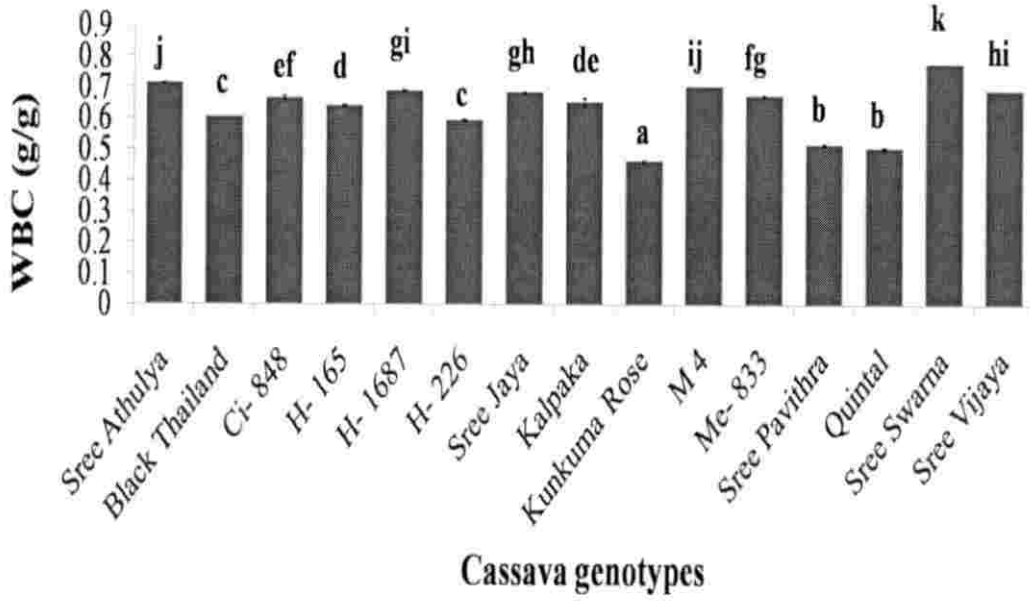


Figure-10: Water binding capacity of starch extracted from stems of different cassava genotypes

4.6.4 *In vitro* starch digestibility

In vitro starch digestibility ranged from 12.43 to 31.8% (Figure-11). *In vitro* starch digestibility was found to maximum for M4 and minimum for Quintal.

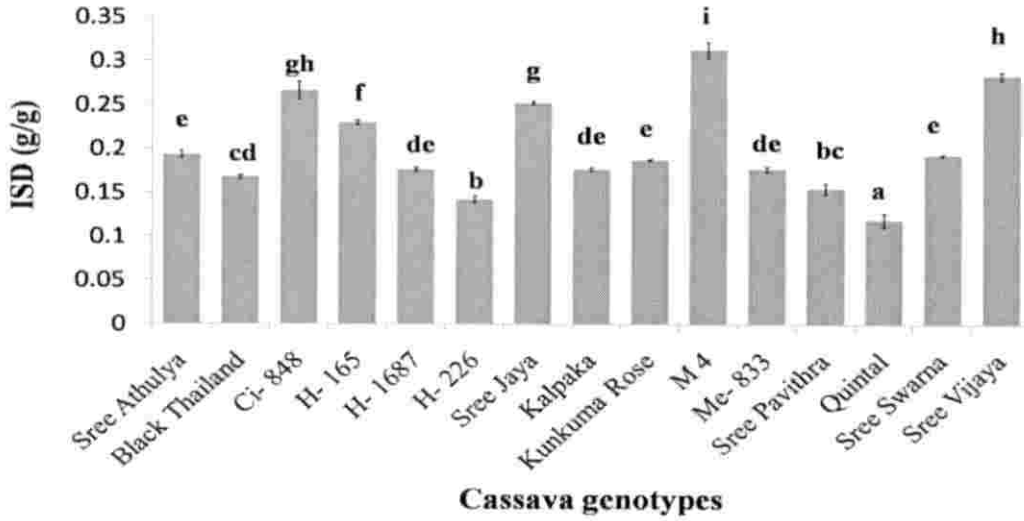


Figure-11: *In vitro* starch digestibility of starch extracted from different varieties of cassava stem

4.6.5 Pasting properties

Stem starches from different cassava varieties exhibited variations in viscosity characteristics when analyzed by a Rapid Visco Analyzer. The viscosity of stem starches ranged between 309 and 1651cP. Pasting time ranged between 5.33 and 6.93 min and pasting temperature ranged from 84.70 to 94.25°C.

Viscosity value was highest in Black Thailand followed by Sree Athulya and the lowest for Sree Jaya and Kalpaka. Pasting time and pasting temperature were maximum for Sree Swarna and pasting time was lowest for Sree Jaya but pasting temperature was lowest for Sree Athulya (Table-5).

Table-5: RVA viscosity parameters of different cassava stem starches

SL No.	Sample	Peak viscosity	Breakdown viscosity (cP)	Final viscosity	Set back viscosity	Pasting time (m)	Pasting temp. (°C)
1	Quintal	786.00	96.00	913.00	223.00	6.00	89.40
2	Sree Swarna	369.00	11.00	492.00	134.00	6.53	94.25
3	Sree Vijaya	956.00	113.00	1089.00	246.00	5.80	86.45
4	Sree Jaya	281.00	68.00	309.00	96.00	5.33	92.15
5	Kalpaka	178.00	11.00	324.00	157.00	6.93	89.56
6	Kunkuma Rose	1243.00	280.00	1392.00	429.00	5.40	88.5
7	M4	672.00	104.00	767.00	199.00	5.93	90.55
8	Me-833	846.00	142.00	1075.00	371.00	5.67	85.75
9	Sree Pavithra	299.00	13.00	404.00	118.00	6.13	93.90
10	Sree Athulya	1191.00	190.00	1470.00	469.00	5.73	84.70
11	Black Thailand	1339.00	103.00	1651.00	415.00	6.33	87.25
12	Ci-848	807.00	122.00	1026.00	341.00	6.00	88.40
13	H-165	1002.00	179.00	1121.00	298.00	5.87	89.75
14	H-226	997.00	189.00	1076.00	268.00	5.73	87.25
15	H-1687	639.00	99.00	755.00	215.00	5.93	92.05

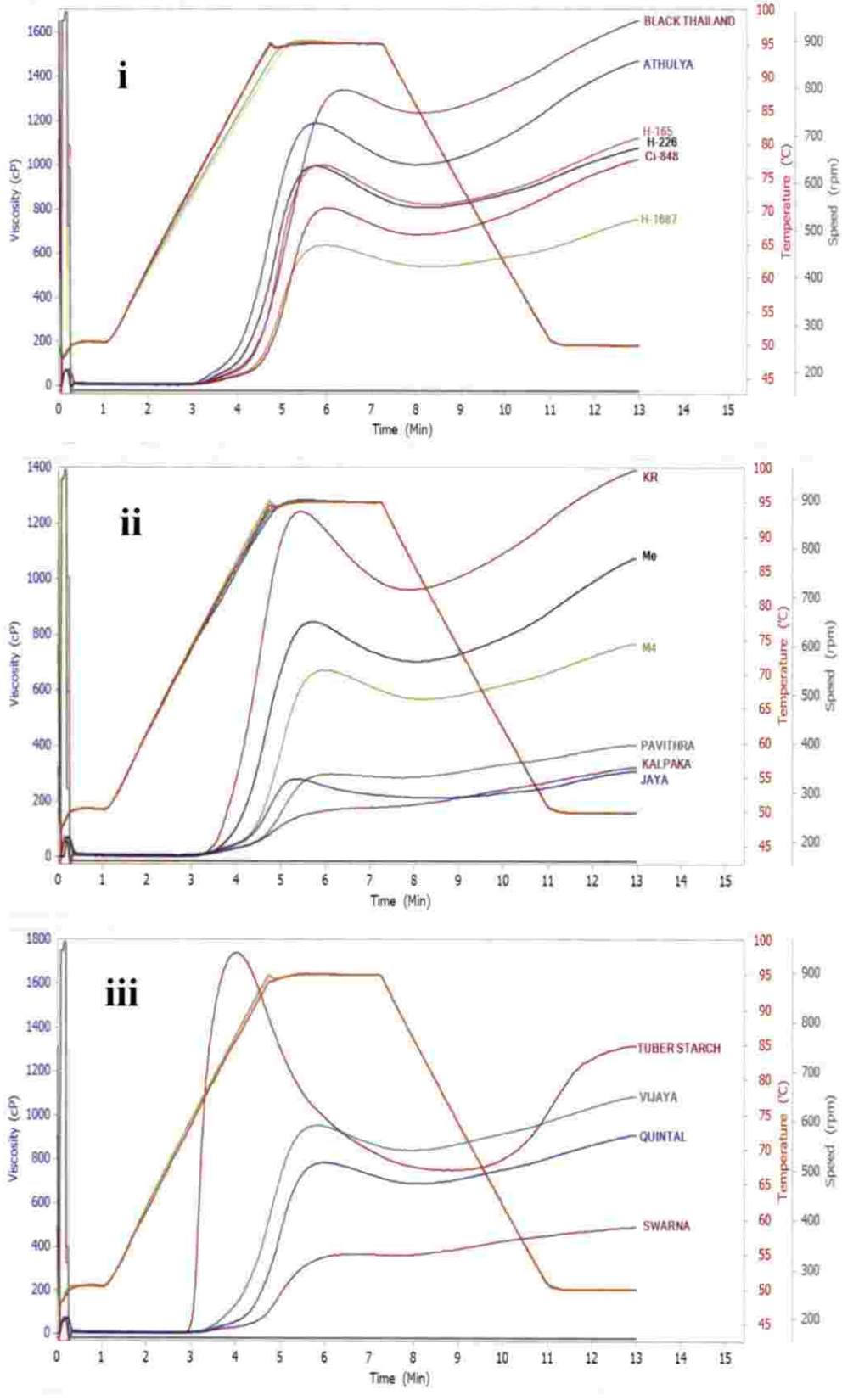


Figure-12 i, ii, iii: RVA pasting profiles of different cassava stem starches

4.7 Enzymological studies

4.7.1 *Estimation of peroxidase activity*

It was found that peroxidase activity was more in Sree Jaya and lowest in Kunkuma Rose. The peroxidase content in cassava stem starches ranges from 0.06-0.12 ng/mg (Figure-13).

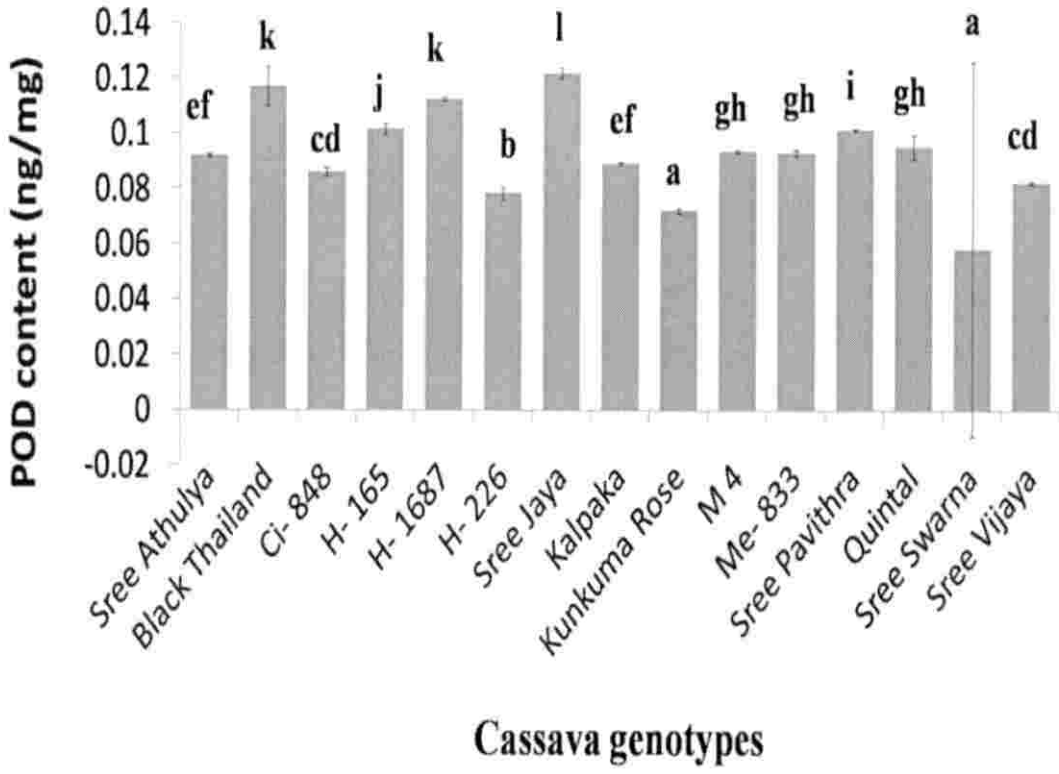


Figure-13: Peroxidase content in starch extracted from different genotypes of cassava stem

4.7.2 *Estimation of polyphenol oxidase*

It was found that poly phenol oxidase activity was highest in Black Thailand and lowest in Quintal. The poly phenol oxidase content in cassava stem starch ranged from 12.6- 29.8 mg/g (Figure-14)

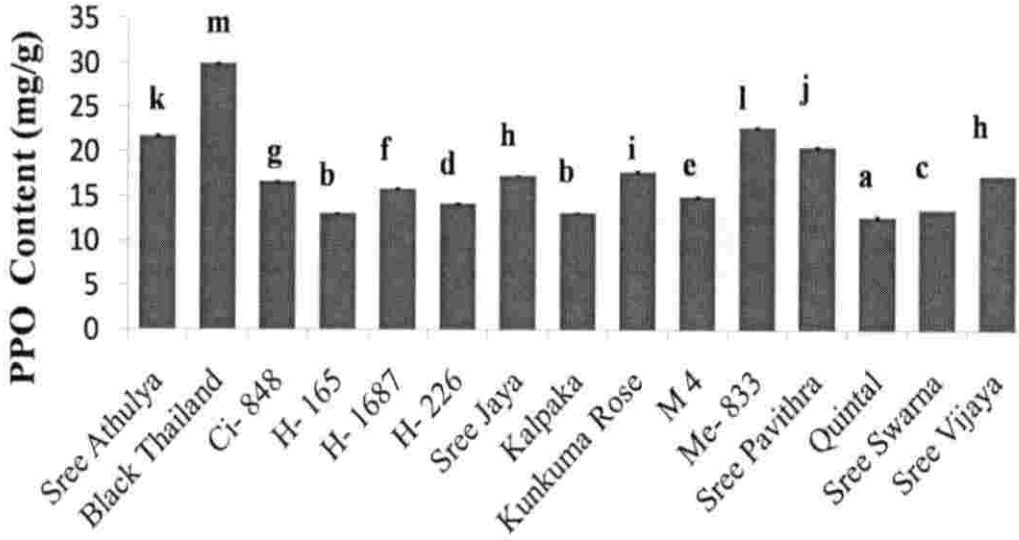


Figure-14: Poly phenol oxidase content in starch extracted from different genotypes of cassava stem

4.8 Identification of suitable stem starch extraction method

For the identification of suitable stem starch extraction method, stem of H-1687 was used. Four methods were tried for the extraction such as, simple water technique, using chemicals (0.2% NaOH and 0.5% $Na_2S_2O_5$), Microwave assisted extraction and Ultra sound assisted extraction. $Na_2S_2O_5$ and NaOH extraction could increase the whiteness of the stem starch but the starch yield was less when compared to water (Table-6). Hence this study shows that water is the simplest and most suitable extraction medium for isolating starch from cassava stem.

Table-6: Extraction of starch using water and chemicals

Extraction method	Starch recovery(g/g) from fresh stem	Starch recovery(g/g) from dried stem powder
Water	0.3008±0.0149	0.282±0.0276
0.2% NaOH	0.2614±0.0380	0.214±0.0191
0.5%$Na_2S_2O_5$	0.1914±0.0191	0.17±0.0173

4.8.1 Microwave Assisted Extraction

Starch extraction by means of microwave assistance was done using microwave oven. It was found that micro wave assisted extraction could positively influence the starch extraction. It was found out that extraction using water gives more extractability than 0.2% NaOH and 0.5% $Na_2S_2O_5$ (Table-7).

Table-7: Microwave assisted extraction of starch

SL No.	Extraction medium	Reaction Time (Sec)	Microwave Frequency (Hz)	Starch yield (g/g)
1	90% Water	60	360	0.275±0.01
2	90% Water	60	720	0.312±0.06
3	90% Water	120	360	0.295±0.01
4	90% Water	120	720	0.30±0.043
5	0.2% NaOH	60	360	0.208±0.08
6	0.2% NaOH	60	720	0.254±0.01
7	0.2% NaOH	120	360	0.275±0.04
8	0.2% NaOH	120	720	0.316±0.01
9	0.5% $Na_2S_2O_5$	60	360	0.164±0.03
10	0.5% $Na_2S_2O_5$	60	720	0.198±0.01
11	0.5% $Na_2S_2O_5$	120	360	0.182±0.01
12	0.5% $Na_2S_2O_5$	120	720	0.218±0.02

4.8.2. Ultra sound assisted extraction

Starch extraction by Ultra sound assistance was done using **Rivotek**™ ultrasonic sonicator with a frequency of 20KHz and it was found that ultra sound assisted extraction could yield more starch than conventional methods. It was found that the extractability of starch increases with time and maximum extractability of 36% found at a time of 50 minute (Figure-15).

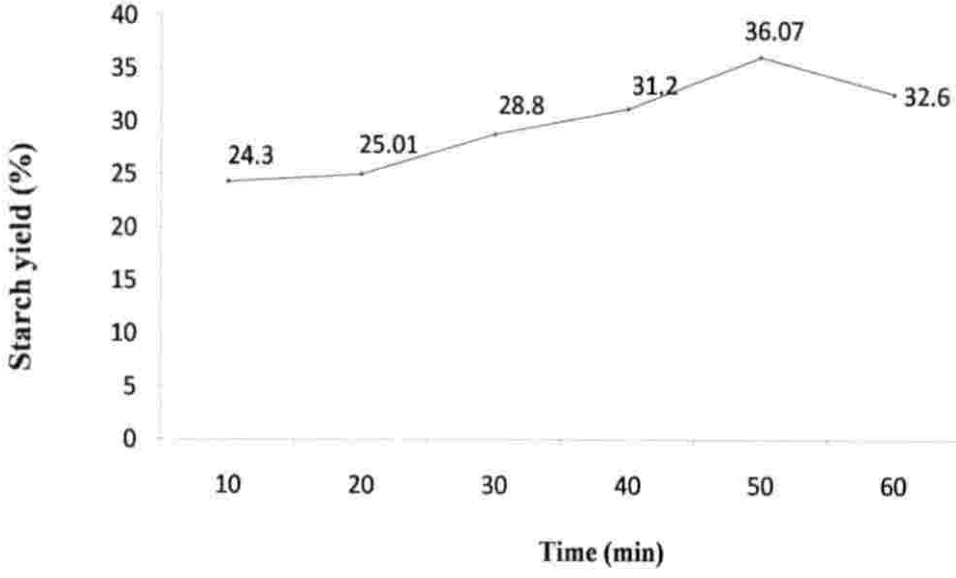


Fig-15: Ultra sound assisted extraction of starch extracted from cassava stem

4.9 Characterization of starch biosynthesis gene

RNA was isolated from very young stem tissues of two different genotypes of cassava Using TRIzol reagent. Cassava genotypes H-1687 and Quintal tissue samples were used in this study. The quantity and quality of RNA was checked using Nano spectrophotometer and Agarose gel electrophoresis respectively. Clear 18S and 28S subunits were observed during electrophoresis (Figure-16). The TRIzol method is simple and less-time consuming protocol for isolating RNA from the plant tissues.

Table 8: Concentration and purity of the isolated RNA from stem samples calculated from the Nanodrop spectrophotometer

SL No.	Sample	RNA yield (ng/µl)	18S	28S	260/230	260/280
1	H-1687	1945.5	YES	YES	1.723	2.314
2	Quintal	1248.96	YES	YES	1.976	1.466

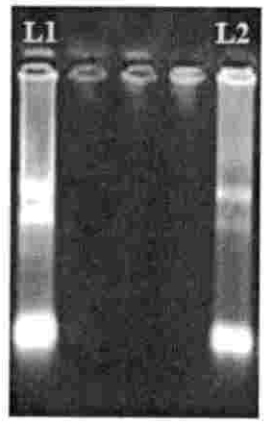
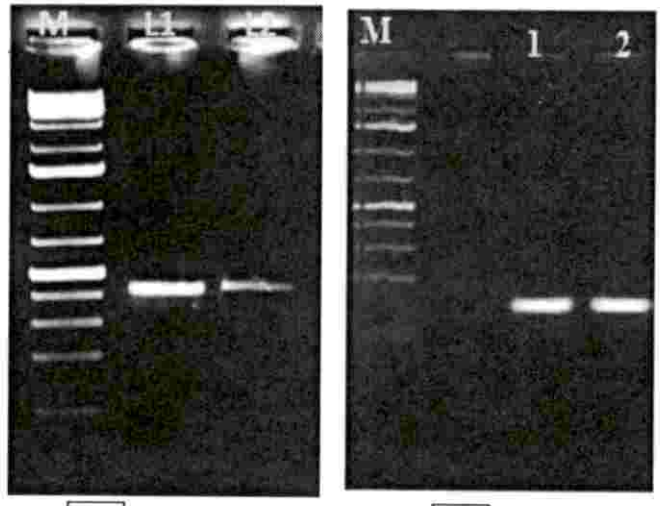


Figure-16: Quality Check of RNA in the stem tissue samples by Agarose gel electrophoresis

(Lane L1 represents H-1687 and L2 represents Quintal)

Two RNA samples (H-1687 and Quintal) were selected to study the expression pattern *Starch synthase* genes (420 Kb band size) and the analysis showed that the genes were expressed in stem tissues of cassava and the expression was more in H-1687 when compared to quintal (Figure -17a). The Tubulin gene (150Kb band size) was used as an internal control (Figure-17b)



a b

Figure-17: a) Expression of *starch synthase* gene in stem tissues of cassava b) Expression of internal control gene (Tubulin) in stem tissues of cassava. (M- 1Kb plus ladder, L1,1- H-1687, L2,2- Quintal)

DISCUSSION

5. DISCUSSION

Cassava is an important staple food crop cultivated primarily within the tropical and sub-tropical areas of the planet. It is grown in a wide variety of environmental and soil conditions and highly tolerant to drought and heat stress. Cassava is mainly cultivated for its root starch and stem is widely considered as waste, but the fact is cassava stem is having a worldwide production of 40 Tg of dry weight in 2014 which contains almost 40% starch (Zhu *et al.*, 2015). So, when compared to other plant stems and types of agricultural waste, the starch content in cassava stems is much higher and this starch can be easily extracted using water. Cassava stem starch is an ideal source to increase the availability of starch without using additional land, water and fertilizers. Hence understanding the structural and functional properties of stem starch is an important aspect before substituting with root starch because there is a lack of knowledge of starch properties when compared to root starch.

In this present study, starch extraction from cassava stems and their functional properties were examined. Starch yield from cassava stem is almost 15-30% by dry weight. Zhu *et al.* (2015) reported that starch content in cassava stems ranged between 22 and 39% which depends on the growing location, variety, stem height and extent of plant senescence. The starch yield varies with the milling size and the starch content increases with the decreasing milling size. The time has no significant effect on starch extraction, but the temperature has a significant and positive effect.

The stem charcteristic were studied for the selected genotypes. The stem length of cassava ranges from 100-300 cm and the stem girth ranges from 2.5 - >8cm. Ramanujam and Indira (1983) reported that cassava plant height ranged from 1.2 – 3.7m and the diameter of cassava stem ranges from 2-8 cm. The fresh weight and dry weight of cassava stem ranges from 0.4-2 kg and 0.2-0.7 kg respectively. Wheatley in 1993 reported that the fresh weight of cassava stem is 0.5-3kg. Howler and Cadavid (1983) reported that in cassava DM accumulation was slow during the first 2 months of cassava crop, but increased rapidly during the next 4 months and slowed down during the final 6 months as DM production was partly offset by leaf fall. The moisture content of cassava stem varied from 62-72%. Oka *et al.*, 1987 in their study reported that the moisture content of cassava stem ranged from 72.7 to 65.9% in Rayong 1 and from 63.6 to 53.1% in Rayong 3. It has been reported that massive amount of starch has been found to be mostly stored in the

xylem tissue of the stems (Zhu *et al*., 2015). An indicative analysis showed that about 95% stem starch is located in xylem tissues, 4% in bark (including phloem), and 1% in pith of a current year stem.

The starch content in cassava stem is varied from 37-55% on dry weight basis. According to Wei *et al*. (2018), the starch content of the crude stem starch isolate was 66.7%, but increased to 69.3% by purification. The starch content of the crude root starch was 83.8%, and increased to 87.1% after purification. Stem starches from different cassava varieties exhibited variations in viscosity characteristics when analyzed by a Rapid Visco Analyzer. The viscosity of stem starches ranged between 309 and 1651cP. Pasting time ranged from 5.33 to 6.93min and pasting temperature significantly varied and ranged from 84.70- 94.25˚C.

Jane *et al*. (2000) reported that amylopectin contributed to the swelling of starch granules and pasting properties, whereas amylase and lipids inhibited the swelling power. Pasting properties are dependent on the amount of amylose leaching out in a solution and on the rigidity of starch granules, which in turn affect the granule swelling potential. Wei *et al*. (2018) reported that thepastingtemperature ishigher forthestem starch (72.6˚C for purified and 75.0˚C for crude) than for the root starch (67.1˚C for the commercial one, 68.7˚C for purified, and 68.2˚C for crude). The differences in viscosity between the samples were mainly influenced by the different starch contents in the isolates, and are thus not possible to relate to differences between the stem and root starch granules. The pasting of the purified stem starch was more pronounced than that of the raw stem starch, showing that impurities that interfered with granule swelling were removed by the purification. These impurities could be proteins that attached to the granule surface before the proteinase treatment during purification.

The moisture content was less than 2% in all stem starches. The swelling volume was same for all the stem starches (15ml) and the solubility was less than 10%. The solubility showed soluble amylose and amylopectin that has leached out of the starch granules. Bijttebier *et al*. (2008) reported that the composition of the soluble starches has generally a much higher ratio of amylose to amylopectin than their native starches. The degradation of amylopectin could cause disruption of granular structure and increase in leaching with the heating of starch in water resulting in higher solubility.

The water binding capacity significantly varied among all the genotypes and ranged from 46.09% to 77.50% for different cassava stem starches. The differences in water binding capacity of starches from different cassava genotypes may be attributed to the variation in granular structure; loose association of amylose and amylopectin molecules in native starch granule has been reported to be responsible for the difference in water binding capacity. For native starches, the functional properties like moisture content, swelling volume, solubility, water binding capacity, *in vitro* digestibility and pasting properties are significantly related to the average granule size of the starches separated from various sources.

Rarely has a high content of starch been reported in crop nonfood residues and their various xylem cells. It is usually considered that starch should accumulate in the phloem, although a small amount of starch has been observed in the sapwood and ray cells.

In the present study starch was extracted using four different methods such as extraction using water, extraction using chemicals, micro wave assisted extraction and ultra sound assisted extraction. It was found that both microwave assisted extraction and ultra sound assisted extraction could increase the extractability of starch from cassava stem. Moreover, both MAE and UAE could be used to reduce extraction time in comparison with conventional methods. Kaufmann and Christen (2002) reported that microwave assisted extraction and ultra sound assisted extraction is used to extract natural products and both of the techniques allow reduced solvent consumption and shorter extraction times but the extraction yields is same or even higher than those obtained with conventional methods. Kamalini (2018) reported that the extraction of starch from cassava stem using microwave assisted extraction with alkaline pretreatment. They used four different variables such as reaction time (60–120 s), NaOH concentration (2–4% w/v), solid to liquid ratio (1:25–1:75 g/ml), and microwave frequency (360–720 Hz) to study the effect and increase the sugar recovery. The results showed that reaction time of 116.4 s, NaOH concentration of 3.21% (w/v), substrate to liquid ratio of 1:62.07 g/ml and microwave frequency of 719.86 Hz is optimum and obtained a maximum yield of 43.60 µg/ml of reducing sugar. In the present study water extraction was found to be more suitable for starch extraction from cassava stem and genotype H1687 is suitable for extraction of starch from stem.

SUMMARY

6. SUMMARY

The study entitled "Investigation on extraction of starch from cassava (*Manihot esculenta* Crantz) stem" was carried out at division of crop utilization, ICAR-Central Tuber Crops Research Institute, Sreekariyam. Thiruvananthapuram during the period 2018-19. The objective of the study was to identify the cassava (*Manihot esculenta* Crantz) genotypes suitable for extraction of starch from stem, to identify suitable method of extraction of starch from cassava stem and to characterize the starch obtained from stem tissue.

In the present study, the physiological characters of selected varieties cassava stem such as stem length, stem girth, stem fresh weight and stem dry weight and stem moisture content were measured. The stem length of cassava ranges from 100-300cm and the stem girth ranges from 2.5 - 8cm.The fresh weight and dry weight of cassava stem ranges from 0.4-2Kg and 0.2-0.7Kg respectively.The moisture content of cassava stem varies from 62-72%.

The moisture content was less than 2% in all stem starches. The swelling volume was same for all the stem starches (15ml) and the solubility was less than 10%. Starches with high swelling volume and solubility and low gelatinization temperature has various applications in food industry.

The water binding capacity varied from 46.09% to 77.50% for different cassava stem starches. The in vitro starch digestibility of cassava stem starches ranges from 0.1-0.3 g/g.

The starch yield from cassava stem ranges from 17% to 30% and the starch content was found to be 38-55% on dry weight basis. The starch yield was found to be maximum for H-1687 and minimum for Quintal.

The peroxidase content in cassava stem starches ranges from 0.05-0.13ng/mg and the poly phenol oxidase content ranges from 10-30mg/g. Both this enzyme content was found to be lowest in Black Thailand and maximum for Sree Swarna and Quintal.

The starch was extracted using four different methods such as extraction using water, extraction using chemicals, micro wave assisted extraction and ultra

sound assisted extraction. It was found that extraction using ultra sound and micro wave assistance could increase the extractability of starch from cassava stem.

The genotypes H-1687 and H-226 was found to be high starch yielding varieties and thus it could be used for the extraction of starch from cassava stem. The functional properties of all the genotypes were analyzed and Starch biosynthesis gene expression was studied using RT- PCR.

REFERENCES

7. REFERENCES

Afolayan, M. O., Adama, K. K., Oberafo, A., Omojola, M. and Thomas, S. 2014. Isolation and characterization studies of ginger (*Zingiber officinale*) root starch as a potential industrial biomaterial. *Am. J. Mater. Sci. 4*(2): 97-102.

Aiemnaka, P., Wongkaew, A., Chanthaworn, J., Nagashima, S. K., Boonma, S., Authapun, J., Jenweerawat, S., Kongsila, P., Kittipadakul, P., Nakasathien, S. and Sreewongchai, T. 2012. Molecular characterization of a spontaneous waxy starch mutation in cassava. *Crop sci.52*(5): 2121-2130.

Alcazar-Alay, S. C. and Meireles, M. A. A. 2015. Physicochemical properties, modifications and applications of starches from different botanical sources. *Food Sci. Technol.* 35(2): 215-236.

Alves, A. A. C. 2002. Cassava botany and physiology. *Cassava: biology, production and utilization*. 1: 67-89.

BeMiller, J. N. 2018. *Carbohydrate chem. for food scientists*. Elsevier.

Benmoussa, M. and Hamaker, B. R., 2011. Rapid small-scale starch isolation using a combination of ultrasonic sonication and sucrose density separation. *Starch-Stärke*, 63(6): 333-339.

Bijttebier, A., Goesaert, H. and Delcour, J. A. 2008. Amylase action pattern on starch polymers. *Biologia*. 63(6): 989-999.

Birt, D. F., Boylston, T., Hendrich, S., Jane, J. L., Hollis, J., Li, L., McClelland, J., Moore, S., Phillips, G. J., Rowling, M., Schalinske, K., Scott, M. P. and Whitley, E. M. 2013. Resistant starch: promise for improving human health. *Adv. in Nutr.* 4 (6): 587–601.

Carvalho, A.J. 2008. Starch: major sources, properties and applications as thermoplastic materials. In *Monomers, polymer and composites from renewable resources*. 321-342.

Cauvain, S.P. 2017. *Baking problems solved*. Woodhead Publishing.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochem.* 162(1): 156-159.

Daiuto, E., Cereda, M., Sarmento, S. and Vilpoux, O. 2005. Effects of extraction methods on yam (*Dioscorea alata*) starch characteristics. *Starch-Starke*. 57(3-4): 153-160.

El-Sharkawy, M.A., De Tafur, S.M. and Cadavid, L. F. 1992. Potential photosynthesis of cassava as affected by growth conditions. *Crop Sci.* 32(6): 1336-1342.

Essers, A.J. A. 1995. *Removal of cyanogens from cassava roots: studies on domestic sun-drying and solid-substrate fermentation in rural Africa.* Essers.

Fagbohun, A., Michael, A., Patrick, I., Olutayo, O., Adedayo, A., Olakunle, F., Ademola, A. and Abayomi, O. 2013. Isolation and Characterization Studies of *Moringa Oleifera* Root Starch as a Potential Pharmaceutical and Industrial Biomaterial. *Int. J. Chem. Applications. Int. Research Publication House. ISSN* :0974-3111.

FAO. 2008.Cassava for Food and Energy Security: Investing in Cassava Research and Development Could Boost Yields and Industrial Uses. Food and Agriculture Organization (FAO) of the United Nations, Rome.

FAO. 2010. 'Climate-smart'agriculture: Policies, practices and financing for food security, adaptation and mitigation.

FAO. 2016. Agriculture organization of the United Nations statistics division. *Agri-Environmental Indicators/Pesticides*.

FAOSTAT. 2016. Agriculture Organization of the United Nations Statistics Division. *Economic and Social Development Department, Rome, Italy.*

Glover, J. D., Reganold, J. P., Bell, L.W., *et al.*2010.Increased food and ecosystem security via perennial grains. *Sci.*25:1638– 1639.

Granham-Rowe, D.2011. Beyand food versus fuel. *Nat.*474:6– 8.

Gu, C., Wang, L., Zhang, L., Liu, Y., Yang, M., Yuan, Z., Li, S. and Han, Y. 2013. Characterization of genes encoding granule-bound starch synthase in sacred lotus reveals phylogenetic affinity of Nelumbo to Proteales. *Plant Mol.Biol. reporter*, 31(5): 1157-1165.

Gunaratne, A. 2004. Analysis of Quality in "Encyclopedia in Grain Science" (Wrigley, CW, Corke H. and Walker C. eds.).

Guo, J. 2004. Processes and equipment to separate liquid and solids during cassava starch production. *J. Longyan Teachers College*.22:83– 84.

Hegenbart, S. 1996. Understanding starch functionality. *Food Prod. Design January*. 23-34.

Howeler, R. H. 2012. Dry matter accumulation and nutrient absorption and distribution during the growth cycle of cassava. In: The Cassava Handbook, (ed. RH Howeler), CIAT, Cali, Colombia.351– 410.

Howler, R. H. and Cadavid, L. F. (1983): Accumulation and distribution of dry matter and nutrients during a 12 –month growth cycle of cassava. *Field Crops Res.* 7: 123 –139.

Jane, J. L. 2000. Characterization of barley starches of waxy, normal, and high amylose varieties. *Carbohydrate polymers* 41(4): 365-377.

Jane, J.J.M.S. 1995. Starch properties, modifications, and applications. *J. of Macromolecular Sci. Part A: Pure and Applied Chem.32*(4): 751-757.

Kamalini, A., Muthusamy, S., Ramapriya, R., Muthusamy, B. and Pugazhendhi, A. 2018. Optimization of sugar recovery efficiency using microwave assisted alkaline pretreatment of cassava stem using response surface methodology and its structural characterization. *J. Mol. Liquids.254*: 55-63.

Karlström, A., Calle, F., Salazar, S., Morante, N., Dufour, D. and Ceballos, H. 2016. Biological implications in cassava for the production of amylose-free starch: impact on root yield and related traits. *Frontiers plant Sci.7*: 604.

Kaufmann, B. and Christen, P. 2002. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochemical Analysis: An Int. J. of Plant Chemical and Biochemical Tech. 13*(2): 105-113.

Kaur, L. and Singh, J. 2009. *Advances in Potato Chemistry and Technology*. Academic Press.

Keeling, P.L. and Myers, A.M. 2010. Biochemistry and genetics of starch synthesis. *Annual review of food Sci. and Technol. 1*: 271-303.

Kleczkowski, L.A., Villand, P., Lonneborg, A., Olsen, O. A. and Luthi, E. 1991. Plant ADP-glucose pyrophosphorylase-recent advances and biotechnological perspectives (a review). *Zeitschrift fur Naturforschung C*, *46*(7-8): 605-612.

Koehorst-van Putten, H.J., Wolters, A.M.A., Pereira-Bertram, I.M., van den Berg, H.H., van der Krol, A. R. and Visser, R.G. 2012. Cloning and characterization of a tuberous root-specific promoter from cassava (*Manihot esculenta* Crantz). *Planta* 236(6): 1955-1965.

Kozlowski, T. T. 1992. Carbohydrate sources and sinks in woody-plants. *Botanical Review* 58:107– 222.

Lesile, T., Xiao, H. and Dong, M. 2005. *J. Petroleum Sci. Eng.* 46(4): 225-232.

Lopez- Molina, J. and El-Sharkawy, M. A.1995. Increasing crop productivity in cassava by fertilizing production of planting material. *Field Crops Res.*44:151– 157.

Macdonald, F.D. and Preiss, J. 1985. Partial purification and characterization of granule-bound starch synthases from normal and waxy maize. *Plant Physiol.* 78(4): 849-852.

Maddever, W. J. and Chapman, G. M. 1989. *Plastics Eng.* 31(7): 450-466.

Moorthy, S. N. 1991. Extraction of starches from tuber crops using ammonia. *Carbohydrate polymers* 16(4): 391-398.

Moorthy, S. N. and Padmaja, G. 2002. Starch Content of Cassava Tubers. *J. Root Crops* 28(1): 30-37.

Myllarinen, P., Partanen, R., Seppala, J. and Forssell, P. 2002. Effect of glycerol on behaviour of amylose and amylopectin films. *Carbohydrate Polymers*. 50 (4): 355–361.

Nattapulawat, A., Purkkao, N. and Suwithayapanth, O. 2009. *AAPS Pharm. Sci. Tech.* 10(1): 193- 198.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153(2): 375-380.

Nuwamanya, E., Baguma, Y., Wembabazi, E. and Rubaihayo, P. 2011. A comparative study of the physicochemical properties of starches from root, tuber and cereal crops. *African J. Biotechnol.* 10(56): 12018-12030.

Nuwamanya, E., Chiwona-Karltun, L., Kawuki, R. S. and Baguma, Y. 2012. Bio-ethanol production from non-food parts of cassava (*Manihot esculenta* Crantz). *Ambio* 41: 262– 270.

Oka, M., Sinthuprama, S. and Limsila, J. 1987. Variations in some characteristics of cassava stems during storage prior to taking cuttings. *Japan Agri. Res. Quarterly*. 3: 233-236.

Pfister, B. and Zeeman, S. C. 2016. Formation of starch in plant cells. *Cellular and Mol. Life Sci. 73*(14): 2781-2807.

Pohja, S., Suihko, E., Vidgren, M., Paronen, P. and Ketolainen, J. *J. Control. Release* 94: 293–298

Preiss, J. 1982. Regulation of the biosynthesis and degradation of starch. *Annual Review of Plant Physiol.* 33(1): 431-454.

Pycia, K., Gałkowska, D., Juszczak, L., Fortuna, T. and Witczak, T. 2014. Physicochemical, thermal and rheological properties of starches isolated from malting barley varieties. *J. Food Sci. Technol*. 52 (8): 4797–4807.

Raemakers, K., Schreuder, M., Pereira, I., Munyikwa, T., Jacobsen, E. and Visser, R. 2001. Progress made in FEC transformation of cassava. *Euphytica*, 120(1): 15-24.

Ramanujam, T. and Indira, P. 1983. Canopy structure effect on growth and development of cassava (*Manihot esculenta* Crantz) [morphology]. *Turrialba*.

Rani, A. and Chawhaan, P. H. 2012. Extraction and scanning electron microscopic studies of *Curcuma angustifolia* Roxb. Starch.

Saboktakin, M. R., Maharramov, A., Ramazanov, M. A. and Mahkam, M. 2007. Modification of carboxymethyl starch as nano carriers for oral drug delivery. *Nat. Sci. 5*(3): 67.

Saithong, T., Rongsirikul, O., Kalapanulak, S., Chiewchankaset, P., Siriwat, W., Netrphan, S., Suksangpanomrung, M., Meechai, A. and Cheevadhanarak, S. 2013. Starch biosynthesis in cassava: a genome-based pathway reconstruction and its exploitation in data integration. *BMC systems Biol. 7*(1): 75.

Sakurai, T., Plata, G., Rodriguez-Zapata, F., Seki, M., Salcedo, A., Toyoda, A., Ishiwata, A., Tohme, J., Sakaki, Y., Shinozaki, K. and Ishitani, M. 2007. Sequencing analysis of 20,000 full-length cDNA clones from cassava reveals lineage specific expansions in gene families related to stress response. *BMC Plant Biol.* 7(1): 66.

Schoch, T.J., 1964. Swelling power and solubility of granular starches. *Methods in carbohydrate chem.* 4:106-108.

Smith, A. M., and Martin, C. 1995. Starch biosynthesis. *The plant cell*, *7*(7): 971.

Smith, A. M., Denyer, K. and Martin, C. 1997. The synthesis of the starch granule. *Annual review plant biol.* 48(1): 67-87.

Tappiban, P., Smith, D. R., Triwitayakorn, K. and Bao, J. 2018. Recent understanding of starch biosynthesis in cassava for quality improvement: A review. *Trends in food Sci.and Technol.*

Tetlow, I. J. and Emes, M. J. 2014. A review of starch branching enzymes and their role in amylopectin biosynthesis. *IUBMB life* 66(8): 546-558.

Viola, R., Davies, H.V. and Chudeck, A.R. 1991. Pathways of starch and sucrose biosynthesis in developing tubers of potato (*Solanum tuberosum* L.) and seeds of faba bean (*Vicia faba* L.). *Planta 183*(2): 202-208.

Wei, M., Andersson, R., Xie, G., Salehi, S., Boström, D. and Xiong, S. 2018. Properties of Cassava Stem Starch Being a New Starch Resource. *Starch-Starke 70*(5-6): 1700125.

Wei, M., Zhu, W., Xie, G., Lestander, T.A. and Xiong, S. 2015. Cassava stem wastes as potential stock for fuel ethanol production: A basic parameter study. *Renewable Energy*. 83: 970-978.

Wheatley, C.C. 1993. Cassava. *Encyclopedia of Food Sci. Food Technol. and Nutrition*. 734-743.

Witono, J. R., Santoso, H., Miryanti, Y. A. and Tan, D. 2013. Integration of physical and chemical treatment on the extraction of starch from *Canna edulis* Ker. rhizome. *Agricul. Sci.* 4(09): 51.

Wurzburg, O. B. and Szymanski, C. D. 1970. *J. Agr. Food Chem.* 18(6): 997-1001.

Yang, J. and Zhang, P. 2011. Expression profiling of cassava storage roots reveals an active process of glycolysis/gluconeogenesis. *J. Integr. Plant Biol.* 53 (3): 193-211

Yazid, N. S. M., Abdullah, N., Muhammad, N. and Matias-Peralta, H. M. 2018. Application of Starch and Starch-Based Products in Food Industry. *J. Sci. Technol.* 10(2):345-360.

Zaldivar, J., Nielsen, J. and Olsson, L. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiol. Biotechnol.* 56(1-2): 17-34.

Zhao, S.S., Dufour, D., Sanchez, T., Ceballos, H. and Zhang, P. 2011. Development of waxy cassava with different biological and physico-chemical characteristics of starches for industrial applications. *Biotechnol. and Bioeng.108*(8): 1925-1935.

Zhu, F. 2015. Isolation, composition, structure, properties, modifications, and uses of yam starch. *Comprehensive Reviews in Food Science and Food Safety*, 14(4): 357-386.

Zhu, W., Lestander, T.A., Orberg, H., Wei, M., Hedman, B., Ren, J., Xie, G. and Xiong, S. 2015. Cassava stems: a new resource to increase food and fuel production. *Gcb Bioenergy* 7(1): 72-83.

APPENDICES

APPENDIX I

TBE Buffer (10 X)	
Tris base	107g
Boric acid	55g
0.5M EDTA (pH 8.0)	40ml

APPENDIX II

70% Ethanol	
100% Ethanol	70 mL
Distilled water	30 mL

APPENDIX III

Sodium phosphate buffer (pH-6.9, 0.02 M)

Solution A 0.2 M Mono sodium phosphate 3.12g in 100ml Distilled water

Solution B 0.2 M Di sodium phosphate 3.56g in 100ml Distilled water

(Mix 45ml of solution A and 55ml of solution B and make up to 1L with distilled water. Add 17.85 g NaCl to the solution and mix well.)

APPENDIX IV

Citrate phosphate buffer (pH-7.0, 0.1 M)

Citric acid	0.1 M
Dibasic sodium phosphate	0.2M

INVESIGATION ON EXTRACTION OF STARCH FROM CASSAVA (*Manihot esculenta* Crantz) STEM

By

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(2014-09-102)

Abstract of Thesis

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9. ABSTRACT

The study entitled "Investigation on extraction of starch from cassava (*Manihot esculenta* Crantz) stem" was carried out at the Division of Crop Utilization, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during the year 2018- 2019. Cassava stem starch is an ideal source to increase the availability of starch without using additional land, water and fertilizers. Hence understanding the structural and functional properties of stem starch is an important aspect before substituting with root starch because there is a lack of knowledge of starch properties when compared to root starch.

In this study, starch from cassava stem were extracted by four different methods such as extraction using water, extraction using chemicals such as Sodium hydroxide and sodium meta bi sulphate, microwave assisted extraction and ultra sound assisted extraction. The cassava genotypes suitable for extraction of starch from stem and functional properties of this stem starches were investigated.

In the present study, the physiological characters of such as stem length, stem girth, stem fresh weight and stem dry weight and stem moisture content were measured in the selected genotypes of cassava. The results showed that stem length of cassava ranges from 100-300cm and the stem girth ranges from 2.5-8cm. The fresh weight and dry weight of cassava stem ranges from 0.4-2Kg and 0.2-0.7Kg respectively.The moisture content of cassava stem varies from 62-72% but the moisture content was less than 2% in all stem starches. The swelling volume was same for all the stem starches (15ml) and the solubility was less than 10%. Starches with high swelling volume and solubility and low gelatinization temperature has various applications in food industry.

The water binding capacity varied from 46.09% to 77.50% for different cassava stem starches. The *in vitro* starch digestibility of cassava stem starches ranges from 0.1-0.3 g/g. The starch yield from cassava stem ranges from 17% to 30% and the starch content was found to be 38-55% on dry weight basis. The starch yield was found to be maximum for H-1687 and minimum for Quintal.

The peroxidase content in cassava stem starches ranges from 0.05-0.13ng/mg and the poly phenol oxidase content ranges from 10-30mg/g. Both this enzyme content was found to be lowest in Black Thailand and maximum for Sree Swarna and Quintal.

It was found that extraction using ultra sound and micro wave assistance could increase the extractability of starch from cassava stem and the genotypes H-1687 and H-226 was found to be high starch yielding varieties and thus it could be used for the extraction of starch from cassava stem. Cassava stem contain more than 30% of starch (dry mass), hence the wasted cassava stem starch can be utilized for both food and non-food applications. More over cassava can increase both food and fuel resources where cassava roots are for food and stems for fuel and even reduce poverty without using additional land.