International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

Comparative study using banana peel and male flower as substrates for amylase production using Aspergillus nigerin Kerala

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Submitted: 01-07-2022 Accepted: 08-07-2022

ABSTRACT
Amylases are well known for applications ranging from starch and food processes industry to medical applications. The increased demand for these enzymes in various industries has led to an enormous interest in developing enzymes with better properties such as raw starch degrading amylases. It is suggested that banana peel and male inflorescence could employ as a promising substrate for the production of amylase by Aspergillus niger. Further, solid state fermentation is a better choice for amylase production. The addition of external growth medium is also found beneficial for increasing enzyme production. The present study was undertaken to isolate, identify and characterize the Aspergillus niger in the culture medium followed by amylase production and extraction. The banana parts used here as substrates are ripe fruit peel and male inflorescence from locally cultivated species Ethan (Nendran), Palayamkodan (Palayanthodan), Rasakadali (NjaliPoovan) and Sundari. The result shows that amylase from sundari peel have the best activity followed by Ethan peel. Ethan flower bud shows the least activity among the eight substrates under study. **Keywords:**Aspergillus niger; Solid state fermentation; Musa; Banana; Submerged fermentation; Peel; Male flower.

I. INTRODUCTION

Banana is grown extensively in the tropical and subtropical countries. At the global level, the banana production is estimated around 72.5 million metric tonnes, of which 21.77 million metric tonnes is contributed by India. Since storage and postharvest losses are critically high, considerable interest has been generated, in the recent years, for value addition to banana, such as, the production of banana juice, banana wine, banana powder, banana chips, etc. During the production of these products, the banana peel and flower bud accumulates in bulk posing serious environmental problems. To prevent this, it has become necessary to develop alternative, commercial application of these agro-industrial wastes.

There are so many methods to utilize these waste products, solid state fermentation is only an example for this. These waste products can be used as substrate for the production of enzymes, antibiotics and other bio-products. In this project we are utilizing peels and male inflorescences as a substrate for the production of α-amylase enzyme. To this endeavour, efforts have been made to use banana peel for large scale production of alcohol (Tewari et al., 1986) and enzymes, such as, laccases (Johannet al., 2007) and amylases (Shaista et al, 2003).

α-Amylase is one of the important and well-known industrial enzymes that can cause the breakdown of starch or glycogen. Use of microorganisms for the production of amylases is economical as microbes are easy to manipulate to obtain enzymes of desired characteristics (Aiyer, 2005). Amylases stand out as a class of enzymes useful in the food, brewing, textile, detergent and pharmaceutical industries (Rosell et al., 2001; Pandeyet al., 2000). They are mainly employed for starch liquefaction, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. They are also applied during detergent production to improve cleaning effect and are also used for starch de-sizing in textile industry (Lonsane and Ramesh, 2002).

Amylases can be produced either by submerged fermentation (SmF) (Aguilaret al, 2002; Mathew et al., 2016) or solid-state fermentation (SSF) procedures. Although the conventional

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

amylase production was carried out by SmF, SSF system offers advantages, such as, high volumetric productivity, high concentration of product with less effluent generation and utilization of simple fermentation equipment (Pandey et al., 1999).

A wide range of microbes, such as, bacteria and fungi, are used for the industrial production of amylases. However, fungus is preferred over bacterium for enzyme production because of its filamentous nature, which helps in its penetration through the solid substrate (Ramachandranet al., 2004). Another advantage associated, especially with Aspergillus niger (an acidifying mould), is the control over bacterial contamination due to its capacity to tolerate high degree of acidity (pH<3) (Raimbault, 1983). The present study was aimed to exploit locally available and inexpensive banana peel and male flower bud for amylase production using A. niger.

1.1 Biological evolution and nomenclature

Banana is widely cultivated over 130 countries along the tropics and sub tropics (Mohapatra et al., 2010). Original bananas were seeded and mostly non edible forms. The slow decline in seed fertility, increases in parthenocarpy as well as human selection of characters (pulpiness, fruit colour and taste) may leads to the evolution of edible banana varieties (Uma et al., 2005a;Uma et al., 2005b). Most of the edible bananas present now a days are derived solely from MusaaccuminataColla or Musa balbisianaColla or a hybrid between the two wild diploid species. These two diploid ancestral parents contribute to A and B genomes respectively and considered as the Adam and Eve of present day bananas (Uma et al., 2005a;Uma et al., 2005b; Mohapatra et al., 2010; Simmonds and Shepherd, 1955). The banana plant seems to be originated from India as well as eastern Asian regions (Malaysia and Japan). Polyploidy, hybridization and various combinations of A and B genome has resulted in the development and emergence of broad spectrum of genomic groups; diploid (AA, AB, BB), triploid (AAA, AAB, ABB, BBB) and tetraploid (AAAA, AAAB, ABBB, AABB) varieties of banana.

Various other varieties also co-evolved or exist naturally with theses genomes and have slightly different nomenclatures (Simmonds, 1962; Robinson, 1996). Three common species of Musa (M. cavendishi, M. paradisiaca and M. sapientum) are widely cultivated across the world. Musa cavendishi is the pure triploid acuminate (AAA) is also known as desert banana characterized by sweeter and less starchy than M. paradisiaca. Musa sapientum is known as the true banana could be eaten raw when fully mature. Both M. paradisiaca and M. sapientum belongs to AAB group characterized by higher starch content compared to pure acuminate group (Mohapatra et al., 2010; Stover and Simmonds, 1987). Cooking bananas falls under ABB and BBB genome with prominent M. bulbisiana genes. A great diversity of dessert banana exist due to plant size and various morphological characters. Productivity is high for Cavendish bananas and giant French plantains (>30 t/ha/yr).

1.2 Indian production scenario

Banana is the second most important fruit crop in India after mango, good export potential and popular among all classes of people due to its year round availability, affordability, varietal range and nutritional properties. Out of more than 50 varieties of banana cultivated across India, around 20 are commonly grown in various Indian States(Duran et al., 2007).

1.3Musa (banana)

The bispecific origin of edible banana first mentioned by Kurz(1867) and experimentally proved by Simmonds and Shepherd (1955) by cross the two parent varieties; M. acuminate and M. bulbisiana. Supported by morphological and cytological evidences, it was assumed that the edible bananas were evolved from the two ancestors in five main stages. The triploids were formed by the fertilization of diploid egg cell with haploid pollen leads to the formation of triploids as a main step in the banana evolution process. The triploids were popular among farmers and breeders due to many beneficial traits especially sturdiness, robustness and pulpiness. Parthenocarpy, sterility, polyploidy and vegetative propagation for perpetuation of useful traits has played a major role in the evolution of current banana varieties (Uma et al., 2005b).

The generic name Musa is rooted in Sanskrit word Moca or may have derived from Arabic worldMauz, Mouz or Mauwz, which is used for banana (De Candolle, 1886; Nayar, 2010; Hakkinen et al., 2013). The Arabic name for banana ‘Mauz’ is also mentioned in Rheede’s ‘Hortus Malabaricus’.

The earliest scientific classification of banana was made by the famous taxonomist Linnaeus in 1783. According to his classification, all dessert banana were known as Musasapientium;

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

which is sweet during ripening and consumed fresh. The name Musa paradisiaca was assigned to the plantain group which are cooked and consumed while starchy. These two apparent species are not species at all, but considered to be closely related interspecific triploids hybrids of the AAB group. The modern method of classifying edible bananas was devised by Simmonds and Shepherd (1955), most modern edible bananas originally come from two wild species, Musa acuminateColla (A genome) and Musa balbisianaColla (B genome). The classification proposed by Simmonds and Shepherd (1955) based on the relative contribution of the parent character to the constitution of the cultivar and to the ploidy or chromosome number of the cultivar. The original characters used by Simmonds and Shepherd (1955) were amended and updated by many taxonomists (Purseglove, 1972; Stover and Simmonds, 1987; Valmayor et al., 1991).

By using 15 separate characters, with strong diagnostic differences between the two ancestors, the contribution of the two species could be clearly distinguished. For each character in which a cultivar agreed completely with wild acuminate, a score of 1 was given, and for each character in which the cultivar agreed with balbisiana, a score of 5 was given. The intermediate expression of the character were assigned as score of 2, 3 or 4, according to intensity.

Concerning ploidy, edible bananas belonging to the section Eumusa have 22, 33 or 44 chromosomes. The basic haploid number is 11, thus cultivars can only be diploid, triploid or tetraploid. Of the 200-300 clones which are thought to exist, more than half are triploids, with the remaining being mostly diploids. Tetraploid clones are very rare. The planted area of triploid bananas is more than 100 times greater than that of diploids. Triploids are hardier, more vigorous and easier to grow. Morphologically, triploids and tetraploids are larger and more robust than diploids. Also leaf thickness and cell size increases with increasing ploidy.

The scoring technique based on 15 plant characters allows for a range of total score from 15 (pure Musa acuminate) to 75 (pure Musa balbisiana). Scores in between would be based on the relative contribution of the two species plus the level of ploidy in the interspecific hybrid. Simmonds and Shepherd (1955) and Stover and Simmonds (1987) used the groups and scores to classify a range of edible bananas. Silayoi and Chomchalow (1987) classified 137 accessions in the Thai banana gene bank on the same basis. Recognizing some deficiencies, they later modified the classification.

The main difference between these two classification is the introduction of almost pure balbisiana clones in the Thai grouping, which did not appear in the original classification. Espino and Pimental (1990) used isozyme technology to differentiate clones of pure acuminate, pure balbisiana and their hybrids from one another. They found broad bands of malate dehydrogenease activity which were unique to pure balbisiana, and other bands which indicated an acuminate genome. They concluded that BB and BBB cultivars were unique and distinct from hybrid ABB clones. The cooking plantain Saba (BBB) is very close to pure balbisiana (73 to 75 points).

Valmayor et al. (1991) endorsed the continued adaptation of Simmonds and Shepherd's classification scheme with amendments to accommodate South-east Asian varieties.

All banana taxonomist agree that no single scientific name can be given to all the edible bananas. Musa acuminate could be applied to the pure, seedless diploid (AA) and triploid (AAA) forms of dessert bananas such as Pisang Mas and Grand Nain respectively. Similarly Musa balbisiana could be applied to the pure seedless diploid (BB) and triploid (BBB) forms of cooking bananas such as Abuhon and Saba respectively. However, the many hybrids cannot carry a specific name due to their mixed composition and differences in ploidy. To avoid confusion, it is internally accepted that all banana cultivars should be referred to by genus Musa followed by a code denoting the genome subgroup and ploidy level, followed by subgroup name (if any), followed by the popular name of the cultivar.

Musa AAA (Cavendish subgroup) Grand Nain
Musa (AAB) (plantain subgroup) Horn
Musa BBB Saba
Musa AB Ney Poovan

The significance of somatic mutations in bananas is very great because of the number of clones has gradually increased in this way. Many somatic mutations have remained unrecognized, especially when morphological changes has been small. Some better known somatic mutants have been selected, utilized and names are Extra Dwarf Cavendish from Giant Cavendish; Williams from Giant Cavendish; Highgate from Gros Michel; Cocos from Gros Michel; Dwarf French Plantain from French Plantain; Sliver Bluggoe from

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Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

Bluggoe and Green Red from Red. The natural rate of somatic mutations are very low with banana propagated conventionally. The levels are significantly increased during propagation by in vitro techniques and considered as somoclonal variations.

1.4 Objectives

The main objectives of this study is the isolation of a good Aspergillus niger isolates and optimise the production using bananapeel and male flower.

1.5 Scope of the study

The study would enlighten the amylase production using underutilized substrates especially banana peel and male flowerwhich could be further explored.

II. REVIEW OF LITERATURE

Amylases are group of enzymes which posses 25% of the global enzyme production (Mojsov, 2012). The first commercially produced enzyme from microbial source was an amylase in 1894(Tiwariet al., 2015). Amylases are omnipresent and distributed in throughout in plants, animals and micro-organisms (Pandeyet al., 2000).Amylases are classified into endo- and exo-amylases based on their mode of action. The endo-amylases randomly hydrolysis -1,4 glycosidic bonds present in the structural components (amylose or amylopectin chain of starch). This results in the formation of linear and branched oligosaccharides of different chain lengths. The exo-amylase hydrolyses from non-reducing end and result in the production of short end products. Exo-amylase such as β-amylase hydrolysis α-1,4 glycosidic linkage and glucoamylase catalyse cleavage of α-1,4 and α- 1,6 glycosidic bonds(Agrawalet al.,2005). α-amylase, the utilizer of high abundance of starch on earth is one of the highly versatile enzymes in commercial areas, with numerous uses such as production of sugar syrups, biofuel and pharmaceutically important cyclodextrin (Gupta et al.,2003). Due to easiness in production, ubiquitous occurrence and wide range of uses α-amylase is a highly valuable commercial biocatalyst (Sundarram et al., 2014). Amylases are used as processing agent in processed food industry including baking, beverages, starchsyrups. (Gurung et al.,2013). Amylases, alone or in a mixture of enzyme are added to the flour to extend freshness and shelf life of the baking products(Singet al.,2016).Amylases are used to clarify juices to produce maximum clear juice(Vaillant,2001).In detergent industry amylases hydrolyses the glycosidic linkages in stain and remove the starchy glue present along with other stains and dirt(Guptaet al.,2003).The α-Amylase, an endo-amylase, is utilized primarily for laundry detergents but exo-amylase activity is inefficient for stain removal (Guptaet al.,2003).The α-amylases from Bacillus and Aspergillus species exhibit perfect compatibility with detergent condition. So, these enzymes are added in laundry and dishwashing detergent formulations to remove residues of starch based food products such as cakes, chocolate, pasta, potato and gravies(Souzaet al., 2010).The applications of amylases in the paper industry are coating of starch, de-inking, drainage improvement and cleaning of paper (Singet al.,2016).The primary application of α-amylase in paper industry is the production of high molecular weight starch with low viscosity through modification of starch of coated paper (Gurunget al.,2013).In the textile industry, amylases are used for desizing process; removal of starch to improve uniform wet processing (Aiyer,2005).Amylases are treated with cocoa slurries to produce chocolate syrup to make chocolate starch dextrinize and thus syrup does not become thick (Ismail et al., 1992).Amylases such as alpha amylase and glucoamylase are important to produce fermentable sugars to produce ethanol (Kirk et al., 2002).Besides applications of amylase in food, leather, alcohol, paper; its uses has been expanded to many otherfields. Amylases are used in animal feed to enhance digestibility (Marcet al.,2002).For analytical purposes, amylase are used to detect oligosaccharides(Guptaet al.,2003).

Aspergilla are widespread in nature and being found on fruits, vegetables, and other nutrient substrates. They are economically important because of their uses in the production of citric acid and gluconic acid, both produced in abundance by Aspegillusniger (Pelcza et al.,2002). Aspergilli have septate, branching mycelia with the vegetative portions submerged in nutrient. The conidiophores (or fertile hyphae) are also submerged. Conidiophores, which may be septate or non-septate have vesicle at the apex. Vesicle gives rise to sterigmata and sterigmata give rise to conidia (Pelcza et al.,2002). Aspergillus niger, the mould that is rarely reported as a cause of pneumonia (Personet al.,2010).

SSF can be defined as; " A solid porous matrix which can be biodegradable or not, but with

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Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

a large surface area per unit volume, in the range of 103 to 106 m^2/ cm^3, for a ready microbial growth on the solid/gas interface"(Maurice,1998). Typical examples of SSF are traditional fermentations such as: Japanese "koji" which uses steamed rice as solid substrate and mould Aspergillus oryzae is inoculated into it. Another example is Indonesian "tempeh" or Indian "ragi" which use steamed and cracked legume seeds as solid substrate and a variety of non-toxic moulds as microbial seed. French "blue cheese" which uses perforated fresh cheese as substrate and the selected moulds like Penicillium roquefortii are inoculated. Composting of ligno-cellulosic fibres by a large variety of organisms including cellulolytic bacteria, moulds and Streptomyces sp. etc. is another SSF process"(Maurice.,1998).In addition to traditional process, new applications of SSF have been suggested for the production of antibiotics (Barrios et al., 1988), secondaries metabolites (Trejo-Hernandez et al., 1992;Trejo-Hernandez et al.,1993), enriched foodstuffs (Senez et al., 1980).Now a days, SSF has been used in large-scale industrial processes mainly in Japan. Traditional koji process is changed from small wooden and bamboo trays into to more sophisticated processes like fixed bed room fermentations, rotating drum processes and automated stainless steel chambers or trays with microprocessors, electronic sensors and servo mechanical stirring, loading and discharging. The usual scale in sake or miso factories is around 1 or 2 metric tons per batch even though reactors can be made and delivered by engineering firms to a capacity as large as 20 tons (Maurice, 1998). Outside Japan, Kumar in 1987 has reported the medium scale production of enzymes, such as pectinases, in India. Koji type processes are widely used in small factories of the Far East (Hesseltine, 1972). Koji fermentation has been adapted to local conditions in USA and other Western countries like Cuba. In France, blue cheese production is being modernized by improving the mechanical conditioning of cheeses, by the production of mould spores and through the control of environmental conditions. Composting, which was for the small-scale production of mushrooms, has been modernised and grew up in Europe and USA. Various firms in Europe and USA produce mushroom spawn by using the organisms like Agaricus, Pleurotus or Shii. New versions for SSF reactors have been developed in the countries like; France (Durand et al., 1988; Roussos et al., 1993, Durand et al., 1997), Cuba (Cabello, 1985; Enríquez, 1983 and Rodríguez, 1986), Chile (Fernández et al., 1996).Tempeh and Koji are the most important applications of SSF using filamentous fungi. The first step of soy sauce or citric acid fermentation is growing of Aspergillus oryzae on wheat bran and soybean for Koji production. Koji is a concentrated hydrolytic enzyme medium required for the further steps of the fermentation process. Tempeh is an Indonesian fermented food produced by the growth of Rhizopus oligosporus by using soybeans as substrates. The fungal fermentation provide better nutritive quality and degrades some antinutritional compounds in the crude soybean (Raimbault, 1981).Bacteria, yeasts and fungi can find application in SSF processes by growing on solid substrates. Filamentous fungi are best for SSF and dominate in research works. Bacteria are mainly used in composting, ensiling and some food processes (Doelle et al., 1992). Yeasts are used for ethanol and food or feed production (Saucedo-Castañeda et al., 1992a; 1992b).

Tone of banana peel is produced during post-harvest by improper handling and the eradication of its pulps becomes a problem. However, the usage of banana peel is limited because of the limited knowledge on its properties(Syarifah et al.,2018).Tones of banana peel generates due to the massive consumption of banana pulp and this peel gives 40 % of the total weight of fresh bananas (Wachirasiet al.,2009).The pH value of banana peels found to be 5.79 to 6.18, which indicates that pH value for stage three banana peels will be neutral and hence might be less sour in taste (Syarifah et al., 2018).High carbohydrate content is beneficial in breakfast meal and can be good source of energy for breakfast formulations (Davidet al.,2015).The carbohydrate content in Rastali banana peel was higher while compared to others. The range of total carbohydrate was between 64.62 % and 72.84 %.There was no remarkable difference of protein content between all the banana peel. The amount of crude protein of banana peel at stage 1 and 5 were between 6.3% and 9.8 % and 6.6 %-11.2 % respectively.The banana peel can be kept for a long time since they have low moisture content. Each variety of banana peels possess slightly different nutritional content and functional properties. These could be an added value to the food industry especially in developing a high fiber product by exploiting a cheap and continuous source of ingredient (Syarifah et al., 2018).

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2.1 Plant morphology

In the publications of Simmonds (1962), Barker and Steward (1962), Purseglove (1972), Morton (1987), Ross (1987), Simmonds and Weatherup (1990), Espino et al., (1992), Karamura and Karamura (1995), Rieger (2006), Pillay and Tripathi (2007); detailed morphological description of banana plant is provided.

Banana plant is a perennial monocotyledon with an approximate height of about 2-9m. The part above the ground is called pseudostem (false stem), which is composed of concentric layers of leaf sheath and the part below the ground is called corm (also known as true stem). The meristem of apical bud initially gives rise to leave before it elongates to the pseudostem. Each pseudostem produces inflorescence only once. According to Barker and Steward (1962), leaves around the Musa gets tightly rolled from the centre of the pseudostem in a clockwise manner. The petiole is formed as the leaf sheath taper on the both sides. The can be erect, intermediate or dropping on the basics of the Musa sp.

According to Méndez et al.(2003), the biochemical composition of the fruit depends on its cultivator, abiotic factors, like climate, method of cultivation and the nature of soil. The fruit contains high level of potassium whereas the level of vitamin A is low. Banana doesn't exist any toxic properties but it contains high levels of biogenic amines. The intake of high amount of banana can cause endomyocardial fibrosis(Foy and Parratt, 1960).

2.1.1 Musaaccuminata

Stools are moderate in which pseudostem attains a height of 3-8m, is slenderer than of cultivated banana. Presence of brown black blotches marked on pseudostem. Its petiole canal is erect in position, with short hairy peduncle of about 1 cm. Ovules are arranged in 2 regular rows in each of the locules. The shape of the bractis lanceolate, after the opening of the bracts it roll back and its colour varies from reddish-purple to pink purple. Usually one Bract falls daily, prominent scars are present on its bract. Presence of creamy white male flower with rich yellow or orange stigma is an important feature. The approximate length of the fruit variesfrom 8-13 cm with dull black smooth seeds.

2.1.2Musabalbisiana

Stools are free in which the pseudostem attains an average height of 6-7m and is robust in nature. Presence of green or yellowish green blotches often black blotches in its upper part. Its petiole margins are curved inside with long hairy of about 1-2 cm. Ovules are arranged in Four irregular locules. The bracts lift up without rolling them back. The colour of the bract varies from crimson purple to bright crimson purple. Scarcely prominent scars are present on the bract. The colour of the male flower is variably flushed with pink within it, with cream or pale-yellow stigma. The length of the fruitis 7-15 cm long with black seeds. For the growth of the plant it requires nutrients, both macronutrients and micronutrients are essential. Macronutrients are those which are required in large amounts and in large qualities. These include nutrients like nitrogen, phosphorous, potassium,magnesium, calcium, and sulphur. The chief promoter for the growth of the plant is nitrogen,which induces the growth of the pseudostem and leaves. For the production of healthy rhizome and a strong root system phosphorus play an important role. Potassium stimulates the early shooting and helps in significantly shortening the time required for fruit maturity.Nutrients that are required in very small quantities are called micronutrients, it includes boron, iron and zinc. The deficiency boron results in the reduction in weight and size of the bunch, which affects the filling of the bunch. Iron deficiency are commonly seen in the plants that are grown in alkaline soils. Plants that are grown on zinc deficient soils are found to be zinc deficient. Symptoms like narrow pointed and chlorite young leaves etc due to zinc deficiency.

2.1.3Taxonomical classification (Musa acuminata; banana)

Kingdom: Plantae-- planta, plantes, plants, vegetal
Subkingdom: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Liliopsida
Order: Zingiberidae
Family: Musaceae
Genus: Musa L
Species: Musa acuminata

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 4 July-Aug 2022, pp: 124-150 www.ijprajournal.com ISSN: 2456-4494

Table 1. Different vernacular names of Musa paradisiacaaround the globe and India.

Language	**Names**
Scientific names	Musa paradisiaca
Name in various global languages	
French	Bananier
German	Banane
English	Banana
Name in various Indian languages	
Sanskrit	Kadali
Hindi	Kela
Urdu	Bonana
Marathi	Kela
Kannada	Baale
Gujarati	Kelphool
Malayalam	Vazha
Tamil	Vazhai

Table 2. Classification of edible bananas.

Genomic group	**Score**	**References**
AA diploid	15-23	Simmonds and Shepherd (1955); Stover and Simmonds (1987)
AAA triploid	15-23	
AAB triploid	24-46	
AB diploid	49	
ABB triploid	59-63	
ABBB tetraploid	67	
AA/AAA	15-25	Silayoi and Chomchalow (1987)
AAB	26-46	
ABB	59-63	
ABBB	67-69	
BB/BBB	70-75	

Table 3. Important banana varieties cultivated in different states of India.

State	**Varieties grown**
Andhra Pradesh	Dwarf Cavendish, Robusta, Rasthali, Amritpant, Thellachakrakeli, KarpooraPoovan, Chakrakeli, Monthan and YenaguBontha
Assam	Jahaji (Dwarf Cavendish), ChiniChampa, Malbhog, Borjahaji (Robusta), Honda, Manjahaji, Chinia (Manohar), Kanchkol, Bhimkol, Jatikol, Digjowa, Kulpait, Bharat Moni
Bihar	Dwarf Cavendish, Alpon, Chinia , ChiniChampa, Malbhig, Muthia, Kothia , Gauria
Gujarat	Dwarf Cavendish, Lacatan, Harichal (Lokhandi), Gandevi Selection, Basrai, Robusta, G-9, Harichal, Shrimati
Jharkhand	Basrai, Singapuri
Karnataka	Dwarf Cavendish, Robusta, Rasthali, Poovan, Monthan, Elakkibale
Kerala	Nendran (Plantain), Palayankodan (Poovan), Rasthali, Monthan, Red Banana, Robusta
Madhya Pradesh	Basrai
Maharashtra	Dwarf Cavendish, Basrai, Robusta, Lal Velchi, Safed Velchi, RajeliNendran, Grand Naine, Shreemanti, Red Banana
Orissa	Dwarf Cavendish, Robusta, Champa, Patkapura (Rasthali)
Tamil Nadu	Virupakshi, Robusta, Rad Banana, Poovan, Rasthali, Nendran, Monthan,

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	Karpuravalli, Sakkai, Peyan, Matti
West Bengal	Champa, Mortman , Dwarf Cavendish, Giant Governor, Kanthali, Singapuri

HYPOTHESIS

The current research work is based on the following hypothesis

1) The isolated Aspergillus niger strain differ in their amylase production capabilities.
2) Underutilized substrates especially banana peel and male flower could be used for the amylase production.

III. MATERIALS AND METHODS

4.1 Study area

Kerala state covers an area of 38,863 km^2 with a population density of 859 per km^2 and spread across 14 districts. The climate is characterized by tropical wet and dry with average annual rainfall amounts to 2,817 ± 406 mm andmean annual temperature is 26.8°C (averages from 1871-2005; Krishnakumaret al., 2009). Maximum rainfall occurs from June to September mainly due to South West Monsoon and temperatures are highest in May and November.

4.2Banana varieties selected

4.2.1Nendran

It is the one of the most commonly cultivated and largely grown variety, also known as Ethavazha. These fruits are mostly eaten as ripe, raw or used as vegetable in the mature unripen condition. Pseudostem of this specific variety possess a colouration. Each bunch may weigh up to 12-15 kg. The fruits are usually large, long, thick, peels are thick and leathery. These fruits remain starchy even on ripening. It is highly susceptible to Banana Bract Mosaic Virus (BBMV), nematodes, and borers.

4.2.2Palayamkodan

It is the most widely cultivated banana variety in Kerala, and can be grown at any particular climatic condition, which is cultivated for both fruit and vegetable purpose. This variety is adjusted as the suitable cultivar of banana for intercropping in coconut gardens. The fruit is usually small or medium with yellow skinned. It has a good shelf quality. It is a sweet variety of banana and the cheapest of all forms of banana. This variety is also known as Mysore Poovan. The average height of the plant is about 2-6 m and the number of the fruits in its mid hand is about 10-12. It is a small, blunt tipped, round fruit. The peel colour varies from green to yellow and is susceptible to bunchy top and is resistant to panama wilt.

4.2.3Njalipoovan

Due to its fruit quality it has a high demand among consumers. Fruits of this specific variety which is small are used more than the raw ones, it is very sweet and soft within the peel.it has the poor keeping quality and it falls off bunches. Usually it is cultivated as intercrops between coconut gardens. soft leaves of this variety are commonly used as plates. It is less susceptible to most of the pests and diseases.

4.2.4Sundari

It is a type that look likes fingers in shape; the fruit colour changes from green to light yellow at the of maturity. This fruit is not so sweet as compared to the other varieties. The height of the plant is about 2.8 m with slender pseudostem aspect. The average number of fruits in its mid hand is about 15. The fruit shape is curved in S shape.

4.3 Substrate selection

The banana peel and plantain blossoms used in this study were from four varieties of bananas trees. All the varieties were obtained from two districts in Kerala, India. The choice of each variety was done on those which were not used or found in literature. The banana peel and blossoms were washed and chopped into particles. They were dried at 55°C for 48 h, and then stored in polypropylene bags in the desiccators before use. The selected varieties are Ethan (Nendran), Palayamkodan (Palayanthodan), Rasakadali (NjaliPoovan) and Sundari.

4.4 Isolation of Aspergillus niger

A piece of bread was kept in a moist condition at room temperature in dark for 2 days. The bread sample was serially diluted and different dilutions were inoculated on potato dextrose agar (PDA) medium. The slants were incubated at 30°C for 4 days. Fungal cultures were observed on PDA medium. The fungal strain was subjected to lactophenol cotton blue staining for studying the morphology. The fungal culture was confirmed as

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Aspergillus niger by studying the morphology and the spore colour.

4.5Lactophenol cotton blue staining

Place a drop of Lactophenol Cotton Blue Solution on a slide. Using an inoculating needle carefully spread the fungal culture into a thin preparation. Place a cover slip edge on the drop and slowly lower it. Observe under low to high power objectives of microscope.

Lactic acid acts as a preservative for fungi. The phenol portion kills the fungi. The cotton blue stains the fungal elements. Fungal elements are stained a deep blue; background is pale blue(Aneja, 2003).

4.6 Screening for high yielding strain

The Aspergillus niger isolate was tested for amylase production by starch hydrolysis. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase and the fungal isolate was taken for amylase production.

4.7 Establishment of mother culture

Using sterile techniques, Aspergillus niger were selectively grown on potato dextrose agar (PDA) medium for 3 days and are used as mother culture for inoculation.

4.8 Enzyme production by Solid State fermentation (SSF)

The Aspergillus niger was subjected to solid state fermentation in different substrates like peels of Ethan (TMP1), Palayamkodan (TMP2), Njalipoovan (TMP3), and Sundhari (TMP4) and male-inflorescence or flower bud of Ethan (TMM1), Palayamkodan (TMM2), Njalipoovan (TMM3) and Sundhari (TMM4) which was used as solid substrates for fermentation. Each substrate was taken in about half inch thickness in all the fermentation trays and hydrated with 40ml of basal salt solution and adjusted with moisture content from 43-81%. 1% of inoculums was inoculated after sterilization and incubated at room temperature for 10 days.

4.9 Enzyme extraction

100 ml of 0.1M phosphate buffer saline (pH 7) was added to each of the inoculated substrate beds and was vigorously shaken in rotary shaker for 15 minutes at 120rpm. The mixture was filtered through cheese cloth and centrifuged at 8000rpm at 4°C for 15min. The supernatant was filtered through cheesecloth and the filtrate was used as the crude enzyme preparation. Enzyme amylase was assayed by Dinitrosalicyclic acid (DNS) method.

4.10 Enzyme assay

Enzyme assay was carried out by DNS method of (Miller, 1959) in which 0.5ml enzyme was reacted with substrate (1% starch in 100mM Tris buffer) under standard reaction conditions and the reaction was stopped by adding DNS reagent, amount of maltose released was determined by comparing the absorbance reading of the test enzyme at 540 nm with the standard graph plotted by reacting the known concentration of maltose ranging from 0.05mg/ml to 0.5mg/ml. One-unit amylase activity was defined as amount of enzyme that releases 1 micromoles of maltose per minute under standard reaction conditions.

The culture supernatant was collected separately. 15 test tubes were taken and marked S1-S9, pure blank (PB), substrate blank (SB) and enzyme blank (EB). With the help of a micropipette, 2ml of phosphate buffer was transferred to all the tubes. 1ml of starch was added to all tubes except PB & SB. 1% sodium chloride was added to all the test tubes. 1ml of distilled water was added to PB & SB. The contents of the test tubes were mixed well and then incubated for 5mins at 37^0 C. After incubation crude enzyme was added to all the test tubes except PB & EB, and distilled water is added to PB & EB .The contents of the test tubes were mixed well and incubated for 10mins at 37°C. After incubation 1ml of 2N sodium hydroxide (NaOH) were added to all the test tubes. The reducing sugars liberated were assayed calorimetrically by the addition of 1ml DNS reagent. The contents of the test tubes were mixed well and incubated in boiling water bath for 10mins.Intensity of the colour developed was read at 520nm using a spectrophotometer. A standard graph was plotted and the enzyme activity was calculated. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of sugar per minute under the standard assay conditions and enzyme activity is expressed in terms of micromoles per second on fermented substrates. (Appendix 1).

4.11 Statistical analysis

The results were analyzed and descriptive statistics were done using SPSS 12.0 (SPSS Inc., an IBM Company, Chicago, USA) and graphs were

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generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

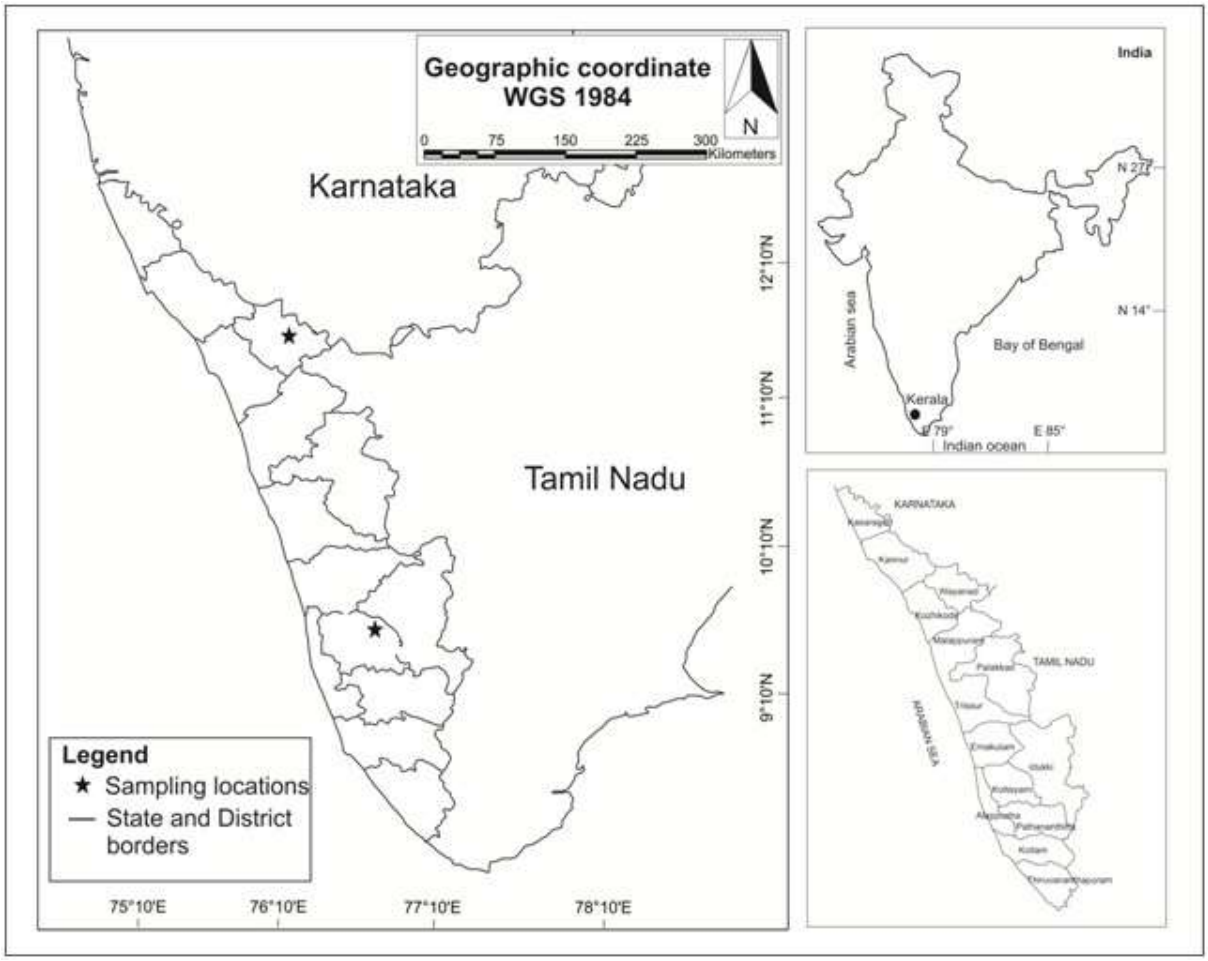

Figure 1. Map of Kerala showing the sample collection point.

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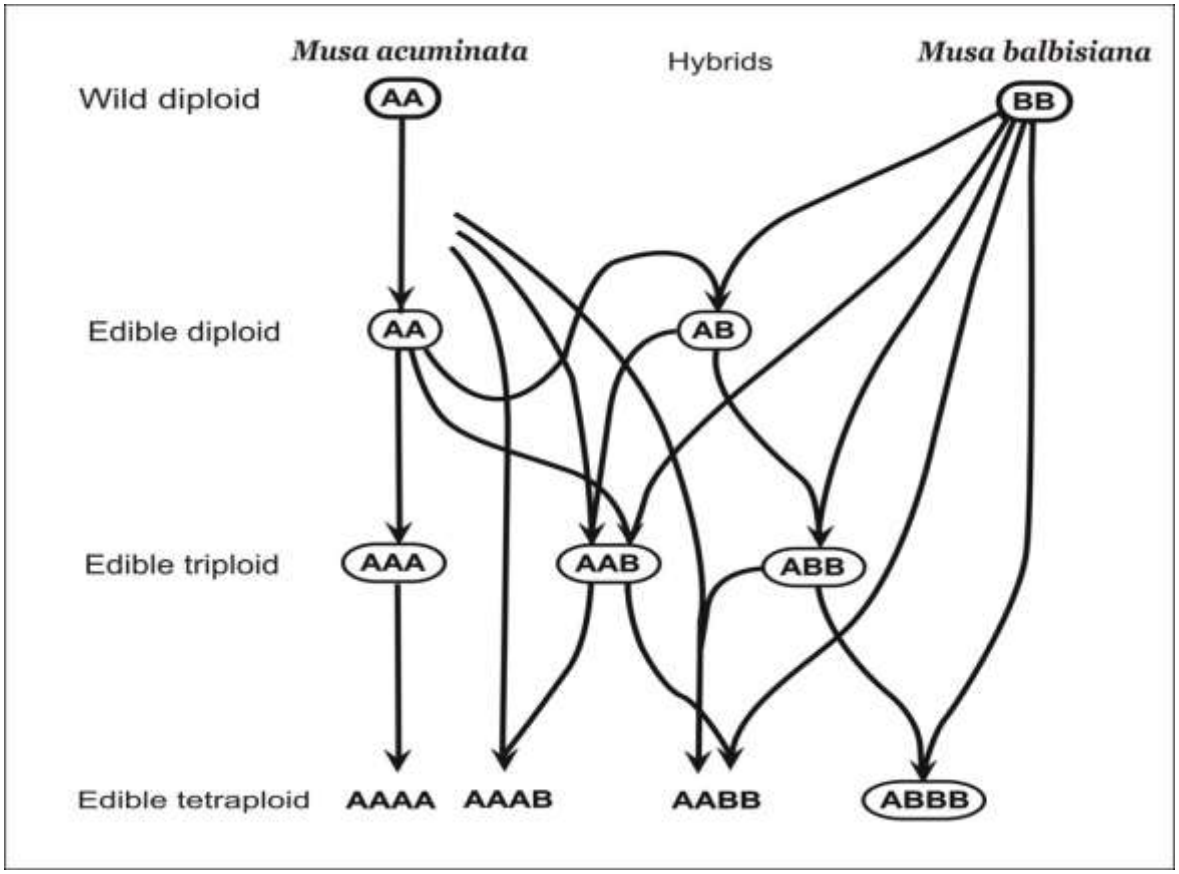

Figure 2. The evolution of the banana complex: A, M. acuminata; B, M. balbisiana. Genotypes known to occur naturally are encircled, those known only from experiment are not encircled (adopted from Simmonds and Shepherd, 1955).

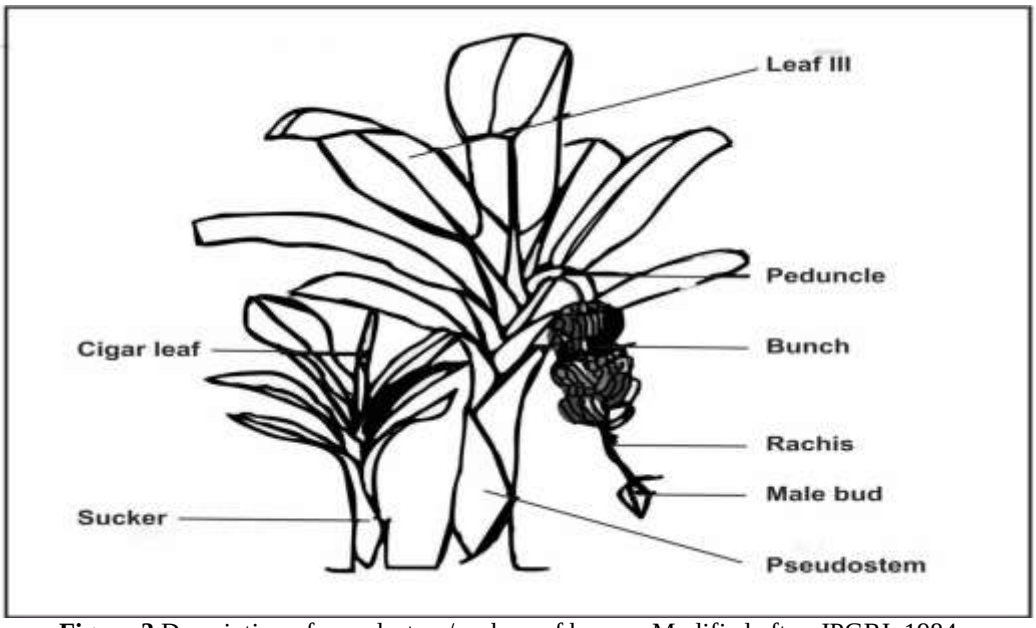

Figure 3. Description of pseudostem/suckers of banana.Modified after: IPGRI, 1984.

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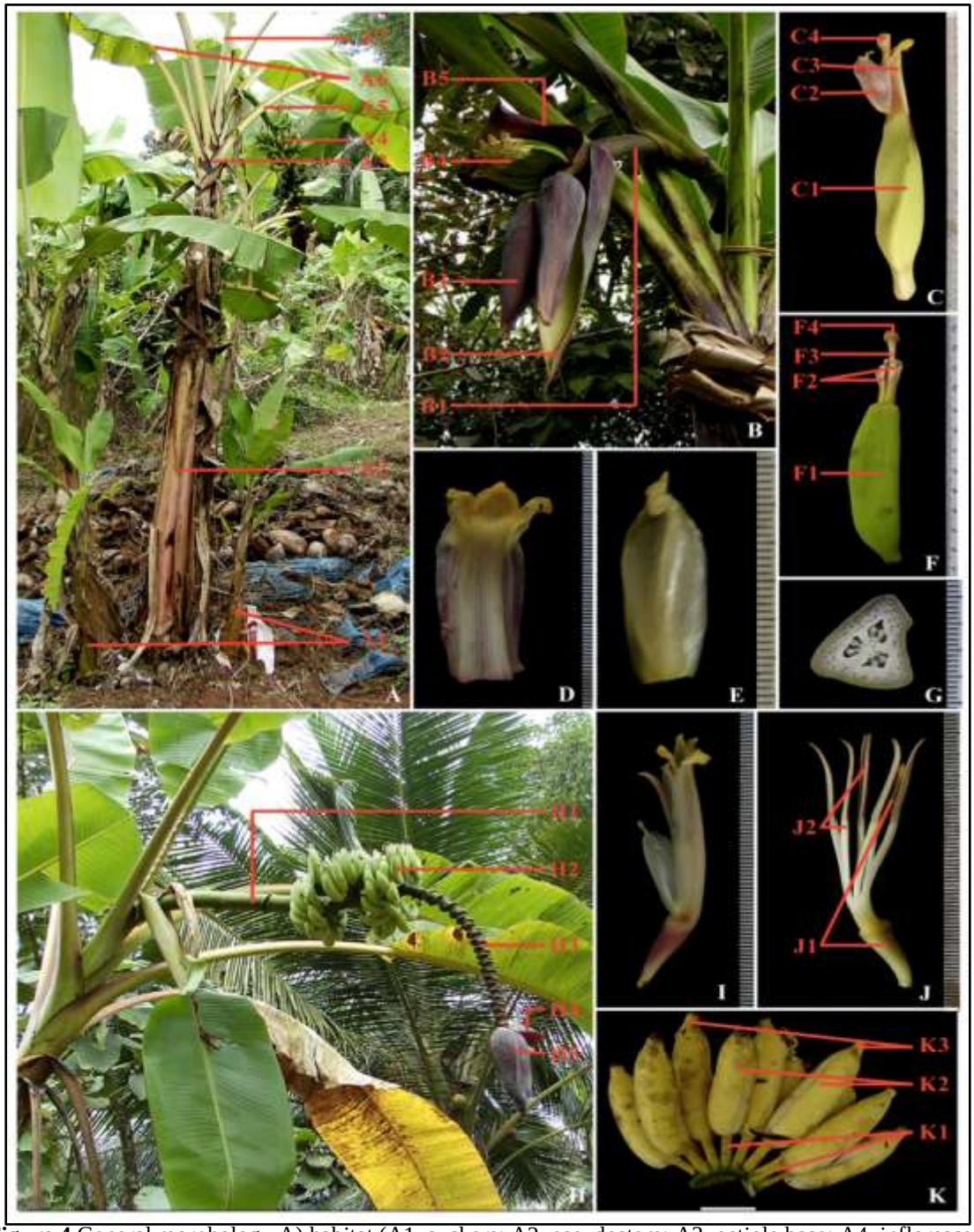

Figure 4. General morphology A) habitat (A1, suckers; A2, pseudostem; A3, petiole base; A4, inflorescence; A5, petiole; A6, leaf base; A7, 3rd leaf), B) inflorescence at early stages (B1, peduncle; B2, sterile bract; B3, female bud; B4, female flowers; B5, female bract), C) female flower (C1, ovary; C2, free tepal; C3, compound tepal; C4, stigma), D) compound tepal, E) free tepal, F) pistil with staminodes (F1, ovary; F2, staminodes; F3, style; F4, stigma), G) c.s of ovary, H) infructescence (H1, peduncle; H2, fruits; H3, rachis; H4, male bract; H5, male bud), I) male flower, J) rudimentary pistil with stamens (J1, rudimentary pistil; J2, stamens), K) fruit hand (K1, pedicel; K2, fruit; K3, fruit apex).

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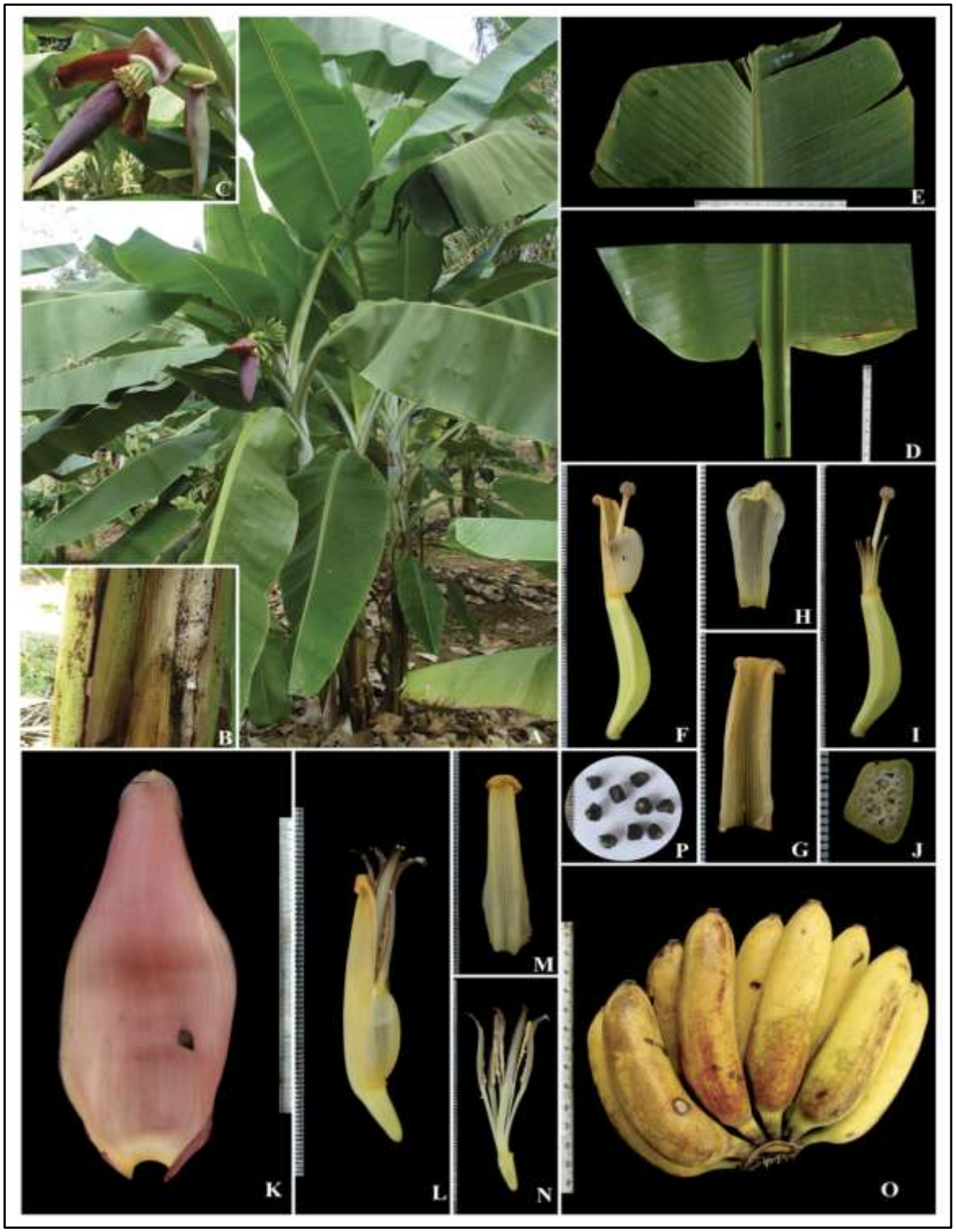

Figure 5. Musa acuminataColla A) habitat, B) pseudostem coloration, C) inflorescence at early stage, D) leaf base, E) leaf apex, F) female flower, G) compound tepal, H) free tepal, I) pistil with staminodes, J) c.s of ovary, K) male bract abaxial surface, L) male flower, M) compound tepal, N) rudimentary pistil with stamens, O) ripened fruit hand, P) seeds.

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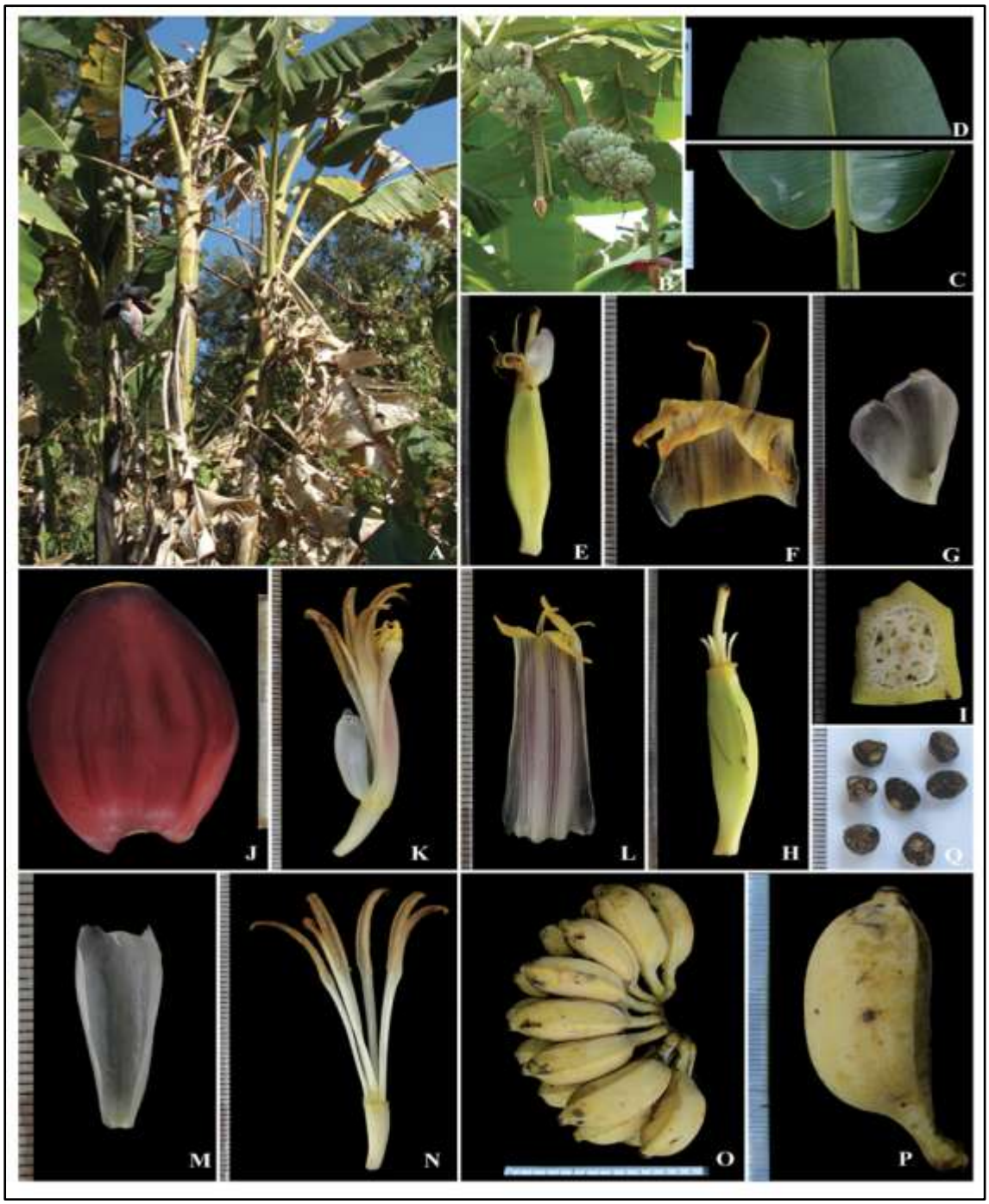

Figure 6. MusabalbisianaCollaA) habitat, B) infructescences with advanced stage of male bud, C) leaf base, D) leaf apex, E) female flower, F) compound tepal (female), G) free tepal (female), H) pistil with staminodes, I) c.s of ovary, J) male bract, K) female flower, L) compound tepal (male), M) free tepal (male), N) rudimentary pistil with stamen, O) ripened fruit hand, P) single fruit, Q) seeds.

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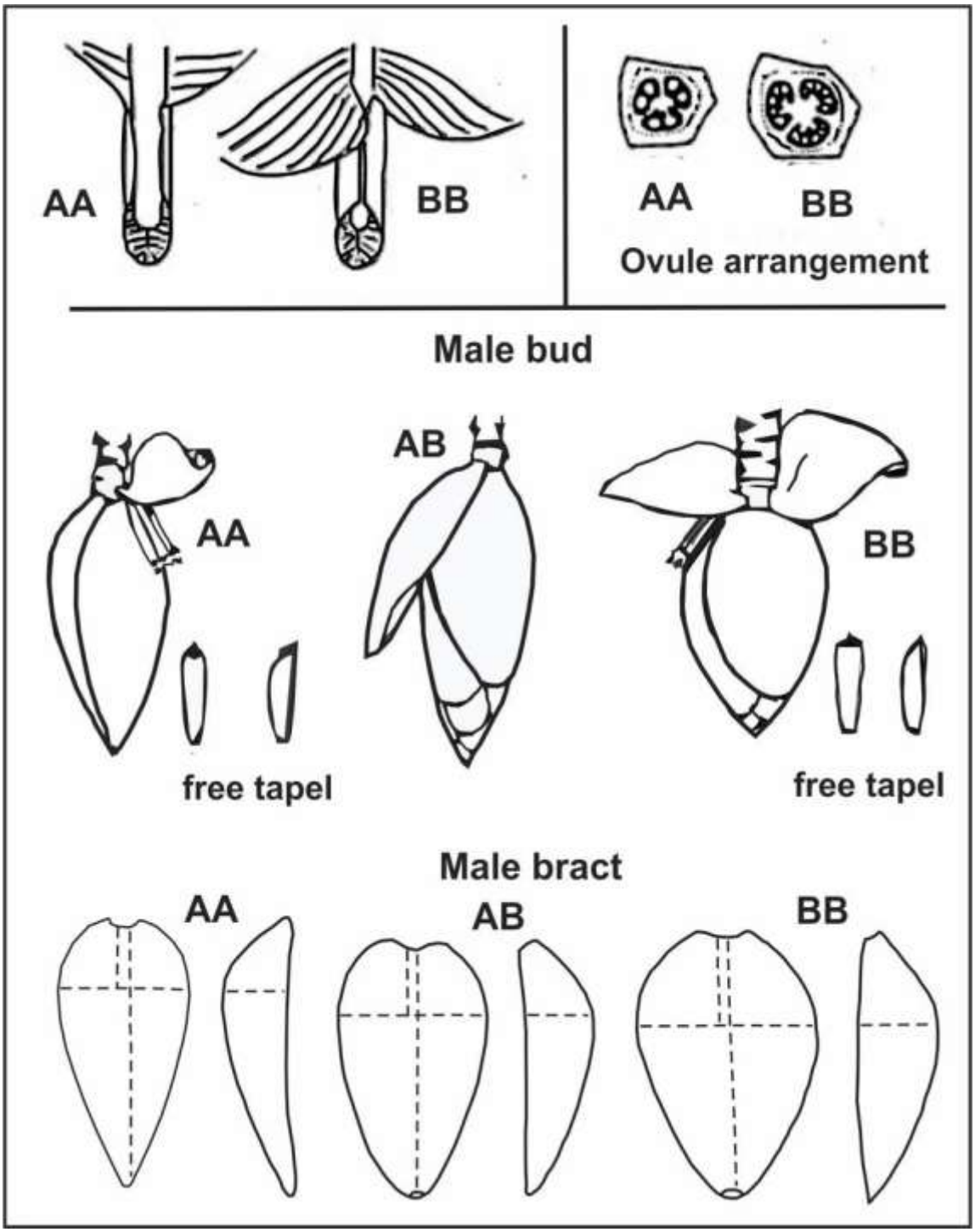

Figure 7. Important characters used in species and genome groups of edible banana. Modified after: IBPGR, 1984.

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Figure 8. Selected substrates used for Solid State Fermentation.

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Figure 9. Selected substrates used for Solid State Fermentation after inoculation.

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Table 4. Characters used in the classification of bananas through a taxonomic scorecard. Modified after (Simmonds and Shepherd, 1955).

Character	Musa acuminata	Musabalbisiana
Pseudostem colour	More or less heavily marked with brown or black blotches	Blotches slight or absent
Petiolar canal	Margin erect or spreading, with scarious wings below, not clasping pseudostem	Margin inclosed, not winged below, clasping pseudostem
Peduncle	Usually downy or hairy	Glabrous
Pedicels	Short	Long
Ovules	Two regular rows in each loculus	Four irregular rows in each loculus
Bract shoulder	Usually high (ratio < 0.28)	Usually low (ratio > 0.30)
Bract curling	Bract reflex and roll back after opening	Bracts lift but do not roll
Bract shape	Lanceolate or narrowly ovate, tapering sharply from the shoulder	Broadly ovate, not tapering sharply
Bract apex	Acute	Obtuse
Bract colour	Red, dull purple or yellow outside, pink, dull purple or yellow inside	Distinctive brownish-purple outside; bright crimson inside
Colour fading	Inside bract colour fade to yellow towards the base	Inside bract colour continuous to base
Bract scars	Prominent	Scarcely prominent
Free tapel of male	Variably corrugated below tip	Rarely corrugated
Male flower colour	Creamy white	Variably flushed with pink
Stigma colour	Orange or rich yellow	Cream, pale yellow or pale pink

Table 5. Taxonomic score card of Musa.

Genome Group	Score
AA/AAA	15-25
AAB	26-46
AB/AABB	47-49
ABB	59-63
ABBB	67-69
BB/BBB	70-75

Table 6. Banana varieties and its parts used as substrates.

SL. NO.	**Selected banana varieties**	**Part used as substrate**	**Code**
1	Ethan	Fruit peel	TMP1
		Male flower	TMM1
2	Palenkodan	Fruit peel	TMP2
		Male flower	TMM2
3	Njalipoovan	Fruit peel	TMP3
		Male flower	TMM3
4	• Sundari	Fruit peel	TMP4
		Male flower	TMM4

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Table 7. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Ethan fruit peel (TMP1) as substrate.

Ethan fruit peel	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.6x10^{-3}$	$0.7\ x10^{-3}$
Trial 2	$0.7\ x10^{-3}$	
Trial 3	$0.8\ x10^{-3}$	

Table 8. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Palenkodan fruit peel (TMP2) as substrate.

Palenkodan fruit peel	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.3x10^{-3}$	$0.3\ x10^{-3}$
Trial 2	$0.3\ x10^{-3}$	
Trial 3	$0.3\ x10^{-3}$	

Table 9. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Njalipoovan fruit peel (TMP3) as substrate.

Njalipoovan fruit peel	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.62x10^{-3}$	$0.62\ x10^{-3}$
Trial 2	$0.63\ x10^{-3}$	
Trial 3	$0.61\ x10^{-3}$	

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Table 10. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Sundhari fruit peel (TMP4) as substrate.

Sundhari fruit peel	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.7x10^{-3}$	$0.8\ x10^{-3}$
Trial 2	$0.9\ x10^{-3}$	
Trial 3	$0.8\ x10^{-3}$	

Table 11. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Ethan male flower (TMM1) as substrate.

Ethan male flower	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.025x10^{-3}$	$0.025x10^{-3}$
Trial 2	$0.024\ x10^{-3}$	
Trial 3	$0.026\ x10^{-3}$	

Table 12. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Palenkodan male flower (TMM2) as substrate.

Palenkodan male flower	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.17x10^{-3}$	$0.17\ x10^{-3}$
Trial 2	$0.17\ x10^{-3}$	
Trial 3	$0.17\ x10^{-3}$	

Table 13. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Njalipoovan male flower (TMM3) as substrate.

Njalipoovan male flower	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.18x10^{-3}$	$0.17\ x10^{-3}$

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Trial 2	0.16×10^{-3}	
Trial 3	0.17×10^{-3}	

Table 14. Activity of enzyme (Amylase) produced by Apsergillusniger using dried Sundhari male flower (TMM4) as substrate.

Sundhari male flower	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	0.10×10^{-3}	0.10×10^{-3}
Trial 2	0.09×10^{-3}	
Trial 3	0.11×10^{-3}	

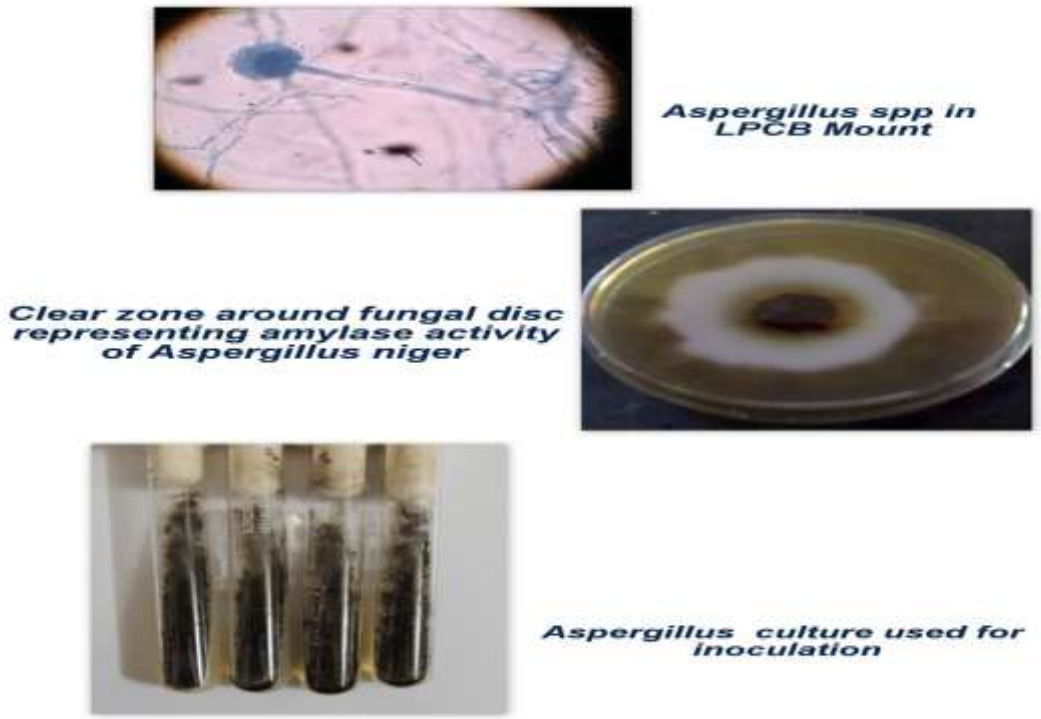

Figure 10. Isolation and screening of Apergillusnigerproduction strain.

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Figure 11. Substrate before inoculation with Aspergillus niger.

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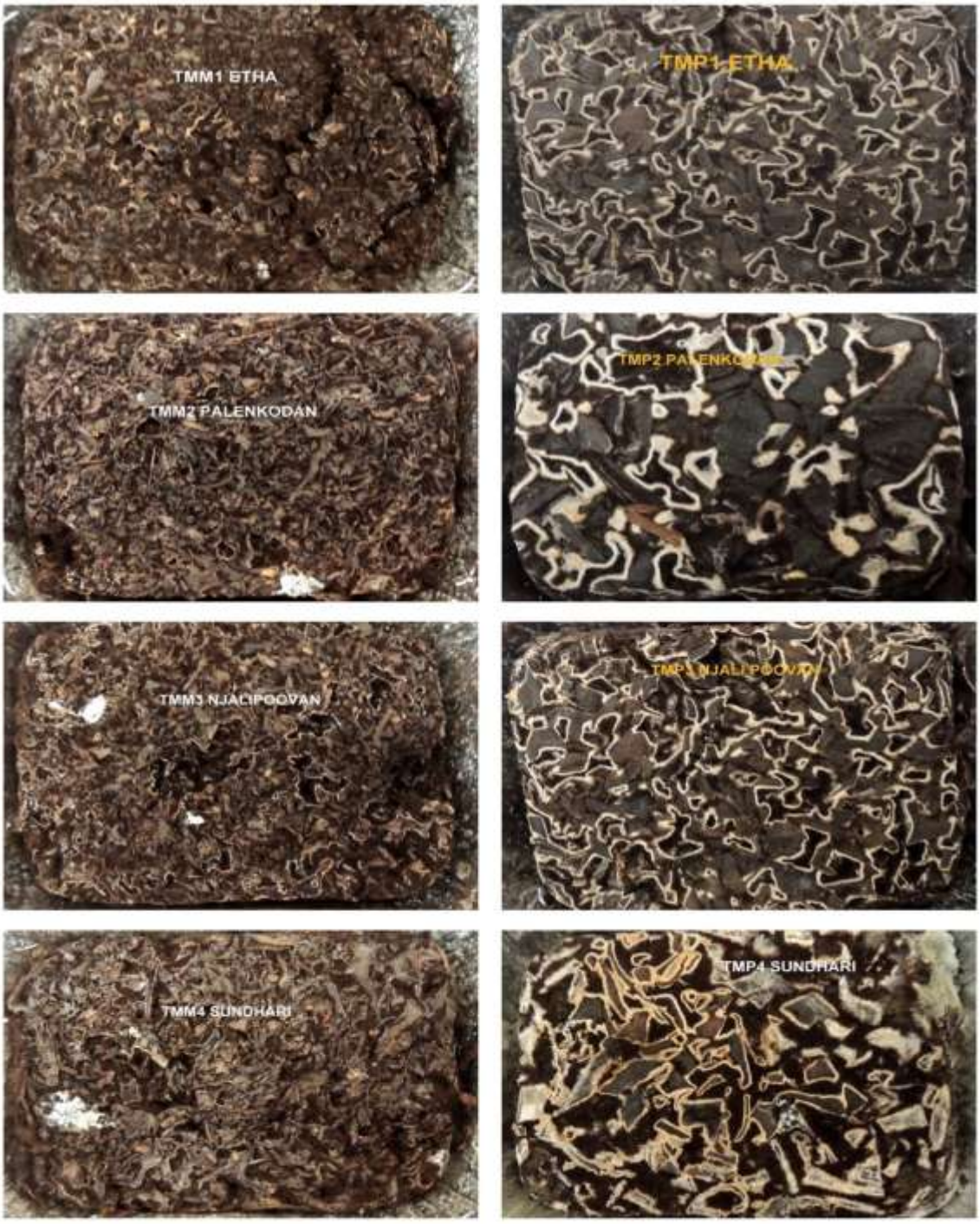

Figure 12. Substrate after incubation of 10 days at 37°C

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IV. RESULTS AND DISCUSSION

5.1 Isolation and identification of strain

Three different fungal isolates were differentiated on the basis of colony morphology obtained after spreading. Based on morphological studies, and Lactophenol cotton blue staining characteristics the isolates were identified as Aspergillus niger. All the three isolates were subcultures by point inoculation and used for further studies.

5.2Screening and identification of the isolate showing maximum hydrolysis

All the three fungal isolates were subjected to screening procedure using starch-agar plate method and after completion of incubation period plates were flooded with iodine solution and observed for zone of starch hydrolysis. The strain which have the maximum zone of starch hydrolysis (clear zone) was considered as the maximum producer and selected for fermentation.

5.3Evaluation of fruit peel and male flower from different banana varieties as substrates for SSF

Enzyme activity in the extracted enzymes from different substrates was determined by DNS assay. All the eight samples were found to be good substrates as the alpha amylase activity was seen in all the eight boxes.Notably, the maximum amylase activity were seen in Sundari peel (TMP4) ($0.8x10^{-3}$µ mols/min)followed by Ethan peel (TMP1) ($0.7x10^{-3}$µ mols/min), Njalipoovan peel (TMP3) ($0.62x10^{-3}$µ mols/min), Palayamkodan peel (TMP2) ($0.3x10^{-3}$ µ mols/min).Palayamkodan male flower (TMM2) andNjalipoovan male flower (TMM3) shows the same activity ($0.17x10^{-3}$µ mols/min).Sundari male flower (TMM4) and Eathan male flower (TMM1) shows the least activity. Dried Sundari fruit peel is the most efficient substrate which produced amylase with maximum activity under the culture condition.

V. CONCLUSIONS

Isolation of fungi from bread sample and the rapid screening by plating on starch agar plates led to the finding of fungal strains capable of producing amylase. These strains were confirmed as Aspergillus niger by as by lacto phenol cotton blue staining. Lignocellulosic substrates like dried banana peels and flower bud was used as solid substrates for SSF. After six days of incubation under room temperature it was found that all substrates are covered with fungal hyphae. The crude enzymes are extracted from the substrates and assayed for the activity of amylase. Among them dried Sundhari peels showed higher activity (0.8 x 10-3 µ mols / s) than any other substrates. The results obtained in the present study suggest that the Sundhari peels may act as a potent substrate for industrial production of α-amylase and subjected for further explorations regarding industrial applications.

Acknowledgements

The authors are grateful for the cooperation of the management of Mar Augusthinose college for necessary support. Technical support from Binoy A Mulanthra is also acknowledged.

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