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Comparative study using banana pseudostem and leaf vein as substrates for amylase production using Aspergillus nigerin Kerala

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ABSTRACT
Solid state fermentation holds tremendous potentials for the production of the enzyme amylase by Aspergillus niger. Different solid substrates like banana pseudo-stem and leaf vein are rich in starch. These agro-industrial residues are cheap raw materials for amylase production. Aspergillus niger isolated from the bread was identified to be the best producer of amylase. When A. niger was incubated for 10 days at 37°C on pseudo-stem and leaf vein of locally available banana varieties like Ethan, Poovan, Palayamkodan and Kaali, as substrate in solid state fermentation. It showed high yield of amylase in Ethan leaf vein, followed by Palayankodan vein. All other substrate also showed moderate amount of amylase production.

Keywords: Aspergillus niger; Solid state fermentation; Musa; Banana; Submerged fermentation; Pseudostem.

I. INTRODUCTION

Amylolytic enzymes from numerous sources degrade starch, the primary storage polysaccharide in plants. Amylase is one of the most important enzymes and of great significance in present day biotechnology and having approximately 25% of the enzyme market (Rao et al., 1998). New amylases could be potentially useful in the pharmaceutical and fine chemical industries of enzymes with suitable properties could be identified (Nigamet al., 1995). With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields such as clinical, medicinal and analytical chemistry (Pandeyetal., 2000) and other applications.

The amylases can be derived from several sources, such as plants, animals and micro-organisms. Because of their short growth period, the enzymes from microbial sources generally meet industrial demands (Odeeet al., 1997; Reddyet al., 1999). At present, Bacillus, Aspergillus, Rhizopus and rhizobial isolates specified are considered to be the most important sources of industrial amylases (Pandeyet al., 2000; Gupta et al., 2003). Nevertheless, various other sources of microbial amylases are been investigated in the world.

Growth conditions and nutrients promote high yields of microbial amylases. However, carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of these enzymes (Gupta et al., 2003; Haqet al., 2002). These expensive products can be replaced in the medium with economically available agricultural by-products (Ghosh et al., 1984) or industrial amylaceous substances as carbon substrates. In this sense, brans, straws and flours of different grains and tubers, such as barley, corn, cassava, potato, rice, sorghum and wheat, have been used in the fermentation medium to increase the productivity of amylases from bacteria and fungi (Ramesh,et al., 2001; Mathew et al., 2016). Banana (Musa sapientum) is grown extensively in tropical and subtropical countries, and 14.37% of the world's production is shared by India. From the banana produce, in addition to the fruit wastes, the pseudo-stem and leaf vein are also accumulated as wastes in the environment, posing serious environmental problems. Several attempts have been made to utilize these wastes through ensilaging, and to eliminate or reduce the negative nutritional effects (Le Dividichet al., 1976).

Protein enrichment of banana waste, skin and pulp using the yeast Pichia spartinae (Chung et al., 1979), enrichment of dry green banana pulp with protein by solidstate fermentation (SSF) employing Aspergillus niger (Baldenspergeret al., 1985), the production of biogas (Del Rosario et al.,

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1985), ethanol (Iizukaet al., 1985) and biomass production utilizing banana skin with Saccharomyces uvarum (Enwefaet al., 1991) and fermentation of whole banana waste liquor for the production of lactic acid (Lopez-Baca,et al., 1992) have been reported. It has been reported by Krishna and Chandrasekaran (Krishnaet al., 1996) that banana fruit stalk waste can be used for the production of α-amylase employing Bacillus subtilis (CBTK 106) bySSF. However, there is no information available on the use of banana pseudostem and leaf vein as solid substrates for the production of α-amylase by fermentation.

It has been reported that banana pseudo-stem contained 56.8% total sugar, 27.0% starch, 4.65% reducing sugar and 4.3% protein on a dry weight basis (Krishnaet al., 1996). Hence, in the present investigation an attempt was made to develop a suitable process for the production of α-amylase, using banana pseudostem and leaf vein as solid substrate and employing Aspergillus 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 Musa accuminata Colla or Musa balbisiana Colla 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 world Mauz, 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 Musa sapientium;

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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 acuminate Colla (A genome) and Musa balbisiana Colla (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 Bluggoe and Green Red from Red. The natural rate of somatic mutations are very low with banana

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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 banana pseudostem and leaf vein.

1.5 Scope of the study

The study would enlighten the amylase production using underutilized substrates especially banana pseudostem and leaf veinwhich could be further explored.

II. REVIEW OF LITERATURE

Recent discoveries on the use of microorganisms as a source of industrially relevant enzymes had led to an increased application of microbial enzymes in various industrial process. Nowadays, the new potential of using microorganisms as a biotechnological source of industrially relevant enzymes has led to an interest in exploration of extracellular enzyme activities in many microorganisms. (Akpan et al., 1999; Bilinski and Stewart, 1995; Buzzini and Martini, 2002). Amylases are one of the most widely used enzymes in the world. It hydrolyses starch and used commercially for the production of sugar syrups from starch which consist of glucose, maltose and higher oligosaccharides (Hagiharaet al., 2001). Amylases are of great significance in biotechnological applications ranging from, food, fermentation, detergent, pharmaceutical brewing and textile to paper industries (Miller, 1959; Kathireshan and Manivannan, 2006). To meet the higher demands of these industries, low cost production of amylase is required.

Amylases represent about 25-33% of the world market enzymes.Amylase enzymes are important enzymes employed in starch processing industries for hydrolysis of polysaccharides such as starch into simple sugars (Akpan et al., 1999; Fogarty and Kelly,1980;Nigam and Singh 1995).Starch degrading enzymes like amylase have important in industrial scale because of their perceived technological significance and economic benefit. Amylase is also used for commercial production of glucose. In storage tissues such as seeds, starch which is a polysaccharide of glucose is hydrolyzed by growing seedling to meet its energy requirement. Amylase of fungal origin was found to be more of stable than the bacterial enzymes on a commercial scale. Many attempts have been made to optimize culture conditions and suitable strains of fungi (Abu et al., 2005). Studies on fungal amylases especially Aspergillus niger has been concentrated because of their ubiquitous nature and non-fastidious nutritional requirement (Abu et al., 2005).

Moulds are capable of producing high amounts of amylase; Aspergillus niger is used for commercial production of alpha-amylase. Studies on fungal amylases especially in developing countries have concentrated mainly on Aspergillus niger, probably because of their ubiquitous nature and non-fastidious nutritional requirements of these organisms (Abu et al., 2005). It is possible to enlist the use of amylases under extreme condition of pH and temperature using thermo-acidophilic and alkaline amylases. Since the most effective preparation of some applications contain other enzymes, especially amyloglucosidases and submerged methods give a narrow spectrum of additional enzymes and it is worthwhile to isolate suitable strains of Aspergillus niger for efficient mechanism. The production of alpha-amylase by moulds has been greatly affected by cultural and nutritional requirement.

The amylases can be derived from several sources, such as plants, animals and microorganisms. Because of their short growth period, the enzymes from microbial sources generally meet industrial demands (Odeeet al., 2000). The first enzyme produced industrially was an amylase from a fungal source in 1994, which was used for the treatment of digestive disorders (Cruegeret al., 1989). At present, Bacillus, Aspergillus, Rhizopus and rhizobial isolates specified are considered to be the most important sources of industrial amylases (Guptaet al., 2003). Nevertheless, various other sources of microbial amylases are been investigated in the world.

Growth conditions and nutrients promote high yields of microbial amylases. However, carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of these enzymes (Crugeret al., 1989). These expensive products can be replaced in the medium with economically available agricultural by-products (Ghoshet al., 1984) or industrial amylaceous substances as carbon substrates.

Production of alpha amylases has been investigated through SmF andSSF (Perez-Guarreet al., 2003). However, the contents of a synthetic

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medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by-products, as they are considered to be good substrates for SSF to produce enzymes (Kunamneniet al., 2005). In recent years the technique of SSF process has been developed and used more extensively. It has advantages over SmF like simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties, lower levels of catabolic repression and better product recovery (Baysal et al., 2003). The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculums concentration and moisture level of the substrate, temperature and pH. Thus it involves the screening of a number of agro-industrial materials for microbial growth and product formation (Sodhiet al., 2005). Temperature and pH are known to be important parameters in the production of enzymes from bacteria; hence, the thermal and the pH stability of the enzyme, which is a function of the exposure time, must also be taken into account

Solid state fermentation holds tremendous potentials for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Canel and Moo, 1980; Tunga and Tunga, 2003). The free water is indispensable to the microorganism's growth and is adsorbed on a solid support or complexed into the interior of a solid matrix (Soccel, 1992). This method has economic value for countries with abundance of biomass and agro industrial residues, as these can be used as cheap raw materials (Tunga and Tunga, 2003).

Before describing the action pattern and properties of amylolytic enzymes, it is essential to discuss the features of the natural substrate, starch. Starch is a major reserve carbohydrate of all higher plants. In some cases it accounts for as high as 70% of the undried plant material. It occurs in the form of water insoluble granules. The size and shape of the granules are often characteristic of the plant species from which they are extracted. When heated in water the hydrogen bonds holding the granules together begin to weaken and this permits them to swell and gelatinize. Ultimately they form paste or dispersion, depending on the concentration of polysaccharide. Starches are produced commercially from the seeds of plants, such as corn, wheat, sorghum or rice; from the tubers and roots of plants such as cassava, potato, arrowroot and the pith of sago palm. The major commercial source of starch is corn from which it is extracted by a wet milling process (Berkhout, 1976). Starch is a heterogeneous polysaccharide composed of two high molecular weight entities called amylose and amylopectin.

There are mainly two methods which are used for production of alpha Amylase on a commercial scale. These are: 1) Submerged fermentation and 2) Solid State fermentation. The latter is a fairly new method while the former is a traditional method of enzyme production from microbes which has been in use for a longer period of time.

The SmF used free flowing liquid substrates, like molasses and broths. The products yielded by fermentation are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence the substrates need to be constantly added for the action. This fermentation technique is suitable for microorganisms such as bacteria that require high moisture content for their growth. The SmF is primarily used for the extraction of secondary metabolites that need to be used in liquid form (Kunamneni et al., 2005). This method has several advantages; SmF allows the utilization of genetically modified organisms to a greater extent than SSF. The sterilization of the medium and purification process of the end products can be done easily. Also the control of process parameters like temperature, pH, aeration, oxygen transfer and moisture can be done easily (Couto and Sanroman, 2006).

Solid state fermentation is a method used for microbes which require less moisture content and the absence of free flowing water for their growth.

The main advantage of using solid substrate is that nutrient-rich waste materials can be easily recycled and used as substrates in this method. Unlike submerged fermentation, in this fermentation technique, the substrates are utilized very slowly and steadily. Hence the same substrate can be used for a longer duration, thereby eliminating the need to constantly supply substrate to the process (Couto and Sanroman, 2006). Other advantages that solid state fermentation offers over submerged fermentation are simpler equipment's, higher volumetric productivity, higher concentration of products and lesser effluent generation.

Fungal sources have been investigated for α-Amylase production through submerged and solid state fermentation. However, studies reveal that SSF is the most appropriate process in developing countries due to the advantages it offers

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which make it a cost effective production process (Couto and Sanroman, 2006). Also SSF provides a medium that resembles the natural habitat of fungal species, unlike SmF which is considered a violation of their habitat (Ladermanet al., 1993).

Recently, it has gained importance in the production of microbial enzymes owing to several economic advantages over conventional submerged fermentation. The content of synthetic media is very expensive and uneconomical, so they need to be replaced with more economically available agricultural by-products to reduce the costs. Most agricultural wastes contain three major components; cellulose (30-50%), hemicellulose (20-35%) and 4-35% lignin. Starch based agro industrial residues are generally considered the best substrates for the SSF of α-amylases. (Arasaratnamet al., 2001; Kauret al., 2003).

However, the contents of a synthetic medium are very expensive and uneconomical, so they need to be replaced with more economically available agricultural and industrial by-products, as they are considered to be good substrates for SSF to produce enzymes (Kunamneniet al., 2005). In recent years the technique of SSF process has been developed and used more extensively. It has advantages over SmF like simple technique, low capital investment, cheaper production of enzyme having better physiochemical properties, lower levels of catabolite repression and better product recovery (Baysalet al.,2003). The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculums concentration, and moisture level of the substrate, temperature and pH. Thus it involves the screening of a number of agro-industrial materials for microbial growth and product formation (Sodhiet al., 2005). Temperature and pH are known to be important parameters in the production of enzymes from bacteria; hence, the thermal and the pH stability of the enzyme, which is a function of the exposure time, must also be taken into account.

Bananas are the most tropical fruits in the world market. India being the largest producer of banana produces about 39000 tons. Banana pseudostem is the stem of banana plant; it produces a single bunch of banana before dying and then is replaced by new pseudostem (Anhwange et al., 2009). Since each plant produces only one bunch of bananas and cannot be used for the next harvest, this agricultural activity generates a large amount of residue (Cordeiro et al., 2004). It has been reported that banana is the second largest produced fruit in terms of quantity, contributing about 16% of the world's total fruit production (Mohapatra et al., 2010). Therefore, every year after harvesting, a large amount of bare pseudostem is cut and left behind as waste worldwide, which ultimately causes contamination of water sources as well as can affect the environment and health of living microorganisms (Aziz et al., 2011, Hossain et al., 2011).According to Souza et al.(2010), for each ton of bananas harvested, around four tons of lignocellulosic wastes are generated, among which 75% consists of banana plant pseudo-stem, which remain at the harvest location undergoing natural degradation. Making use of this biomass as a substrate for the production of alpha amylase could be a very attractive alternative by not only contributing to the preservation of the environment through removing this waste from the land but also by adding value to the fruit production matrix, transforming the residue into a commodity.

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 Musa accuminata

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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.

2.1.2Musa balbisiana

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:MusaL
Species: Musa acuminata

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

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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, Karpoora Poovan, Chakrakeli, Monthan and Yenagu Bontha
Assam	Jahaji (Dwarf Cavendish), Chini Champa, Malbhog, Borjahaji (Robusta), Honda, Manjahaji, Chinia (Manohar), Kanchkol, Bhimkol, Jatikol, Digjowa, Kulpait, Bharat Moni
Bihar	Dwarf Cavendish, Alpon, Chinia , Chini Champa, 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, Rajeli Nendran, Grand Naine, Shreemanti, Red Banana
Orissa	Dwarf Cavendish, Robusta, Champa, Patkapura (Rasthali)
Tamil Nadu	Virupakshi, Robusta, Rad Banana, Poovan, Rasthali, Nendran, Monthan, Karpuravalli, Sakkai, Peyan, Matti
West Bengal	Champa, Mortman , Dwarf Cavendish, Giant Governor, Kanthali, Singapuri

III. 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 pseudostem and leaf vein could be used for the amylase production.

IV. 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

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mainly due to South West Monsoon and temperatures are highest in May and November.

4.2Banana varieties selected

4.2.1Nendran (N2) Musa x paradisiaca (AAB)

It is the one of the most commonly cultivated and largely grown variety, also known as Etha vazha. 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

4.2.2Poovan (N3) Musa x paradisiaca (AAB)

It is a leading cultivar which is grown throughout the country. Produced year round in large quantities. Usually this is tall and vigorous. Fruits are medium in size with pronounced nipple, which weigh up to 25 kg, it turns to attractive golden yellow on ripening. It is highly susceptible to BBMVand Banana Streak Virus (BSV). It also possess some medicinal properties.

4.2.3Palayamkodan (N7) Musa x paradisiaca (AAB)

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 2-6m and the number of the fruits in its mid hand is about10-12. 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.

4.2.4Kali (N10) Musa x paradisiaca (AAB)

This variety attains an average height of 3.8m, with normal pseudostem aspect. The number of fruits on its mid hand is13. The fruit colour changes from dark green to green at the maturity period.

4.3 Substrate selection

The substrates used are banana pseudo-stem and banana leaf vein. Four different types of banana varieties were chosen based on the availability. The pseudo-stem and leaf vein of Ethan, Poovan, Palayamkodan and Kaali werecollected from the local organic farm from Kottayam Taluk, Manarcad Village of Kottayam district, Kerala state, India. They are cut into smaller pieces and they were dried under sunlight.

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 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 pseudo-stem and leaf vein of different banana varieties (Ethan, Poovan, Palayamkodan and Kaali, AMS1, AMS2, AMS3, AMS4, AMV1, AMV2, AMV3, AMV4) 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.

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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 37^0C. 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 generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

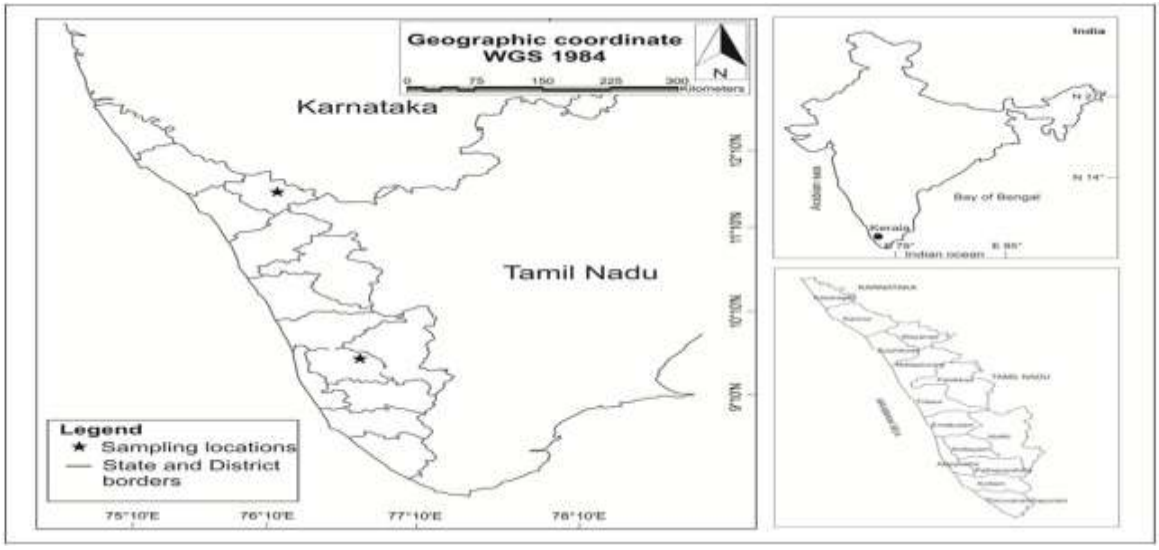

Figure 1. Map of Kerala showing the sample collection point.

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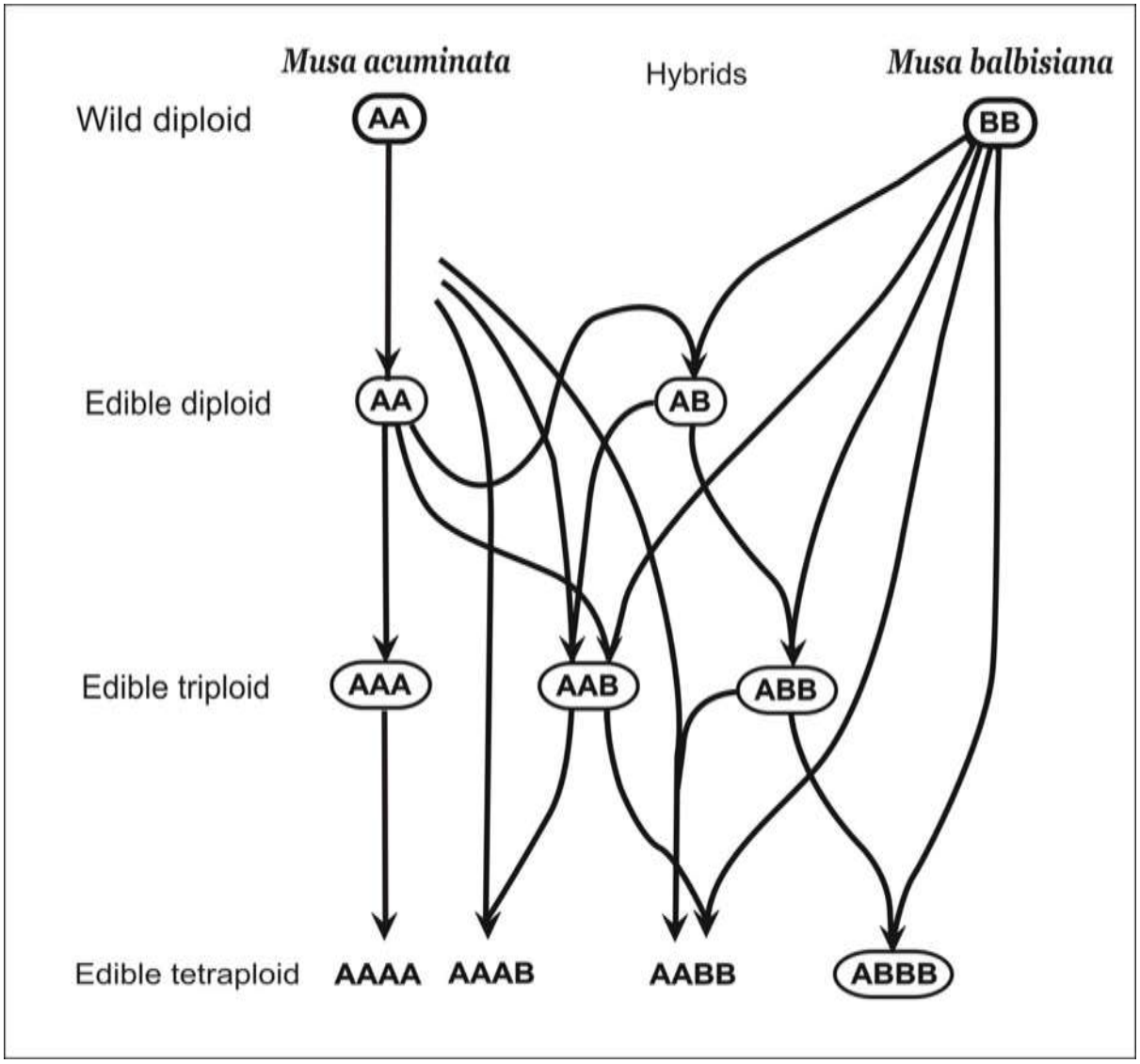

FIG 2.The evolution of the banana complex: A, M. acuminata; B, M. balbisiana. Genotypes known to occur naturally are encircled, those known only from experiment are not encircled (adopted from Simmonds and Shepherd, 1955).

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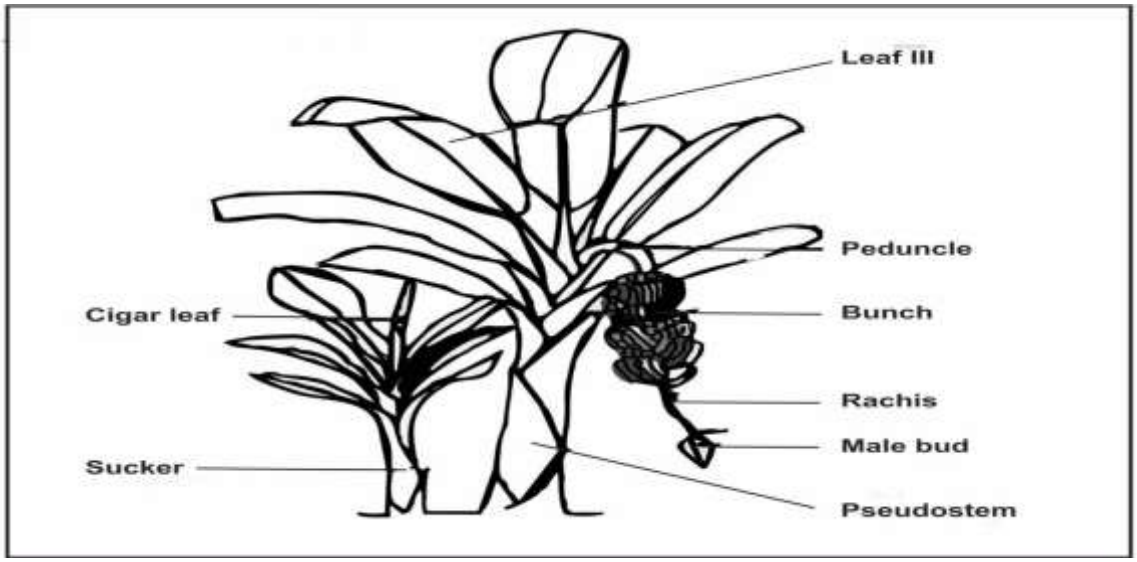

Figure 3. Description of pseudostem/suckers of banana.Modified after: IPGRI, 1984.

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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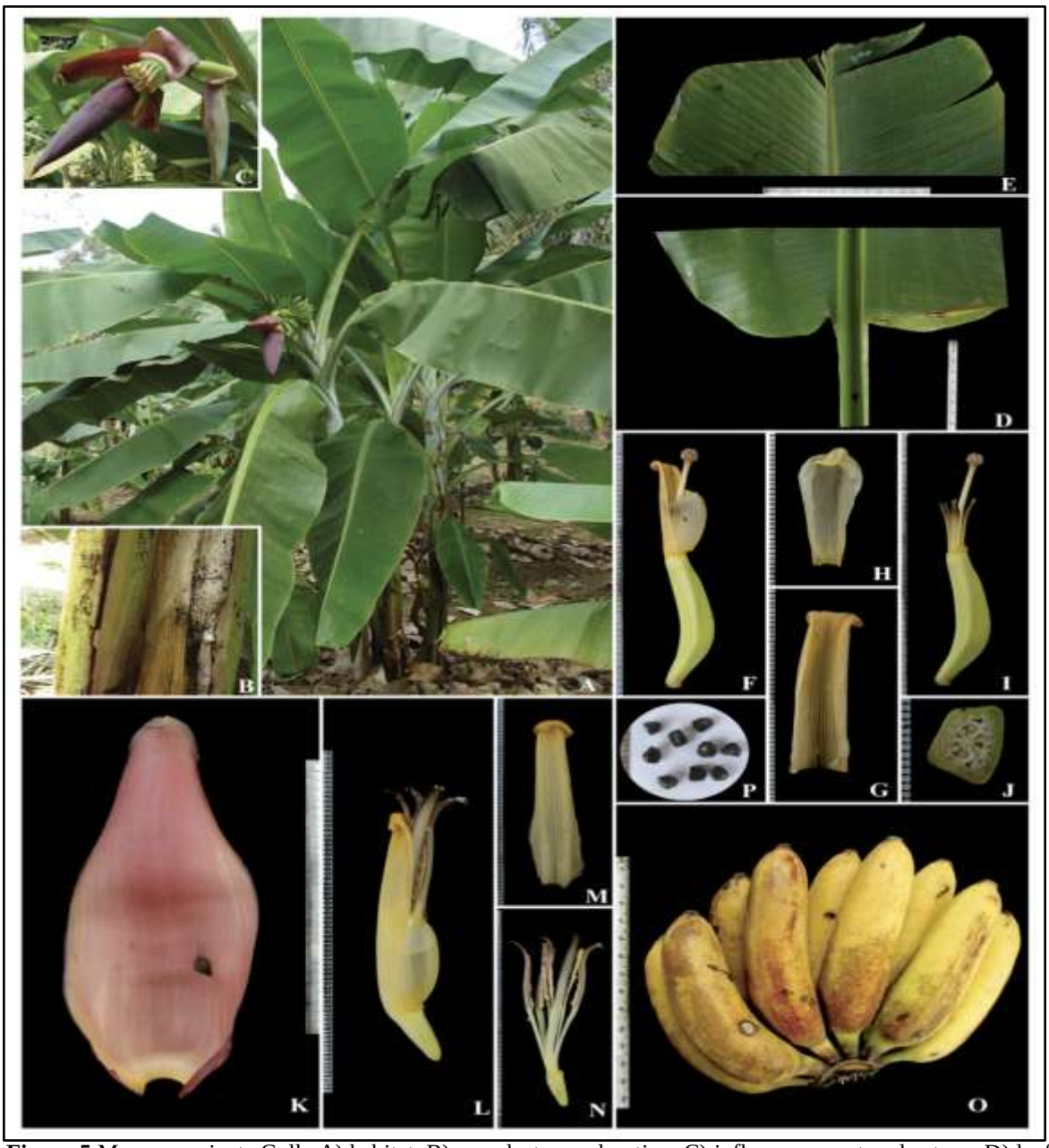

Figure 5. Musa acuminata Colla A) habitat, B) pseudostem coloration, C) inflorescence at early stage, D) leaf base, E) leaf apex, F) female flower, G) compound tepal, H) free tepal, I) pistil with staminodes, J) c.s of ovary, K) male bract abaxial surface, L) male flower, M) compound tepal, N) rudimentary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

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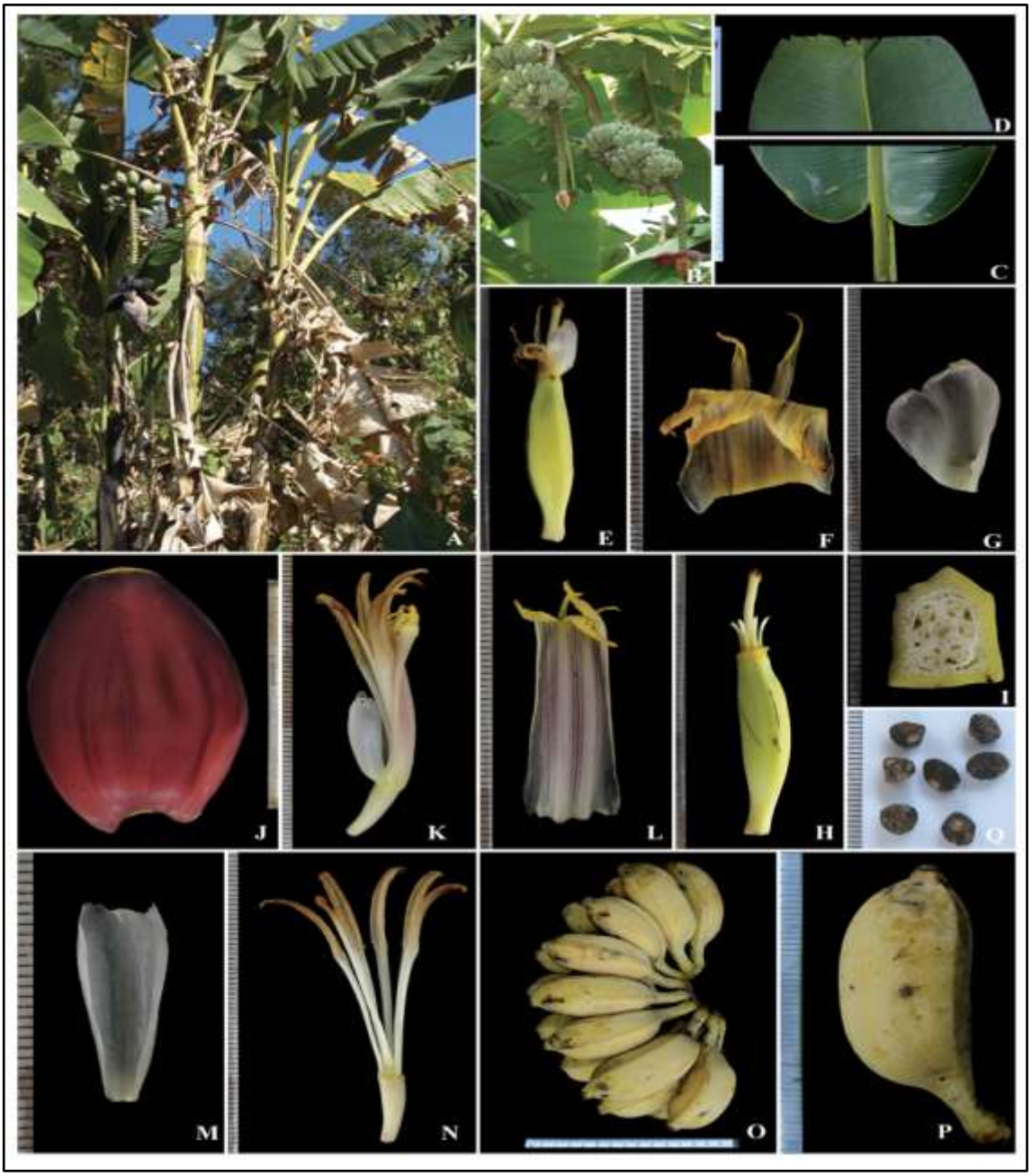

Figure 6. Musa balbisiana CollaA) habitat, B) infructescences with advanced stage of male bud, C) leaf base, D) leaf apex, E) female flower, F) compound tepal (female), G) free tepal (female), H) pistil with staminodes, I) c.s of ovary, J) male bract, K) female flower, L) compound tepal (male), M) free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

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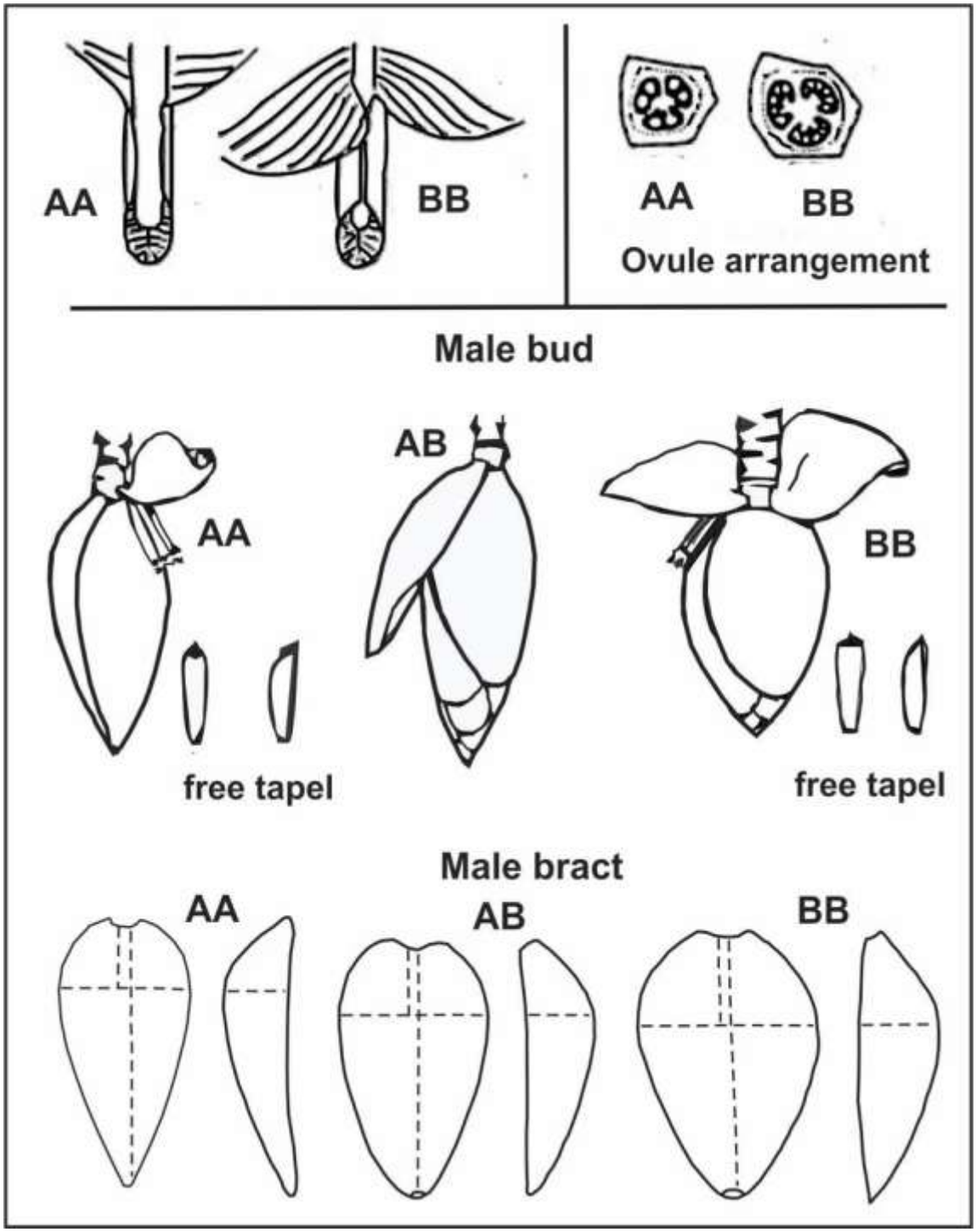

Figure 7. Important characters used in species and genome groups of edible banana. Modified after: IBPGR, 1984.

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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	Musa balbisiana
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	Pseudostem	AMS1
		Leaf vein	AMV1
2	Poovan	Pseudostem	AMS2
		Leaf vein	AMV2
3	Palayamkodan / Palayanthodan	Pseudostem	AMS3
		Leaf vein	AMV3

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4	• Kaali	Pseudostem	AMS4
		Leaf vein	AMV4

Table 7. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Ethan pseudostem (AMS1) as substrate.

Ethan pseudostem	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.45x10^{-3}$	$0.45 x10^{-3}$
Trial 2	$0.47 x10^{-3}$	
Trial 3	$0.43 x10^{-3}$	

Table 8. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Poovan pseudostem (AMS2) as substrate.

Poovan pseudostem	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.29x10^{-3}$	$0.28 x10^{-3}$
Trial 2	$0.28 x10^{-3}$	
Trial 3	$0.27 x10^{-3}$	

Table 9. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Palayamkodan pseudostem (AMS3) as substrate.

Palayamkodan pseudostem	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.46x10^{-3}$	$0.48 x10^{-3}$
Trial 2	$0.50 x10^{-3}$	
Trial 3	$0.48 x10^{-3}$	

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Table 10. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Kaalipseudostem (AMS4) as substrate.

Kaali pseudostem	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.25x10^{-3}$	$0.25\ x10^{-3}$
Trial 2	$0.24\ x10^{-3}$	
Trial 3	$0.25\ x10^{-3}$	

Table 11. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Ethan vein (AMV1) as substrate.

Ethan vein	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$1.1x10^{-3}$	$1.07x10^{-3}$
Trial 2	$0.99\ x10^{-3}$	
Trial 3	$1.12\ x10^{-3}$	

Table 12. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Poovan vein (AMV2) as substrate.

Poovan vein	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.43x10^{-3}$	$0.43\ x10^{-3}$
Trial 2	$0.42\ x10^{-3}$	
Trial 3	$0.44\ x10^{-3}$	

Table 13. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Palayamkodan vein (AMV3) as substrate.

Palayamkodan vein	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	$0.59x10^{-3}$	$0.60\ x10^{-3}$

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Trial 2	0.61×10^{-3}	
Trial 3	0.60×10^{-3}	

Table 14. Activity of enzyme (Amylase) produced by Apsergillus niger using dried Kaali vein (AMV4) as substrate.

Kaali vein	**Activity of each trail (µ mols/min)**	**Average activity (IU)**
Trial 1	0.55×10^{-3}	0.54×10^{-3}
Trial 2	0.53×10^{-3}	
Trial 3	0.54×10^{-3}	

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

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V. 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 pseudostem and leaf vein from different banana varieties as substrates for SSF

Enzyme activity in the extracted enzymes from different substrates was determined by DNS assay and the results of the same can be seen in table 2-9 and chart 1-8. The histogram in chart 9 shows a comparison between the alpha amylase activities in the trays containing different substrates used in the present study. 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 Ethan vein (AMV1) ($1.1x10^{-3}$µ mols/min) followed by Palayamkodan-vein (AMV3) ($0.60x10^{-3}$µ mols/min), Kaali-vein (AMV4) ($0.54x10^{-3}$µ mols/min), Palayamkodan pseudo-stem (AMS3) ($0.48x10^{-3}$ µ mols/min), Ethan pseudo-stem (AMS1) ($0.45x10^{-3}$µ mols/min), Poovan vein (AMV2) ($0.43x10^{-3}$µ mols/min), Poovan pseudo-stem (AMS2) ($0.28x10^{-3}$µ mols/min), Kaali pseudostem (AMS4) ($0.25\ x10^{-3}$µ mols/min). Dried Ethan vein is the most efficient substrate which produced amylase with maximum activity under the culture condition.

VI. CONCLUSIONS

Microorganisms had significant contribution in production of various enzymes. Now a day's, the production of many industrial enzyme using microorganisms is practiced. The starch- degrading amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile, paper, pharmaceutical to sugar industry. Conversion of starch into sugar, syrup forms the major part of starch processing industry. Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological and economic benefits. 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 amylase is economical and less expensive.Bacteria and fungi are the main producer of these enzymes; however fungal amylase is more economical than the bacterial amylase. Amylase production is done by solid state fermentation (SSF) and submerged fermentation (SmF). Aspergillus niger is used for commercial production of α-amylase.

In this study Isolation of Aspergillus from bread sample and the rapid screening by plating on starch agar plates led to the finding of Aspergillus strains capable of producing amylase. These strains were confirmed as Aspergillus niger by as by lacto phenol cotton blue staining. The Aspergillus niger was subjected to solid state fermentation in different banana substrates like pseudo-stem (AMS1, AMS2, AMS3, AMS4), leaf vein (AMP1, AMP2, AMP3, AMP4) which was used as solid substrates for SSF. Enzyme assay was carried out by DNS method of. All the eight samples were found to be good substrates. Notably, the maximum amylase activity was seen in Ethan vein-AMV1($1.1x10^{-3}$µ mols/min) followed by Palayamkodan vein-AMV3($0.60x10^{-3}$µ mols/min). Dried Ethan vein is the most efficient substrate which produced amylase with maximum activity under the culture condition. Further studies include purification of amylase from crude enzyme extract, the various factors affecting enzyme production which include temperature, pH, substrate concentration and the nutritional content analysis of banana pseudo-stem and leaf vein which influence rate of enzyme production.

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