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An Analysis of the Chemical Composition of Different Varieties of Betel Leaves, and Purification and Characterization of Invertase and Polyphenol Oxidase from Doga Variety

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AN ANALYSIS OF THE CHEMICAL COMPOSITION OF DIFFERENT VARIETIES OF BETEL LEAVES, AND PURIFICATION AND CHARACTERIZATION OF INVERTASE AND POLYPHENOL OXIDASE FROM DOGA VARIETY

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DEDICATED TO MY BELOVED PARENTS AND MY SUPERVISOR

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DECLARATION

I hereby declare that the materials included in this thesis are the original research works and have not previously been submitted for the award of any degree or diploma and does not contain any materials previously published or written by another person except when due reference is made in the text of this thesis.

Md. Murad Hossain

(Md. Murad Hossain)
Signature of the candidate

CERTIFICATE

This is to certify that the materials included in this thesis are the original research works conducted under my supervision and have not previously been submitted for the award of any degree or diploma by the candidate.

To the best of my knowledge and believe, this thesis does not contain any material previously published or written by another person except when due reference is made in the text of the thesis.

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ABSTRACT

Betel leaf (*Piper betel*) is a tropical creeper and most important herb in Bangladesh. The nutrient compositions such as protein, lipid, total sugar, reducing sugar, non-reducing sugar, starch, chlorophyll, crude fibre, ash, moisture, phenol, vitamin-C, vitamin-B_1, vitamin-B_2, β-carotene and minerals such as iron, phosphorus and calcium of four varieties of betel leaves were compared. The enzymes such as amylase, invertase and cellulase activities in the betel leaves were also investigated at different maturity stages. Activities of protease, β-galactosidase, polyphenol oxidase, catalase and peroxidase were also measured.

In general premature betel leaf contained about 86.87-87.99 gm% moisture, 2.96-3.08 gm% ash, 1.848-1.93 gm% fibre, 0.492-0.672 gm% phenol, 143.24-186.16 mg% chlorophyll, 1.29-1.43 gm% total sugar, 1.226-1.359 gm% non-reducing sugar and 4.98-6.28 gm% starch 2.87-3.34 gm% protein and 0.175-0.37 gm% lipid while mature betel leaf contained about 85.33-85.94 gm% moisture, 3.22-3.26 gm% ash, 2.095-2.11 gm% fibre, 0.547-0.704 gm% phenol, 255.65-320.76 mg% chlorophyll, 3.74-4.92 gm% protein, 0.98-1.63 gm% lipid, 3.22-3.58 gm% total sugar, 0.497-0.576 gm% reducing sugar, 2.587-2.900 gm% non-reducing sugar, 5.60-7.05 gm% starch, 297-341 mg% vitamin-C, 120-131 mg% β-carotene, 32.24-36.02 mg% vitamin-B_1, 0.63-0.78 mg% vitamin-B_2, 216-231 mg% calcium, 9.15-9.78 mg% iron and 145-157 mg% phosphorus. The amount of total sugar, non-reducing sugar and strach contents were increased moderately from premature to over mature stage. No detectable amount of reducing sugar was found in premature stage but its content was increased thereafter upto over mature stage.

The highest amount of invertase was found in Doga variety (64.00-82.32 Units per gm) and the lowest in Kal Bangla variety (26.67-40.32 Units per gm) in all the maturity stages whereas the activity of invertase was increased with the changes of maturity. The activities of amylase was maximum in Kal Bangla variety (premature 60.23 Units, mature 75.78 Units and over mature 70.51 Units per gm) and minimum in Dudhswar variety (premature 32.49 Units, mature 58.70 Units and over mature 53.35 Units per gm). Shail variety contained the highest cellulase activity while Kal Bangla variety contained the lowest. The activities of amylase and cellulase increased upto mature stage and then decreased drastically in over mature stage. The activities of protease and β-galactosidase were lowest in Dudhswar variety and highest in Doga variety but the protease activity increased with the increase in maturity while β-galactosidase activity decreased from premature to over mature stage. The activities of catalase, peroxidase and polyphenol oxidase were found to be varied between 49.50 to 57.08 Units, 28.24

to 37.45 Units and 20.9 to 22.4 Units per gm respectively in different varieties of betel leaves at mature stage.

Two invertases (AIV I and AIV II) as well as polyphenol oxidase were purified from Doga variety betel leaves at mature stage by successive chromatographies on DEAE-Cellulose followed by CM-Cellulose and Sephadex G-75 column. The molecular weights (Mr) were found to be 94 kDa and 93.5 kDa for the enzyme AIV I, 72 kDa and 71.5 kDa for AIV II and 48 kDa and 45.5 kDa for polyphenol oxidase (PPO) as measured by Gel filtration and SDS-Polyacrylamide gel electrophoresis respectively. In the presence of 2-mercaptoethanol, AIV I showed two identical subunits with Mr of about 46 kDa indicating that the enzyme is a homodimer while the enzyme AIV II is heterodimer with Mr of 40.5 kDa and 31 kDa. On the other hand, the enzyme polyphenol oxidase is monomer in nature. The purified invertases, AIV I and AIV II were glycoprotein with neutral sugar content of 14.6% and 19.4% respectively. The Km values of AIV I, AIV II and PPO were also determined. The enzymes AIV I, AIV II and PPO showed the following characteristics such as optimum pH 4.5, 5.5 and 6.2; optimum temperature 37^0C, 3^0C and 32^0C respectively.

The activities of all three enzymes were gradually decreased with the increase in concentration of various chemicals studied. Zn^{2+} and Ag^{+} produced inhibitory effects while Ca^{2+} and Mn^{2+} produced no effects on betel leaf invertases. The activities of both AIV I and AIV II were increased moderately in the presence of Mg^{2+}, K^{+} and Cu^{2+} salts. The activities of PPO were increased in the presence of metallic salts of Ca^{2+} and Cu^{2+} but its activity was decreased in the presence of salts of Fe^{2+} and Mg^{2+}. The activities of AIV I, AIV II and PPO were decreased drastically in the presence of $HgCl_2$ and the activities were almost completely inhibited by 5 mM $HgCl_2$ suggesting the -SH group containing amino acids are present at or near the active sites. Remarkably, the betel leaves extract showed antibacterial activities but contained no antifungal activities. The betel leaf extracts are toxic in nature as detected by brine shrimp lethality bioassay and the LD_{50} values for different varieties were found to be as follows: 7.02 μg/ml for Dudhswar, 10.75 μg/ml for Shail, 17.25 μg/ml for Doga and 10.52 μg/ml for Kal Bangla varieties.

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CHAPTER ONE

INTRODUCTION

1.1 General:

Betel leaf (Bengali Name: Pan) a tropical creeper belonging to the pepper family of plants named Piper betel. The Aryans called it tambula and the Arabs tambul. People chew it to sweeten the breath and colour (crimson) the lip, tongue and also to have some narcotic pleasure. Normally pan is chewed with shell-lime paste and areca nut or betel nut. Many eat pan mixing it with additional elements such as coriander-seed, cinnamon, cardamoms and manifold flavoured dusts.

It is one of the most important herbs in Bangladesh and acclaimed widely as a common and essential chewing food item to almost all classes of people in our country. Usually the peoples of South Asia, Gulf states, Southeast Asia and Pacific islands take pan as a chewing item. In Bengal pan is traditionally chewed by all classes of people not only as a habit but also as an item of rituals, etiquette and manners. On a formal occasion offering pan meant signaling the time for departure. In festivals and dinners, in pujas and punyas pan was an indispensable item. During the aristocratic age, pan preparation and the style of garnishing it on a plate (pandani) was indeed a recognised folk art. Quite a good number of people in Bangladesh are in the habit of chewing betel leaf and nut without knowing its bad-effects. It is therefore of much importance to know about the effects of betel leaf on human body, and the orodental system. Other ingredients, such as jarda, khaer, tamak pata (tobacco leaf), etc are also mixed with betel leaf, nuts and lime to make the betel leaf eating tasty. The different ingredients of betel leaf have their respective reactions, of these the most important one is the betel nut. In betel nuts, there are aricolin, tannin, alkaliod, etc. Aricolin is almost like the nicotine in cigarette. It irritates the mucous membrane of mouth and intensifies the respiration. The tanins in the betel nut slightly shrinks the cells. Lime is used with betel leaf, which breaks down the alkaloid in the betel nut by metabolism. The acid juice secreted from the stomach due to chewing betel leaf makes the broken alkaloid active. The etheric oil present in the betel leaf makes the tongue less sensitive. On the whole, eating of betel leaf acts as somewhat stimulant. Those who take betel leaf for the first time may suffer from vertigo.

Regular and excessive eating of betel leaf decreases the taste of the mouth. Certain reactions are found on teeth and oral health due to eating of betel leaf. Tartars are formed at teeth roots of those who take betel leaf regularly, and suffer from gum troubles. The gum decays and in many cases the bone which holds tooth root in jaws gradually decays. As a result, the teeth become unsteady and there is an early loss of teeth. Besides, attrition occurs in the enamel as a result of excessive chewing of betel leaf. In certain cases, the dentin is found to be exposed due to attrition of enamel. This results tingling sensation in teeth and various sorts of troubles occur. There is evidence that the habit of taking betel leaf induce cancer in the mouth. (http://banglapedia.search.com.bd/HT/O_0026.htm)

Herbs and plants have great importance and utilization in human civilization. All animals, including human beings greatly rely and depend upon plant kingdom for many of their good utilities and necessities of our life cycle. They supply food materials for all kinds of animals and provide human race with materials for shelter, clothing and fuel. Both herbs and plants are unique in their ability to synthesize carbohydrates, proteins, fats, vitamins and most essential materials and thus constitute important nutrients of our daily living requirements, or our diet. Starch and sugars provide most of the energy we need to move, work and keep ourselves alive. Nutrients like, fats and oils are concentrated form of energy and recognized as carrier of many vitamins. And of course from plants and herbs, the essential fatty acids, are needed for normal growth. Proteins from plants and herbs play important roles in the diet and provide essential amino acids and nitrogen required. In addition, antibodies, enzymes and many hormones are produced in the body from amino acids available in proteins in the diet. Vitamins, although needed only in minute quantities in the diet, are essential as co-enzyme of many enzymes catalyzing and regulating major and vital biochemical reactions within cells. Besides, herbs also provide animals and us such substances as glycosides, alkaloids, sterols, saponins, essential oils, resins, tannins etc. Herbs also help us maintain our ecological and environmental balance. Herbs containing medicinal ingredients are usually classified as medicinal plants.

Betel leaf is also a medicinal herb. It is often offered to guests as betel morsel (pan-supari) in the Indian sub-continent as a common courtesy. We get it available the whole year, which is often used as antiflatulant.

1.2 Social and economic aspect:

The production and marketing of pan led to the rise of an occupational caste called *Barui*. Barui is a pre-Muslim institution and all Baruis were originally Hindus. Most of them belonged to *Nabasak* caste. Their position was fairly high in the caste hierarchy. Many Baruis became zamindars and tenure holders in the nineteenth century. In the Muslim period they were the richest people among the cultivating classes. It was possibly because demand for pan was quite universal at that time. Most numerous among the pan producing Baruis, according to the censuses of 1872 and 1881, lived in Burdwan, Midnapur, Jessore and Dhaka. Since 1947, great many baroujes (gardens of betel leaf) were abandoned or sold by the original Baruis and their estates were bought off by the entrepreneurial Muslims. At present, pan production is predominantly in the hands of the Muslim farmers. But the technique of production remained unchanged.

Pan production is capital intensive but it also yields high income. Due to its pungency, pan barouj is not vulnerable to attacks from vermin and insects.

Therefore, income from pan barouj is very stable. The economic significance of pan in the past was such that Prince Azim-us-Shan, the subadar of Bengal (1697-1703) made it one of the royal monopolies calling it *saudia khas*. Robert clive, after the acquisition of the diwani in 1765, also made pan and supari a monopoly of the east India company in 1767.

At present, pan has a worldwide market. Two factors are responsible for the expansion. First, the global dispersion of the pan chewing South Asians and second, scientific recognition of the medicinal value of pan. But in competition with India and other pan producing countries, Bangladesh has a very small share of the world pan market. Acreage of pan is decreasing fast, because, from economic point of view, other crops now prove to be more profitable. [Sirajul Islam] http://banglapedia.search.com.bd/HT/P_0052.HTM

1.3 Betel Leaves: Origin and Distribution

Piper betle is the botanical name of betel leaf which belongs to a family of piperaceae. The betel plant is a slender, aromatic creeper, rooting at the nodes. The branches of the plant are swollen at the nodes. The plant has alternate, heart-shaped, smooth, shining and long stalked leaves, with pointed apex. It has five to seven ribs arising from the base; minute flowers and one-seeded spherical small berries. Pan differs in shape of leaves, bleaching quality, softness, pungency and aroma. The stems are semiwoody, climbing by many short adventitious roots, very stout, much thickened at nodes, young parts glabrous. Betel leaves are large (15-20 cm), broadly ovate, slightly cordate and often a little unequal at base, shortly acuminate, acute, entire but margin often rather undulate, usually 7-nerved, glabrous, thick, bright green and shining on both sides. Fruits sparingly produced, quite immersed in the fleshy spike, which is about 5 cm long and pendulous.

The use of betel leaf can be traced as far back as two thousand years. It is described in the most ancient historic book of Sri Lanka, *Mahavasma,* written in Pali.

Betel is a native of central and eastern Malaysia. It spreads at a very early date throughout tropical Asia and later to Madagascar and East Africa. In India, it is widely cultivated in Tamil Nadu, Madhya Pradesh, West Bengal, Orissa, Maharashtra and Uttar Pradesh. It has long been naturalized in Bangladesh and grows nearly throughout the country and is cultivated commercially in almost all districts.

1.4 Betel Leaf Oil

Synonyms: piper betel leaf oil; betel leaf oil
Odor Description: Phenolic Tar Smoky Mate Leaves
Appearence: Brown Liquid
Description: The essential oil is produced by steam distillation from the leaves of Piper Betle, a vine of the pepper family. The plant grows widely over the entire area between South Arabia and Southeast China. Betel Leaf Oil is yellow to brown with a distinctly phenolic, almost tar-like or smoky.
http://www.thegoodscentscompany.com/data/es1068341.html

1.5 Nutritive Value of Betel Leaf

The betel leaves are usually used as a chewing herb in our country with supari, jarda, chun and other masalla of different kinds. The leaf is usually bit sour in taste but is a popular mouth freshner and has lots of nutrients. An analysis of the betel leaf shows it to consist of moisture 85.4 per cent, protein 3.1 per cent, fat 0.8 per cent, minerals 2.3 per cent, fibre 2.3 per cent and carbohydrates 6.1 per cent per 100 grams. Its minerals and vitamins contents are calcium, phosphorus, iron, zinc, sulphur, potassium, carotene, thiamine, riboflavin, niacin and vitamin C. Its calorific value is 44.

Recent studies have shown that betel leaves contain tannins, sugar and diastases and an essential oil. The essential oil is a light yellow liquid of aromatic odour and sharp burning in taste. It contains a phenol called chavicol which has powerful antiseptic properties. The alkaloid arakene in it has properties resembling cocaine in some respects.

A number of amino acids have been observed in betel leaves, including essential one's (Vidhyasekaran *et. al.,* 1971). Citric acid is also a major acid in betel leaves, whilst tartaric acid and palmitic acids are present in smaller amounts (Teaotia *et. al.,* 1961).

1.6 Medicinal Uses

The usage of betel leaf is cited as far back as two thousand years. It was found a place in the most ancient Srilankan historic book 'Mahawamsa' written in Pali. Betel leaves usually used as masticatory. It is stimulant, digestive, carminative, anti flatulent, anti inflammatory, invigorating, anti phlegmatic, pain reliever. It eliminates foul smell and is an antiseptic. According to Hakeem Hashmi, outstanding unani physician, betel leaves have been used from ancient times as an aromatic stimulant and anti flatulent. It is used in several common house hold remedies.

Healing Power and Curative Properties

Betel leaf has been used from ancient times as an aromatic stimulant and antiflatulent. It is useful in arresting secretion or bleeding and is an aphrodisiac. Scanty or Obstructed

Urination

Betel leaf juice is credited with diuretic properties. Its juice, mixed with dilute milk and sweetened slightly, helps in easing urination.

Weakness of Nerves

Betel leaves are beneficial in the treatment of nervous pains, nervous exhaustion and debility. The juice of a few betel leaves, with a teaspoon of honey, will serve as a good tonic. A teaspoon of this can be taken twice a day.

Headaches

The betel leaf has analgesic and cooling properties. It can be applied with beneficial results over the painful area to relieve intense headache. Grounded paste of betel leaves should be applied on temples to relieve headache. One or two drops of betel juice can be put in the nostrils to eliminate headache.

Respiratory Disorders

Betel leaves are useful in pulmonary affection in childhood and old age. The leaves, soaked in mustard oil and warmed, may be applied to the chest to relieve cough and difficulty in breathing.

Constipation

In the case of constipation in children, a suppository made of the stalk of betel leaf dipped in castor oil can be introduced in the rectum. This instantly relieves constipation.

Stomach disorders:

i. Taken betel morsel prepared with catechu, quick lime, betel nuts, cardamom etc. after meals cures digestive problems and eliminates flatulence (caution: do not add tobacco in this).
ii. Fomenting the stomach (in case of children) with heated leaf of betel cures stomach ailments.
iii. Boiling 3 betel leaves with little black pepper in 250 ml water and strain it and taking 2-tea spoon of this decoction twice a day can cure indigestion.

Cold & cough:

i. Eating betel leaves with clove relieves cold & cough.

ii. Eating little bishop weed's seeds with betel leaf eliminate frequent coughing.

iii. Heat some betel leaves, rub with oil (either sesame oil or Castor oil) and keep in layers on the chest. This gives instant relief in cough.

Sore throat

Local application of the leaves is effective in treating sore throat. The crushed fruit or berry should be mixed with honey and taken to relieve irritating cough.

Inflammation

Applied locally, betel leaves are beneficial in the treatment of inflammation such as arthritis and orchitis, that is inflammation of the testes.

Wounds

Betel leaves can be used to heal wounds. The juice of a few leaves should be extracted and applied on the wound. Then a betel leaf should be wrapped over and bandaged. The wound will heal up with a single application within 2 days.

Boils

The herb is also an effective remedy for boils. A leaf is gently warmed till it gets softened, and is then coated with a layer of castor oil. The oiled leaf is spread over the inflamed part: This leaf has to be replaced, every few hours. After a few applications, the boil will rupture draining all the purulent matter. The application can be made at night and removed in the morning.

Lumbago

A hot poultice of the leaves or their juice mixed with some bland oil such as refined coconut oil can be applied to the loins with beneficial results in lumbago.

Problem of Breast Milk Secretion

The application of leaves smeared with oil is said to promote secretion of milk when applied on the breasts during lactation.

Precautions: Cancer of the mouth and lips has been found to be more frequent in areas where the betel chewing habit is widely prevalent, Other ill-effects of pan-chewing like dyspepsia, pyorrhea, cancer of the tongue and cheeks have also been observed amongst excessive chewers.

Other Uses

Aphrodisiac: Pan-supari, especially the pan, is prescribed by Ayurvedic physicians as an aphrodisiac. Partly owing to its deodorant, aphrodisiac, and invigorating properties, *pan-supari* came to form a part of the ritual with which a wife welcomed her husband.

The betel leaves are chewed together with betel nut as a masticatory. In its simplest form, sliced betel nut is wrapped in a betel leaf, smeared with lime and chewed. Often though, a clove and other spices such as cinnamon and cardamom are added. When chewed after meals, it sweetens the breath and acts as a gentle stimulant.

According to Hashmi, to preserve the freshness of mint, coriander leaves and greens etc, for a long time put some betel leaves along with them.
http://www.indoindians.com/lifestyle/paan.htm

1.7 Cultivation of Betel Leaves

In olden times, pan (betel leaf) was produced in all parts of Bengal, though some districts like Dinajpur, Rangpur, Midnapur and Chittagong were particularly famous for its production. The cultivation of pan requires special soil and great attention. Land selected for pan production is generally high, of a stiffish soil, and in the vicinity of a stream or tank. The pan garden is called *barouj,* which is usually from twelve to twenty decimals in area. New land dug up from neighbouring field has to be thrown up and raised the place for making the barouj ground. Oil cakes and cow dungs are the traditional manure for a barouj. Nowadays, chemical manure is also used along with traditional manure. The creeper cuttings are planted after proper dressing in the months of May and June. The plants are neatly arranged in parallel rows about two feet apart, and the saplings are twined around upright sticks of split bamboo and reeds. The barouj is enclosed by a wall of bamboo and reeds, about five or six feet in height and thatched with the same material so as to protect the plants from sun and stray cattle. The plants are regularly watered in the hot months. The leaves of the plant become ready for plucking after one year of planting and the production of the barouj lasts for several years from the date of planting.

Total cultivated area under the crop in Bangladesh is about 14,175 ha and the total annual production is about 72,500 tons. The average yield per acre is 2.27 tons. There are usually three crops during the twelve months and they are locally called by the name of the respective months in which they are harvested. Pan leaf is usually plucked in *Kartik*, *Phalgun* and *Ashad.* The Kartik pan is considered by consumers to be the best and Ashad pan the worst. When plucking, it is a rule to leave at least sixteen leaves on the vine.

Almost every district has a variety of pan to which consumers are well acquainted. In the past, the most elegant and highly prized pan was *kafuri or* camphor-scented pan grown in Sonargaon of Narayanganj district. It was exported to Calcutta and Middle Eastern countries. At present, this variety is yielding fast to other more lucrative crops. The next best is the *sanchi pan* grown in Chittagong hill tracts [Sirajul Islam]. http://banglapedia.search.com.bd/HT/P_0052.HTM

1.8 Betel leaf farming in coastal area

Land preparation is the first task for betel- leaf cultivation in coastal area. A good land preparation is necessary for proper and rapid growth of the crop; lot of time and labour is required for land preparation for betel leaf cultivation. From February to March is considered best time for planting it in coastal area.

In the following table-1, the yield of betel leaf received by the selected growers by the time factor is presented.

Table 1. Yield of betel leaf received by the selected growers

Age of Plant	No. of harvest	Yield kg/harvest	Total yield kg/ha.	Yield md/ha.
After 8-12 months	12	30	360	10.0
12-18 months	18	40	720	19.0
18 - 24 months	18	50	900	24.0
24 - 36 months	36	80	2880	77.0
36 -48 months	36	80	2880	77.0
48-54 months	18	50	900	24.0
54 - 60 months	18	30	540	245.0

Source: Betel-leaf farming in coastal area. By Dr. Ali Muhammed Khushk and M. Ibrahim Lashari; http://dawn.com/2002/05/13/ebr14.htm

1.9 Aim of Present Investigation

Bangladesh is a developing country. It has immense natural resources, its flora and fauna are so plenty and rich that very few countries can vie with it in this regard. It abounds with great numbers of plants of diverse varieties. Its plants resources have great medicinal as well as nutritional value. If its untapped resources are properly utilized, and if its plants are scientifically processed and properly marketted, its economy will be a viable one, and it may soon be aligned with the developed countries of the world. It may soon become poverty free, academically progressing and politically leading country.

Of the many species of its flora betel leaf, which is commonly known as 'Pan', is a most important one. It is of plant origin and is produced abundantly in various districts of Bangladesh. Everyday this leaf is being chewed by millions of people of our country and abroad. In their daily life, the Bangalis use it in welcoming guests and newly married couples, in cementing friendship, in establishing relations and in mediating conflicts in rural Bengal. It has also some religious aura among the Hindus who use it in adoring their gods and goddesses. For ages and generations together betel plants are being cultivated in Bengal and a big segment of its population depends economically on betel leaf production.

In our country, so far I know no such detailed study has been conducted to determine the nutritional quality of betel leaf. This might be due to less interest of researchers in this field as 'Pan' is a perishable commodity. Further, the nutritional quality of betel leaf is also deteriorated abruptly in many times as soon as the betel plants are infected with diseases. Viewing the very limited data on the nutritional quality, statistics of enzymatic activities as well as economic importance of betel leaves, in this study attempts are being made to determine the nutritional quality as well as different enzymatic activities of four different varieties of betel leaves namely 'Shail', 'Doga', 'Dudhswar' and 'Kal Bangla' available in our country specially in Rajshahi region, at different maturity levels.

The main focus of this thesis is in the following areas.

i. Analyze the nutrient contents such as moisture, ash, protein, lipid, carbohydrates, phenolic compounds, chlorophyll, vitamins and minerals.
ii. Estimation of the contents of some hydrolytic and oxidative enzymes such as amylase, invertase, catalase, peroxidase, protease, polyphenoloxidase, cellulase and β-galactosidase.
iii. Purification and characterization of invertase, a sucrose splitting enzyme.
iv. Effects of physical and chemical agents on the enzymatic activities of invertase.
v. Purification and characterization of polyphenoloxidase.
vi. Effects of physical and chemical agents on the activities of polyphenol oxidase.
vii. Determination of antibacterial, antifungal and cytotoxic activities of the betel leaf extract.

Figure-1: Photographic representation of betel leaves (up) and the experimental plot of betel leaf (down).

CHAPTER TWO

A COMPARATIVE STUDY ON THE NUTRIENT COMPOSITIONS OF DIFFERENT VARIETIES OF BETEL LEAVES AT DIFFERENT STAGES

Introduction

Betel leaf is one of the most popular and widely cultivated herbs in Bangladesh. It supplies ready sources of carbohydrates, protein, lipid as well as other nutrients. Betel leaf is also a good source of vitamins and minerals. There are many varieties of betel leaves in our country but their nutritive values are not known clearly. The concentration of these nutrients also varies with the changes of maturity of the betel leaves. The primary aim is to ascertain the changes of biochemical constituents in different varieties of betel leaves at different stages.

M.2.1 DETERMINATION OF MOISTURE CONTENT OF BETEL LEAVES

Moisture was determined by the conventional procedure.

Materials:

a) Porcelain crucible.
b) Electrical balance.
c) Oven.
d) Desiccator.

Procedure:

One to two gm of betel leaves were weighed in a porcelain crucible (which was previously cleaned and heated to 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six hours at 100°C. It was then cooled in a desiccator and weighed again.

Calculation:

Percent of moisture content (gm per 100 gm of betel leaves)

$$= \frac{\text{Amount of moisture obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.2 DETERMINATION OF ASH CONTENT OF BETEL LEAVES

Ash content was determined following the method of A.O.A.C. (1980).

Materials:

a) Porcelain crucible.
b) Electrical balance
c) Desiccator
d) Muffle furnace

Procedure:

Four to six gm of healthy betel leaf were weighed in a porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible with its content was placed in a muffle furnace for 4 hours at about 600°C. These were then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible was again heated at the same temperature in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same.

Calculation:

Percent of ash content (gm per 100 gm of betel leaves)

$$= \frac{\text{Amount of ash obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.3 EXTRACTION AND ESTIMATION OF CHLOROPHYLL IN BETEL LEAVES

Chlorophylls are the green pigments universally present in all photosynthetic tissues. Chlorophyll-a and Chlorophyll-b occur in higher plants. Age, disease and varieties of betel leaves affect not only the total Chlorophyll content but also alters the ratio between the Chlorophyll-a and Chlorophyll-b. A measurement of green pigments may indirectly denote the age of betel leaf. Chlorophyll estimates may also be required to relate other biochemical changes in the plant tissue. Chlorophyll content of different varieties at different stages of betel leaf tissues was estimated following the method described by Mahadevan and Shidhar (1982).

Materials:

a) Mortar and pestle
b) Buchner funnel
c) Whatmann no. 42 filter paper
d) Volumetric flasks (50 ml and 100 ml)
e) Pipette (5 ml and 10 ml)
f) Colorimeter

Reagent:

Acetone, 80%

Procedure:

One gm of betel leaf was cut into small pieces and homogenized well with excess acetone in a mortar with pestle and then filtered the extract through a buchner funnel using Whatman no. 42 filter paper. Then sufficient quantity of 80% acetone

was added and repeated the extraction. The content from the extraction was transferred to buchner funnel and washed with 80% acetone until colorless. The filtrates were pooled and made upto the volume of 100ml in a volumetric flask with 80% acetone. The absorbance of the extract was measured at 645 nm and 663 nm for determination of chlorophyll-a and chlorophyll-b respectively.

The chlorophyll contents were calculated on fresh weight basis employing the following formula as described (Mahadevan and Sridhar, 1982), using the specific absorbtion coefficients for chlorophyll-a and chlorophyll-b at 663 nm and 645 nm in 80% percent acetone respectively.

Calculation:

$$\text{Total chlorophyll (mg / gm)} = \frac{20.2\Lambda_{645} + 8.02\Lambda_{663}}{L \times 1000 \times w} \times v$$

$$\text{Chlorophyll-a (mg / gm)} = \frac{12.7\Lambda_{663} - 2.69\Lambda_{645}}{L \times 1000 \times w} \times v$$

$$\text{Chlorophyll-b (mg / gm)} = \frac{22.9\Lambda_{645} - 4.68\Lambda_{663}}{L \times 1000 \times w} \times v$$

Where, Λ = Optical density in each case

L = length of light path in the cell (usually 1 cm)

v = Volume of the extract in ml, and

w = Fresh weight of the sample in gm.

M.2.4 DETERMINATION OF LIPID CONTENT OF BETEL LEAVES

Lipid contents of betel leaves were determined by the method of Bligh and dyer (1959).

Reagents:

A mixture of chloroform and ethanol (2: 1 v/v)

Procedure:

About one gm of betel leaf was first grounded in a mortar with about 10 ml of distilled water. The grounded flesh was transferred to a separating funnel and 30 ml of chloroform-ethanol mixture was added. The mixture was mixed well and

then kept overnight at room temperature in the dark. At the end of this period 20 ml of chloroform and 20 ml of water were further added and mixed well. Generally three layers were seen. A clear layer of chloroform containing all the lipids, a colored aqueous layer of ethanol with all water soluble materials, and a thick pasty inner phase were seen.

The chloroform layer was carefully collected in pre-weighed beaker (50 ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of lipid.

Calculation:

Percent of lipid content (gm per 100 gm betel leaves)

$$= \frac{\text{Amount of lipid obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.5 DETERMINATION OF TOTAL SUGAR CONTENT OF BETEL LEAVES

Total sugar content of betel leaves was determined colorimetrically by the anthrone method (Jayaraman, 1981)

Reagents:

a) Anthrone reagent: The reagent was prepared by dissolving 2 gm of anthrone in one litre of concentrated sulphuric acid (H_2SO_4).

b) Standard glucose solution: A standard solution of glucose was prepared by dissolving 10 ml of glucose in 100 ml of distilled water.

Preparation of sugar extract from betel leaf

Sugar extract from betel leaves was prepared by the following method, (Loomis and Shull, 1937).

Four to six gm of betel leaves were cut into small pieces and grinded in mortar and immediately plunged into boiling ethyl alcohol and allowed to boil for 5 to 10 minutes (5 to 10 ml of alcohol was used for every gm of betel leaf). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the ground tissue for three minutes in hot 40% alcohol using, 2 to 3 ml of alcohol for every gm of tissue. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatman no. 41 filter paper.

The volume of the extract was evaporated to about $1/4^{th}$ the volume over a steam bath and cooled. This reduced volume of extract was then transferred to a 100 ml volumetric flask and made upto the mark with distilled water. Then 1 ml of the diluted solution was taken into another 100 ml volumetric flask and made upto the mark with distilled water.

Procedure:

Aliquot of 1 ml of extracted solution was pipetted into test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a test tube and treated similarly. The absorbance of the blue green solution was measured at 620 nm in a spectrophotometer. A standard curve of glucose was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of standard glucose solution in different test tubes containing 0.0, 10µg, 20µg, 40 µg, 60µg, 80µg and 100µg of glucose respectively and made the volume upto 1.0 ml with distilled water. Then 4 ml of anthrone reagent was added to each of the test tube and mixed well. All these solution were treated similarly as described above. The absorbance was measured at 620nm using the blank containing 1 ml of water and 4 ml of anthrone reagent.

Calculation:

The amount of total sugar present in the extracts was calculated from the standard curve of glucose (Fig-2). Finally, the percentage of total sugar present in the betel leaf was determined using the formula given below:

Percentage of total sugar (gm per 100 gm of betel leaf)

$$= \frac{\text{Amount of sugar obtained}}{\text{Weight of betel leaf}} \times 100$$

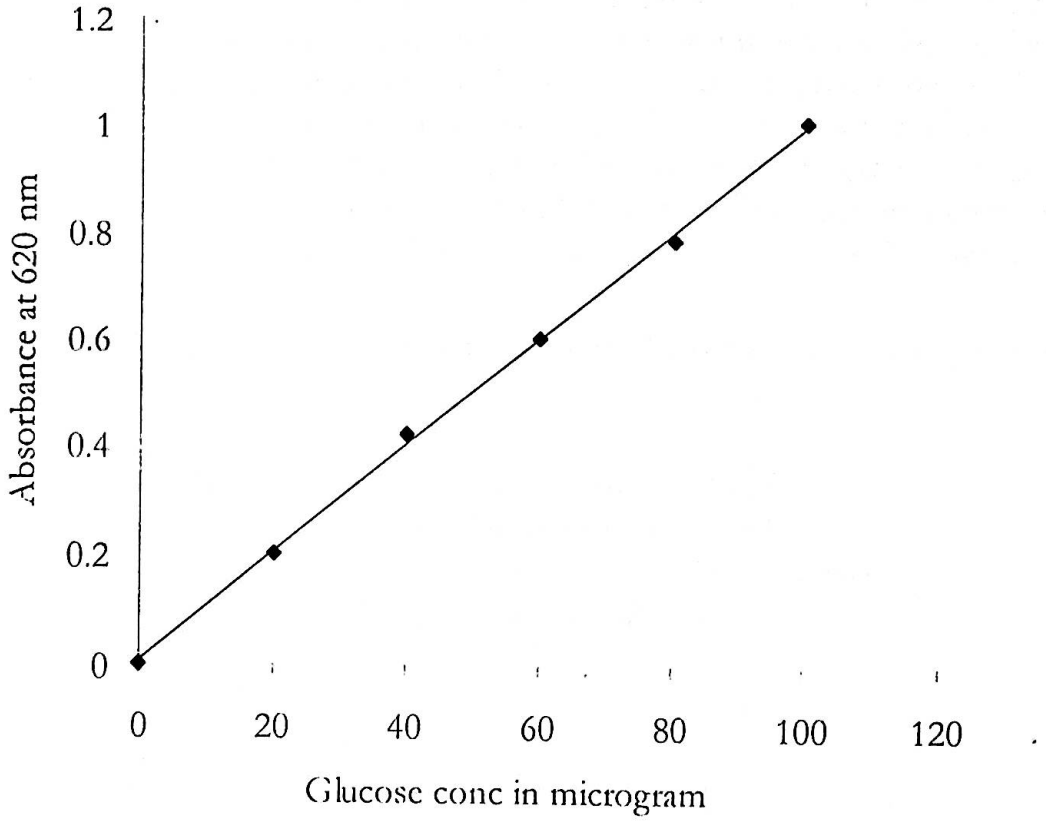


Fig-2: Standard curve of glucose for estimation of total sugar and starch.

M.2.6 DETERMINATION OF REDUCING SUGAR CONTENT OF BETEL LEAVES

Reducing sugar content of four varieties of betel leaves were determined by dinitrosalicylic acid method (Miller, 1972).

Reagents:

a) Dinitrosalicylic acid (DNS) reagent: Simultaneously 1 gm of DNS, 200 mg of crystalline phenol and 50 mg of sodium sulphite were placed in a beaker and mixed with 100 ml of 1% NaOH solution by stirring. If it is stored then sodium sulphite is added just before use.

b) 40% Rochelle salts.

Preparation of sugar extract from betel leaf

Sugar extract from betel leaf was prepared following the procedure as described before.

Procedure

Aliquot of 3ml of the extract was pipetted into test tubes and 3ml of DNS reagent was added to each of the solution and mixed well. The test tubes were heated for 5 minutes in a boiling water bath. After the color has developed, 1 ml of 40% Rochelle salt was added to each of the tubes, when the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3ml of water and 3ml of DNS reagent in a tube and treated similarly. The absorbance of the solution was measured at 575 nm in a colorimeter.

The amount of reducing sugar was calculated from the standard curve of glucose, (Fig-3).

Calculation:

The percentage of reducing sugar (gm per 100 gm of betel leaf)

$$= \frac{\text{Amount of reducing sugar obtained}}{\text{Weight of betel leaf}} \times 100$$

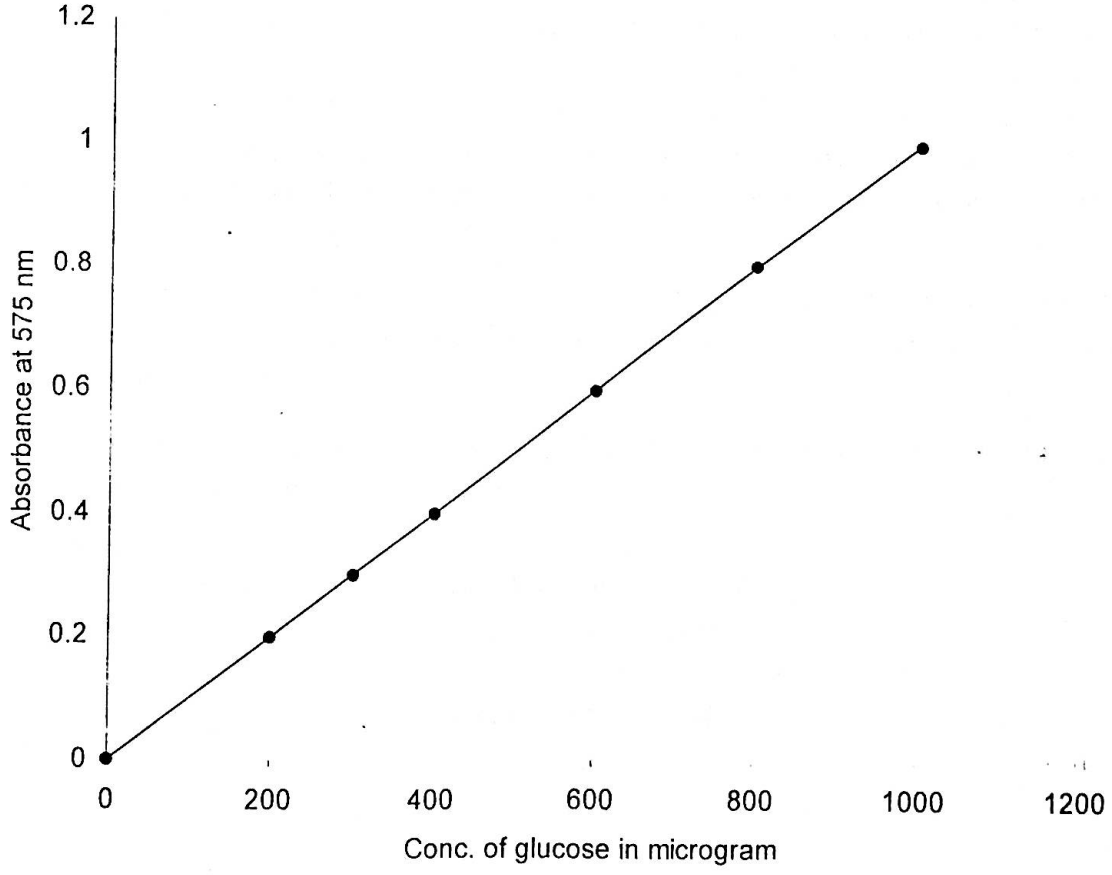


Fig-3: Standard curve of glucose for estimation of reducing sugar.

M.2.7 DETERMINATION OF NON-REDUCING SUGAR (SUCROSE) CONTENT OF BETEL LEAVES

Sucrose content was calculated from the following formula (Ranganna, 1979):

% Sucrose or non reducing sugar = (% Total sugar - % Reducing sugar) × 0.95

M.2.8 STARCH ESTIMATION OF BETEL LEAF

The starch content of the betel leaves was determined by the anthrone method (Morse, 1947 and Loomis & Shull, 1937)

Reagents:

a) Anthrone reagent (0.2% in concentrated H_2SO_4).
b) Standard glucose solution (10mg/100ml)

Procedure:

About five gm of betel leaf were homogenized well with 10ml of distilled water. The content was filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly the starch. After kept it overnight in cold, the precipitate was collected by centrifugation at 3000 rpm for about 15 minutes. The precipitate was dried over a steam bath, then 40ml of 1M hydrochloric acid was added to the dried precipitate and heated to about 70°C. It was then transferred to a volumetric flask and diluted to 100ml with 1M HC1. Diluted solution (1ml) was taken in another 100 ml volumetric flask and made upto 100 ml with 1M HC1.

Aliquots of 1ml of the betel leaf extract from each cultivar were pipetted into test tubes in duplicate and treated in the same manner as described for free sugar estimation, previously.

A standard curve of glucose was prepared and the amount of starch in the betel leaf was calculated from the curve (Fig-2)

Calculation:

Percentage of starch content (gm per 100 gm of betel leaf)

$$= \frac{\text{Amount of starch obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.9 CRUDE FIBRE ESTIMATION OF BETEL LEAF

Crude fibre was determined by the following method (AOAC, 1980).

Reagents:

a) H_2SO_4 (0.26N)
b) NaOH (1.25%)
c) Ethanol
d) Ether.

Procedure:

Three gm of fat free betel leaf were taken into 500ml beaker and 200ml of boiling 0.26N H_2SO_4 was added. The mixture was then boiled for 30 minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker to help smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water till free from acid.

The extract was then transferred into the same beaker and 200ml of boiling 1.25% NaOH was added. After boiling for 30 minutes (keeping the volume constant as before) the mixture was filtered through muslin cloth. The extract was washed with hot water until free from alkali, followed by washing with some ethanol and ether. It was then transferred to a crucible dried overnight at 80°C-100°C and weighed.

The crucible was then heated in a muffle furnace at 600°C for three hours, cooled and weighed again. The difference in the weight represented the weight of crude fibre. The percentage of crude fibre (on dry basis) was calculated from the formula given below.

Calculation:

Crude fibre content (gm per 100 gm of betel leaf)

$$= \frac{\text{Amount of dried extract}}{\text{Weight of betel leaf}} \times 100$$

M.2.10 ESTIMATION OF PHENOLS FROM BETEL LEAVES

Estimation of phenols with Folin-Ciocalteu reagent is based on the reaction between phenols and an oxidizing agent phosphomolybdate, which results in the formation of a blue complex. So, total phenol content of the betel leaves was determined colorimetrically by Folin-Ciocalteu method (Bray and Thorpe, 1954).

Reagents:

a) Folin-Ciocalteu reagent
b) Sodium carbonate, 20%
c) Catechol (0.1mg/ml)

Extraction of phenol from betel leaves:

Extraction of phenols from betel leaves was done following the method as described by Loomis and Shull, (1937).

Four to six gm of betel leaves were cut into small pieces and immediately plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5 to 10ml of alcohol was used per every gm of betel leaf). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the tissue for three minutes in hot 80% alcohol, using 2 to 3ml of alcohol for every gm of betel leaf tissue. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatman no. 41 filter paper. This alcohol extract was used for the estimation of total phenols.

Method:

Aliquot of 1ml of the extract was pipetted into test tubes and 1ml of Folin-Ciocalteu reagent followed by 2 ml of sodium carbonate (Na_2CO_3) solution were added to each tube and mixed well. The test tubes were placed in a boiling water bath for exactly 2 minutes, then removed and cooled. The blue solution was then transferred to a 25ml volumetric flask and made upto the mark with distilled water. Then the solution was filtered. A reagent blank was prepared by taking 1ml of water and 1ml of Folin-Ciocalteu reagent in a test tube and treated similarly. The absorbance of the blue solution was measured at 650nm in a colorimeter. The amount of total phenols was calculated from the standard curve of catechol (Fig-4).

Calculation:

Percentage of total phenol (mg per 100 gm of betel leaves)

$$= \frac{\text{mg of total phenol obtained}}{\text{Weight of betel leaf}} \times 100$$

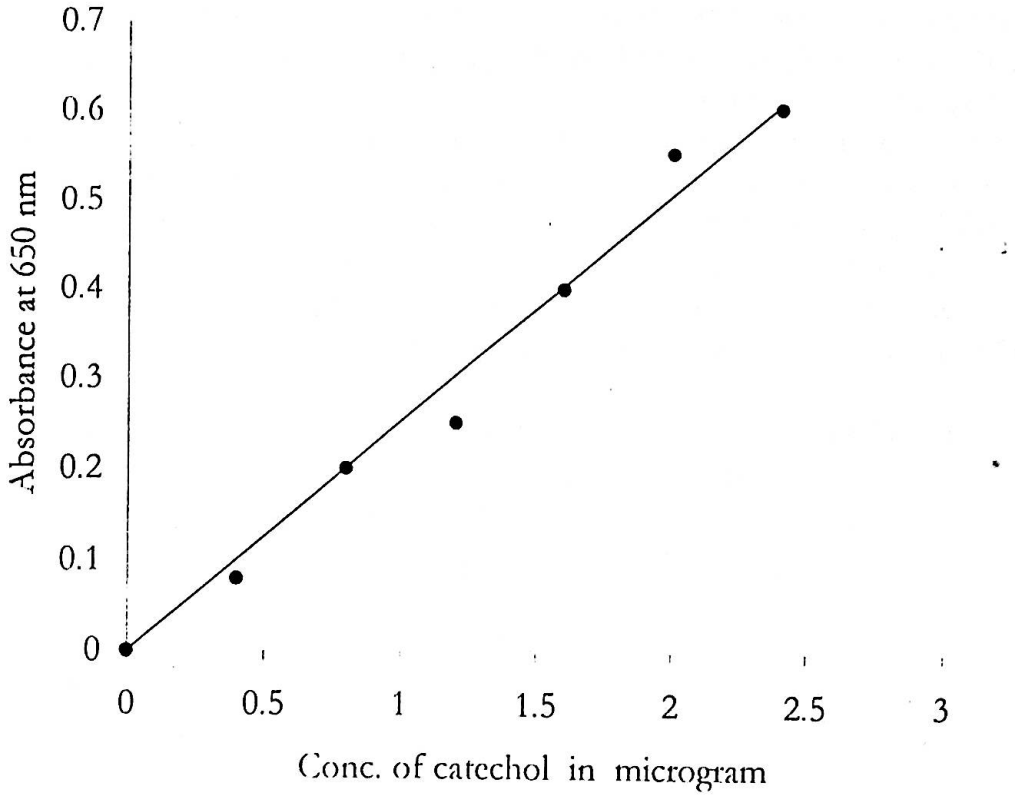


Fig-4: Standard curve of catechol for estimation of total phenol.

M.2.11 DETERMINATION OF PROTEIN CONTENT OF BETEL LEAF

Protein contents of betel leaves were determined by the method of Folin-Lowry (Lowry *et al.* 1951)).

Reagents:

a) Alkaline sodium carbonate solution (20 gm/litre Na_2CO_3 in 0.1M NaOH solution).

b) Freshly prepared copper sulphate and sodium potassium tartrate solution (5 gm/litre $CuSO_4.5H_2O$ in 10 gm/litre Na-K tartrate).

c) Alkaline solution: Mixture of solution a and b in the proportion of 50:1 respectively.

d) Folin-Cicolteau's reagent (Diluted with equal volume of H_2O, just before use).

e) Standard protein (Bovine serum albumin 0.1 mg/ml in dist. H_2O) solution.

Method

From standard protein solution, 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml were taken in different test tubes and made upto the volume 1 ml by distilled water. Then 5 ml of the alkaline solution (solution-c) was added to the protein solution in different test tubes and mixed thoroughly. It was allowed to stand at room temperature for 10 minutes. Then 0.5 ml of diluted Folin-Cicolteau's reagent was added rapidly with immediate mixing and left for 30 minutes. The dark blue color formed was measured at 650 nm against the appropriate blank. By applying the same procedure described above, the absorbance of protein solution was measured and the concentration of protein was determined from the standard curve (Fig-5).

Percentage of protein content of betel leaf (gm per 100 gm of betel leaves)

$$= \frac{\text{Amount of protein obtained}}{\text{Weight of betel leaf}} \times 100$$

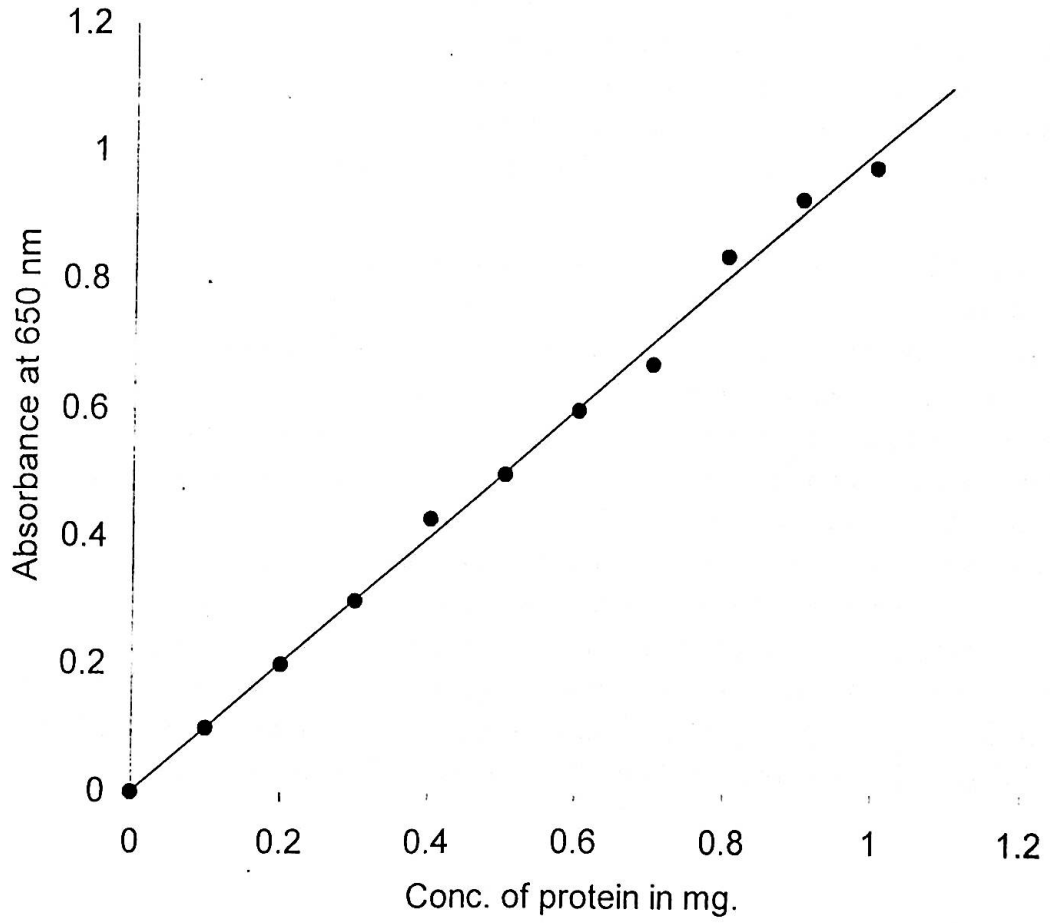


Fig-5: Standard curve of BSA for the determination of protein concentration.

M.2.12 DETERMINATION OF CALCIUM CONTENT OF BETEL LEAF

Calcium content was determined by titrimetric method (Bernard, 1965).

Reagents:

a) Hydrochloric acid (concentrated).
b) Ammonium oxalate (6%).
c) Methyl red indicator
d) Dilute sulphuric acid (2N).
e) Strong ammonia.
f) Potassium permanganate (Jam brand, West Germany) solution (N/100).

Preparation of stock solution:

The ash obtained as described earlier was moistened with a small amount of distilled water (0.5-1.0ml) and then 5ml-concentrated HC1 was added to it.

The mixture was evaporated to dryness on boiling water bath. 5ml of concentrated HC1 were added again and the solution was evaporated to dryness. The residue was dissolved in about 4ml of HC1 and a few ml of water, and the solution was warmed on a boiling water bath. The warmed solution was then filtered using Whatman no. 40 filter paper. After cooling, the volume was made upto 100ml with distilled water and suitable aliquots were used for the estimation of calcium.

Procedure:

25ml of the stock solution was taken in a conical flask and 125ml of double distilled water were added to it. A few drops of methyl indicator were added and the mixture was neutralized with ammonia, till the pink colour changed to yellow. The solution was heated to boiling and 10ml of ammonium oxalate was then added. The mixture was allowed to boil for a few minutes and then glacial acetic acid was added to it till the colour became distinctly pink. The mixture was kept in dark at room temperature for an hour. When the precipitate was settled down, the supernatant was tested with a drop of ammonium oxalate solution to ensure the completion of precipitation. The precipitate was then filtered through Whatman no. 40 filter paper and washed with warm water till the precipitate became free of oxalate (tested with $CaC1_2$). The precipitate was transferred to a beaker by piercing a hole in the filter paper and about 5 to 10ml dil. H_2SO_4 (2N) was poured over it. The solution was then heated to about 70°C and titrated with N/100 $KMnO_4$ solution.

Calculation:

1ml N/100 $KMnO_4$ solution ≡ 0.2004 mg of calcium

Amount of calcium content (mg per 100gm of betel leaf)

$$= \frac{\text{mg of calcium obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.13 DETERMINATION OF PHOSPHORUS CONTENT OF BETEL LEAF

Phosphorus content of betel leaves was determined by method of Vogel (1961).

Preparation of molybdate solution

12.50gm of sodium molybadte was dissolved in 10N H_2SO_4 and diluted to 500ml with 10N H_2SO_4.

Preparation of Hydrazine Sulphate solution

0.75gm of hydrazine sulphate was dissolved in deionized water and diluted to 500ml.

Preparation of standard phoshate solution

Exactly 0.219gm of potassium dihydrogen phosphate was dissolved in deionized water and diluted to one litre.

Then, 1ml solution ≡ 0.05mg phosphous.

Preparation of stock solution:

Same as described previously in case of calcium. Suitable aliquots of stock solution were used for the estimation of phosphorus, potassium, iron, boron, sulphur, manganese and chloride.

Procedure:

One ml betel leaf extract, 2ml of hydrazine sulphate and 5ml of molybdate reagent were taken in a 50ml volumetric flask and made upto the mark with deionized water. The mixture was mixed well. The flask was kept immersed in a boiling water bath for ten minutes, then it was removed and cooled rapidly. The absorbance for each of the solutions was measured at 830nm against reagent blank.

Construction of calibration curve

A calibration curve (Fig-6) was constructed in the usual process by using six standard phosphorus solution containing 1, 2, 3, 4, 5, and 6 ppm of phosphorus. The mg percentage of phosphorus present in each different betel leaf extract was calculated by using the standard curve of phosphorus.

Calculation:

Amount of phosphorus present (mg per 100gm of betel leaf)

$$= \frac{\text{mg of phosphorus obtained}}{\text{Weight of betel leaf}} \times 100$$

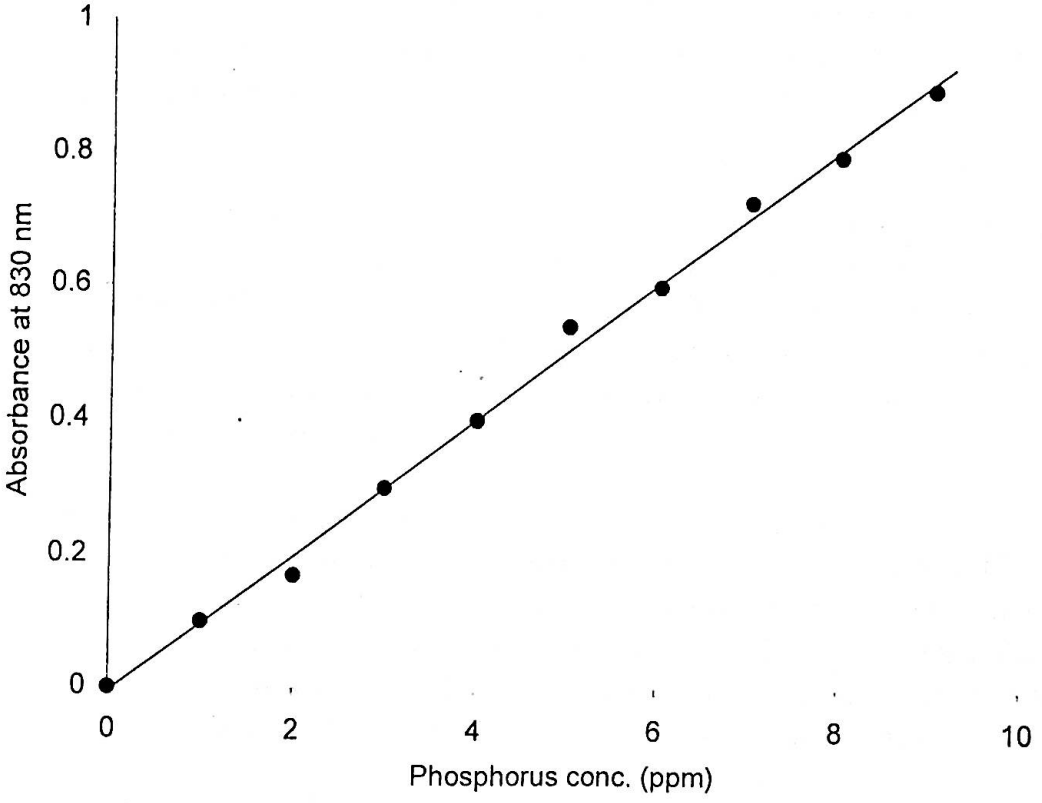


Fig-6: Standard curve for estimation of phosphorus.

M.2.14 DETERMINATION OF IRON CONTENT OF BETEL LEAF

Iron (Fe) content of betel leaf was determined spectrophotometrically by thiocyanate method (Vogel, 1961).

Reagents:

a) 4N HCl

b) Potassium thiocyanate solution (20%)

c) Fe (III) standard solution: Exactly 0.864gm of ammonium iron (III) sulphate was dissolved in distilled water and 10 ml of conc. HCl was added to it and finally made upto one liter. Then, 1ml solution contains ≡ 0.1mg of Fe (III).

Preparation of stock solution

The amount of ash obtained from two gm of betel leaf was taken in a porcelain crucible and 15 ml of conc. HCl was added to it. The resulting solution was evaporated, nearly to dryness to expel excess of acid, diluted slightly with water and made upto 100 ml with distilled water to give the stock solution.

Procedure:

10ml of each of different betel leaf aliquots were taken separately in 50ml volumetric flask; 2 ml of 4N HCl and 5ml of 20% potassium thiocyanate solution were added in each flask. Then the flask was made upto the mark with de-ionized water. The absorbance for each of the solution of the flask was measured at 480nm against a reagent blank. Iron content of this solution was determined by constructing a standard curve.

Construction of standard curve:

A standard curve of iron (Fig-7) was prepared by taking 5ml, 10ml, 15ml, 20ml, 25ml and 30ml of the standard (0.1mg/ml) ammonium iron (III) sulphate solution. To the flask, 2ml of 4N hydrochloric acid and 5ml of 20% potassium thiocyanate solution were added and diluted upto the mark and shaken slowly for uniform mixing. The absorbance for each of the solution was measured at 480nm against a reagent blank.

The mg percent of iron present in each different betel leaf extracts was calculated by using the standard curve.

Calculation:

$$\text{Amount of iron present (mg per 100gm of betel leaf)} = \frac{\text{mg of iron obtained}}{\text{Weight of betel leaf}} \times 100$$

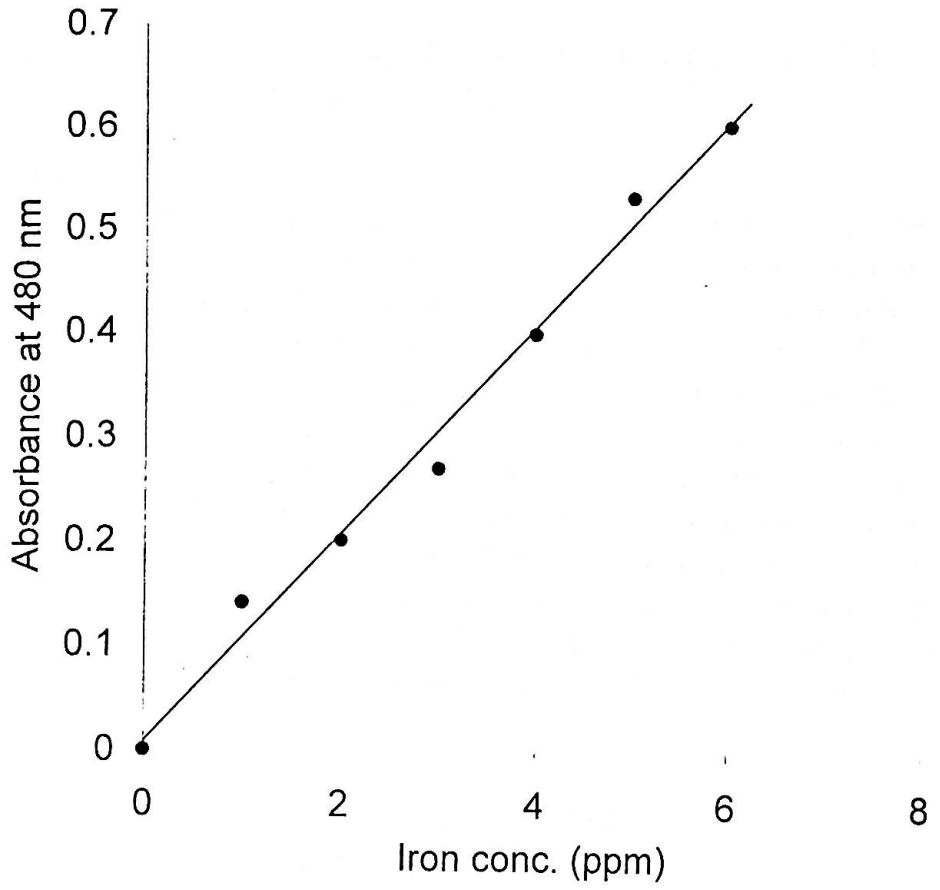


Fig-7: Standard curve for estimation of iron.

M.2.15 DETERMINATION OF β-CAROTENE CONTENT OF BETEL LEAF

β-carotene content of betel leaves were determined according to the procedure reported in the Methods of Vitamin Assay (Anon, 1960) and Methods of Biochemical analysis (Click, 1957)

Reagents:

a) Ammonium sulphate
b) Acetone
c) Petroleum ether (40-60°C)
d) n-Hexane
e) Activated alumina (BDH chemicals Ltd.)
f) Standard solution of β-carotene: A Standard solution of β-carotene (BDH chemicals Ltd.) was prepared by dissolving 50mg of β-carotene in 100ml of petroleum ether.

Column preparation:

A column was prepared by using alumina as a packing material. 10% acetone in petroleum ether was used as an eluant buffer.

Procedure:

Five gm of fresh betel leaf and about four gm of ammonium sulphate were taken in a mortar, and rubbed to an even paste with pestle. The extraction was carried out with acetone and small amount of hexane. Extraction was continued until the acetone extract became colorless. Potassium hydroxide solution (10ml, 5.6%) was added to the extract and it was kept in a dark place for half an hour. The mixture was then transferred to a separating funnel, then 20ml of petroleum ether, a few ml of hexane and 10ml of water were added to the separating funnel and shaked gently. The ether layer was collected and the process was repeated until the petroleum ether layer became colorless. The petroleum ether extract was concentrated by gentle heating. The concentrated extract (1-2ml) was applied on to the top of the alumina column and eluted with 10% acetone in petroleum ether. The absorbance of the eluant was taken at 440nm in a Coleman Junior II spectrophotometer.

Construction of standard curve of β-carotene:

A standard curve (Fig-8) was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0ml standard solution of β-carotene and the volume was made upto 5ml with petroleum ether and mixed well. The absorbance of the solutions was taken at 440nm in a Coleman Junior II spectrophotometer and a standard curve of β-carotene was prepared by plotting the data.

The amount of β-carotene content in each cultivator of betel leaf was calculated by using the standard curve.

Calculation:

mg percent of β-carotene (mg per 100gm of betel leaf)

$$= \frac{\text{mg of β-carotene obtained}}{\text{Weight of betel leaf}} \times 100$$

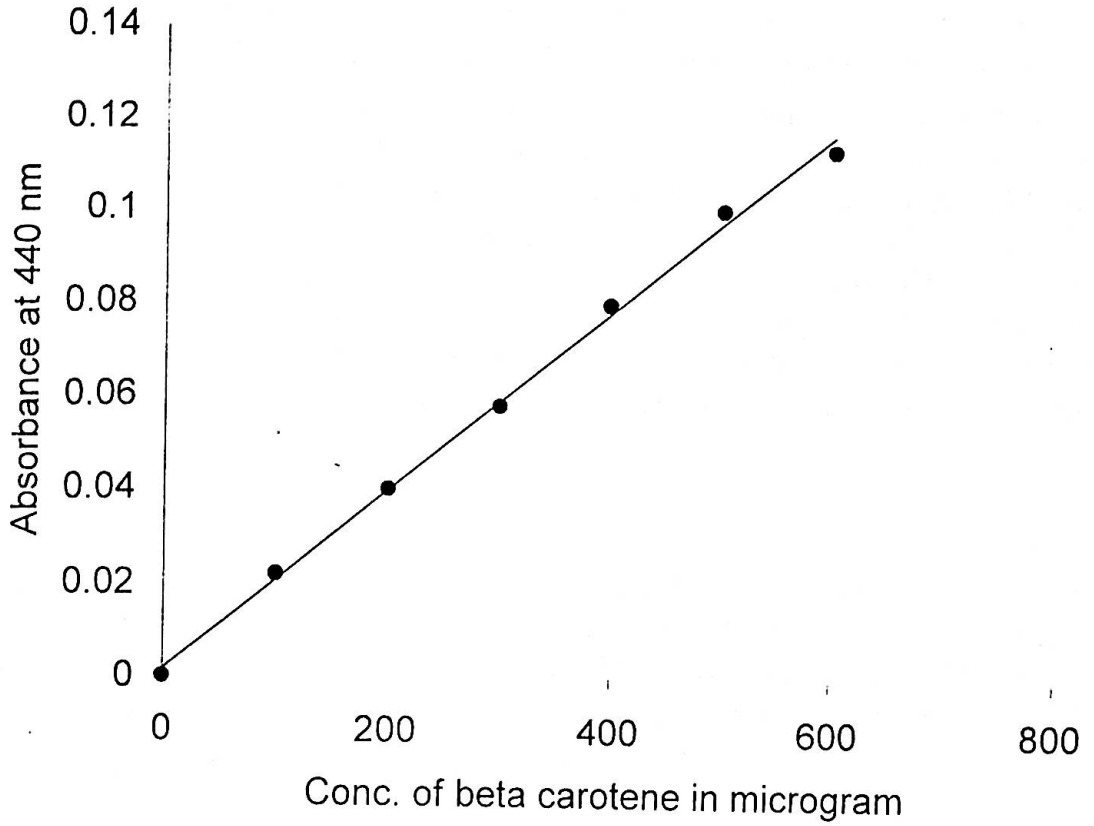


Fig-8: Standard curve for estimation of β-carotene.

M.2.16 DETERMINATION OF VITAMIN-B_1 CONTENT OF BETEL LEAF

Vitamin-B_1 content of betel leaf was determined following the method of Anon (1965).

Reagents:

a) Potassium ferricyanide (2%)
b) Oxidizing reagents : 10ml of 2% potassium ferricyanide was mixed with 3.5N NaOH solution (90ml). The solution might be used within 4 hours.
c) Quinine sulphate
d) 0.2N HC1
e) Alcohol

Standard thiamine hydrochloride preparation:

About 25mg of thiamine hydrochloride was transferred in 100ml volumetric flask and it was dissolved in 30ml of dilute alcohol solution and made upto the mark. The pH was adjusted to 4.0 with dilute HC1 and stored in a light resistant container.

Procedure:

Five to six gm of fresh betel leaves were cut into small pieces and homogenized with 0.2N HCl. The mixture was heated on a steam bath and then cooled.

5ml of standard thiamine-HCl solution was mixed rapidly with 3ml oxidizing reagent and 20ml of isobutyl alcohol was added within 30 seconds, then mixed the mixture vigorously for 90 seconds by shaking the tubes manually. A blank was prepared only by substituting the oxidizing reagent with an equal volume of 3.5N sodium hydroxide and proceed in the same manner. 2ml of dehydrate alcohol was added, swirl for few seconds, allowed the phase to be separated and decanted or drawn off and transferred into cuvettes, then measured the fluorescence. Betel leaf extracts (5ml) were pipetted in different test tubes and treated in the same manner as described above.

Calculation:

The amount (in milligram) of thiamine in each 5ml of the betel leaf extract was calculated from the formula (A-b) / (S-d), in which A and S were the average fluorometer reading of the portions of betel leaf extract and standard preparation with oxidizing reagent, respectively, and b and d were the readings for the blanks of betel leaves extract and standard preparation, respectively.

$$\text{mg percentage of vitamin-B}_1\ (\text{mg per 100gm of fresh leaf}) = \frac{\text{mg of vitamin-B}_1\text{ obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.17 DETERMINATION OF VITAMIN-B_2 CONTENT OF BETEL LEAF

Vitamin B_2 content of betel leaves was determined by the method of Anon (1965).

Reagents:

a) 0.02N acetic acid
b) 0.1N H_2SO_4
c) 0.1N NaOH
d) 0. 1N HCl
e) 4% Potassium permanganate
f) Hydrogen peroxide

Procedure:

Standard Preparation: 50mg of riboflavin was mixed with 300ml of 0.02N acetic acid and the mixture was heated on a steam bath, with frequent agitation until the riboflavin was dissolved. Then cooled and made upto 500ml with 0.02N acetic acid. This solution was diluted appropriately with 0.02N acetic acid to made final riboflavin concentration of 10 µg/ml.

Extraction of riboflavin from betel leaves:

Fresh betel leaves (5-6 gm) were cut into small pieces and homogenized well with 0.1N H_2SO_4 (about 50ml). The mixture was heated in an autoclave at 121-123°C for 30 minutes, then cooled it and filtered through double layer of muslin cloth. The filtrate was made upto 100ml with distilled water and 25ml of this solution was taken in a beaker and 25ml of water was added to it. The mixture was agitated vigorously and adjusted the pH to 6.0 - 6.5 with 0.1N NaOH. Immediately, 0.1N HCl was added until no precipitation occurs. The extract was again filtered and pH of the extract was adjusted to 6.6 - 6.8 with 0.1N NaOH.

10ml of betel leaf extract was taken in the test tube and 1.0 ml of water, 1.0ml of glacial acetic acid were added to it. The mixture was then mixed with 0.05ml of potassium permanganate solution and allowed to stand for two minutes, then 0.5ml of hydrogen peroxide solution was added , where upon the permanganate color was destroyed within 10 seconds. The tube was shaken vigorously until excess oxygen expelled. Then 1ml of standard solution was pipetted in a test tube and treated in the same manner as that described for the leaf extract. In a suitable fluorometer, the fluorescence of the solution was measured. Then to each tubes, 20mg of sodium hydrosulphite was added, mixed well and measured the fluorescence, within 5 seconds.

Calculation:

The quantity in mg in each ml of the betel leaf extract was calculated by the formula, $0.0001\ (I_u - I_B)\ (I_S - I_u)$

Where,

I_u = Average reading for betel leaf extract

I_S = Average reading for standard preparation

I_B = Average reading for mixed with sodium hydrosulphite

mg percentage of vitamin-B_2 content of betel leaf (mg per 100gm of betel leaf)

$$= \frac{\text{mg of vitamin-B}_2\text{ obtained}}{\text{Weight of betel leaf}} \times 100$$

M.2.18 DETERMINATION OF VITAMIN-C CONTENT OF BETEL LEAF

Vitamin-C content of betel leaf was determined by the Bessey's titrimetric method (1933).

Regents:

a) Dye solution: 200mg of 2,6 - dichlorophenol indophenol and 210mg of sodium bicarbonate were dissolved in distilled water and made upto 1000 ml. The solution was filtered.

b) 3% metaphosphoric acid reagent: 3 gm of metaphosphoric acid was dissolved in 80ml of acetic acid and made up to 100ml with distilled water.

c) Standard Vitamin-C solution (0.1mg/ml): 10mg of pure vitamin-C was dissolved in 3% metaphosphoric acid and made upto 100ml with 3% metaphosphoric acid.

Method:

Standard vitamin-C solution was taken in a conical flask and titrated it with the dye solution.

Four to six gm of betel leaves were cut into small pieces and homogenized well with 3% metaphosphoric acid (approximately 20ml) and filtered it through double layer of muslin cloth. The filtrate was centrifuged at 3,000 r.p.m. for 10 minutes and the clear supernatant was titrated with 2,6 dichlorophenol indophenol solution. The amount of vitamin-C present in the extract was determined by comparing with the titration result of standard vitamin-C solution.

Calculation:

Percentage of vitamin-C content (mg per 100 gm of betel leaf)

$$= \frac{\text{mg of vitamin-C obtained}}{\text{Weight of betel leaf}} \times 100$$

RESULTS AND DISCUSSIONS

R.2.1 Moisture content of betel leaf:

Moisture plays an important part in the growth activities of plants, herbs etc. Water is indispensable to the absorption and transportation of food to carry on photosynthesis, metabolism of materials and the regulation of temperature. Moisture is also essential for most of the physiological reactions in plant tissues and in its absence, life does not exist (Rangaswami, 1976).

Moisture contents of four different varieties of betel leaves in three different maturity stages are given in Table-2.

It is found that moisture content is decreased with the changes in the maturity of betel leaf. Among the maturity stages, premature betel leaf contains the highest amount of moisture and over mature betel leaf contains the lowest. The moisture content is varied between 86.87% to 87.99% in premature stage, 85.33% to 85.94% in mature stage and 82.41% to 83.22% in over mature stage. In mature stage, Doga variety contains the highest amount of moisture (85.94%) while Shail variety contains the lowest amount of moisture (85.33%).

Table-2: Moisture and ash contents of betel leaf.

Varieties	Stages	Moisture (gm%)	Ash (gm%)
Shail Pan	Premature Stage	87.95 ± 0.01	2.98 ± 0.02
	Mature Stage	85.33 ± 0.03	3.22 ± 0.04
	Over Mature Stage	82.41 ± 0.01	3.27 ± 0.03
Doga Pan	Premature Stage	86.87 ±0.02	3.08 ± 0.01
	Mature Stage	85.94 ± 0.02	3.24 ± 0.02
	Over Mature Stage	83.22 ± 0.03	3.29 ± 0.01
Dudhswar Pan	Premature Stage	87.99 ± 0.01	2.97 ± 0.01
	Mature Stage	85.40 ± 0.04	3.25 ± 0.03
	Over Mature Stage	82.45 ± 0.01	3.31 ± 0.02
Kal Bangla Pan	Premature Stage	87.70 ± 0.02	2.96 ± 0.02
	Mature Stage	85.44 ± 0.03	3.26 ± 0.04
	Over Mature Stage	82.62 ± 0.01	3.28 ± 0.01

R.2.2 Ash content of betel leaves:

Most of the inorganic constituents or minerals are present in ash. Ash contents of four varieties of betel leaves in three different maturity stages are represented in the Table-2.

The result clearly indicated that the order of ash content is just opposite to the moisture content of betel leaf. Ash content increased proportionally to the maturity of betel leaf. Among the stages, premature betel leaf contains the lowest amount of ash and over mature betel leaf contains the highest amount in all varieties. Among the four varieties, the ash content is varied between 2.96% to 3.08% in premature stage, 3.22% to 3.26% in mature stage and 3.27% to 3.31% in over mature stage. In mature stage, Kal Bangla variety contains the highest amount of ash (3.26%) and Shail variety contains the lowest amount of ash (3.22%).

R.2.3 Crude fibre content of leaves

Cellulose, lignin and pentosan are the components of crude fibre of betel leaves (Anon, 1975). In betel leaves, cellulose is present in cell wall, stimulate the swallowing factor (Ravindra, 1987). Crude fibre has pronounced effect on the digestion and absorption process of nutrients.

Crude fibre contents of four varieties of betel leaves are given in the Table-3. The present data indicated that betel leaves contained about 2% crude fibre. Crude fibre content is lowest in premature stage and the content increased with increasing maturity of betel leaves. In mature stage, crude fibre content ranges from 2.095% to 2.11% and Doga variety contained the highest amount of fibre.

R.2.4 Phenol content of betel leaves

Varieties differ in their response with regard to phenolic changes. Phenolic compounds enjoy a distribution in the plant kingdom and they are particularly prominent in plants where they are important in determining color and flavor (Buren, 1970).

Phenol contents of four varieties of betel leaves in different maturity stages are given in Table-3. Maximum phenol contents were found in mature stage in all the varieties whereas premature stage contained the lowest phenol content. In this study, Dudhswar variety contained the highest amount of phenol (0.704%) and Kal Bamgla variety contained the lowest amount (0.547%) in mature stage.

Table-3: Crude Fibre and phenol contents of betel leaf.

Varieties	Stages	Fibre (gm%)	Phenol (gm%)
Shail Pan	Premature Stage	1.86 ± 0.07	0.522 ± 0.05
	Mature Stage	2.10 ± 0.10	0.576 ± 0.03
	Over Mature Stage	2.12 ± 0.09	0.526 ± 0.01
Doga Pan	Premature Stage	1.93 ± 0.05	0.536 ± 0.05
	Mature Stage	2.11 ± 0.08	0.587 ± 0.02
	Over Mature Stage	2.13 ± 0.05	0.549 ± 0.02
Dudhswar Pan	Premature Stage	1.85 ± 0.09	0.672 ± 0.04
	Mature Stage	2.105 ± 0.11	0.704 ± 0.03
	Over Mature Stage	2.125 ± 0.08	0.683 ± 0.01
Kal Bangla Pan	Premature Stage	1.848 ± 0.06	0.492 ± 0.04
	Mature Stage	2.095 ± 0.09	0.547 ± 0.04
	Over Mature Stage	2.115 ± 0.08	0.521 ± 0.02

R.2.5 Chlorophyll contents of betel leaves

Chlorophyll is the green pigment universally present in all photosynthetic tissues. This pigment is essential for the utilization of light energy to prepare mostly carbohydrate. The maturity is a factor that causes differentiation in chlorophyll contents of leaves. Chlorophyll estimation may also be required to relate other biochemical changes in the plant tissue (Mahadevan and Sridhar, 1982).

Chlorophyll contents of four varieties of betel leaves at different maturity stages are given below (Table-4). In all the varieties, chlorophyll contents were maximum in mature stage suggesting that carbohydrate production in this stage is greater than other stages. Chlorophyll content at over mature stage was less than that of mature stage but the content at over mature stage was very nearer to the mature stage whereas premature stage contained very lower amount of chlorophyll than that of mature stage. It was found that chlorophyll-a content was found to be higher in betel leaves as compared to that of chlorophyll- b. It was concluded from the data that Doga variety contained highest amount of total chlorophyll (320.76 mg%) as well as chlorophyll-a (195.50 mg%) and chlorophyll-b (125.26 mg%).

Table-4: Chlorophyll content of betel leaf:

Varieties	Stages	Total Chlorophyll (mg%)	Chlorophyll-a (mg%)	Chlorophyll-b (mg%)
Shail Pan	Premature Stage	153.40 ± 0.03	101.55 ± 0.03	51.85 ± 0.02
	Mature Stage	273.25 ± 0.02	186.20 ± 0.05	87.05 ± 0.01
	Over Mature	262.35 ± 0.04	180.93 ± 0.04	81.42 ± 0.02
Doga Pan	Premature Stage	186.16 ± 0.02	117.44 ± 0.04	68.72 ± 0.04
	Mature Stage	320.76 ± 0.03	195.50 ± 0.02	125.26 ± 0.02
	Over Mature	301.84 ± 0.01	190.35 ± 0.02	111.49 ± 0.03
Dudhswar Pan	Premature Stage	146.47 ± 0.01	92.65 ± 0.02	53.82 ± 0.03
	Mature Stage	255.65 ± 0.02	174.45 ± 0.03	81.20 ± 0.02
	Over Mature	239.94 ± 0.01	165.27 ± 0.01	74.67 ± 0.01
Kal Bangla Pan	Premature Stage	143.24 ± 0.02	88.86 ± 0.03	54.38 ± 0.02
	Mature Stage	295.46 ± 0.03	178.82 ± 0.01	116.64 ± 0.01
	Over Mature	273.19 ± 0.01	174.96 ±0.02	98.23 ± 0.02

R.2.6 Protein content of betel leaves:

Protein plays an important role in all the biological processes. Protein content of plants and herbs, although occurring in low concentrations, are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must be the full complement of enzymes involved in metabolism during growth, development, maturation and the post harvest of the plant (Hansen, 1970).

Protein content of betel leaf showed to increase gradually with the increased in maturity. Premature stage contained the lowest amount where as over mature stage contained the highest amount of protein. In all the stages, Doga variety contained the maximum amount of protein among the four varieties. The protein content of Doga variety was 3.34% in premature stage, 4.92 % in mature stage and 5.01% in over mature stage.

R.2.7 Lipid content of betel leaves:

Lipid serves as efficient source of energy and insulating material. Dietary fat helps in the absorption of fat-soluble vitamins. Lipo-proteins are important cellular constituents. Phospholipids also proved to be effective for growth improvement (Ito, 1972). Lipids are essential components of cell membrane and source of metabolic energy for cell maintenance (Patton *et al.*, 1941). Lipid contents of the four varieties of betel leaves are given in the Table-5.

The present data indicates that over mature leaves contained maximum lipid while premature leaves contained minimum amount of lipid. Among the varieties

examined, Kal Bangla variety contained the highest and Dudhswar variety contained the lowest amount of lipid. In over mature stage, Kal Bangla variety contained 1.67% lipid and Dudhswar variety contained 1.03% lipid.

Anonymous (1975) reported that crude fat increased in accordance with age of leaves, owing to the increase of the chlorophyll.

Table-5: Protein and Lipid Content of Betel Leaf.

Varieties	Stages	Amount in gm%	
		Protein	Lipid
Shail Pan	Premature	3.29 ± 0.16	0.31 ± 0.08
	Mature Stage	4.27 ± 0.18	1.525 ± 0.12
	Over Mature	4.58 ± 0.17	1.55 ± 0.10
Doga Pan	Premature	3.34 ± 0.14	0.24 ± 0.07
	Mature Stage	4.92 ± 0.16	1.43 ± 0.10
	Over Mature	5.01 ± 0.15	1.446 ± 0.11
Dudhswar Pan	Premature	2.87 ± 0.15	0.175 ± 0.09
	Mature Stage	3.86 ± 0.14	0.98 ± 0.11
	Over Mature	3.91 ± 0.15	1.03 ± 0.10
Kal Bangla Pan	Premature	3.17 ± 0.13	0.37 ± 0.06
	Mature Stage	3.74 ± 0.15	1.63 ± 0.09
	Over Mature	4.81 ± 0.16	1.67 ± 0.08

R.2.8 Total sugar content of betel leaves

The total sugar percentage reflects the physiological activity of the plant, whereas its total weight per leaf represents the accumulated results of the metabolic activity (Hassanein *et. al.*, 1962). Protein or other elements of betel leaves are also synthesized from carbohydrate (Anon, 1975).

Total sugar contents of four varieties of betel leaves are given in the Table-6. Among the four varieties, Doga variety contained highest amount of total sugar and Dudhswar variety contained the lowest amount in all the stages of the leaves. It was also found that premature leaf contained minimum amount of total sugar and sugar content increased with the maturity. In premature stage, Doga variety contained 1.43% of total sugar whereas it contained 3.58% and 3.61% of total sugar respectively in mature and over mature stage. In over mature stage, total sugar contents were ranged from 3.25% and 3.61%.

The present results demonstrated that total sugar contents of the different varieties of betel leaves increased significantly upto mature stage.

R.2.9 Reducing sugar content of betel leaves

Reducing sugar present in all varieties of betel leaves at all maturity stages are shown in Table-6. No detectable amount of reducing sugar was found in premature stage in all the cultivars of betel leaf but increased thereafter upto over mature stage. In mature stage, reducing sugar content was highest in Kal Bangla variety (0.576 gm%) and lowest in Dudhswar variety (0.497 gm%) while in over mature stage, maximum reducing sugar was found in Kal bangla variety (0.583 gm%) followed by Shail variety (0.555 gm%), Doga variety (0.534 gm%) and Dudhswar variety (0.523 gm%).

Table-6: Total Sugar, Reducing Sugar, Non-Reducing Sugar and Starch contents of Betel Leaf

Varieties	Stages	Amount in gm%			
		Total Sugar	Reducing Sugar	Non-Reducing Sugar/Sucrose	Starch
Shail Pan	Premature	1.31 ± 0.01	00 ± 0.02	1.245 ± 0.02	6.17 ± 0.03
	Mature Stage	3.27 ± 0.03	0.545 ± 0.05	2.589 ± 0.05	6.92 ± 0.04
	Over Mature	3.33 ± 0.02	0.555 ± 0.04	2.636 ± 0.03	6.74 ± 0.02
Doga Pan	Premature	1.43 ± 0.02	00 ± 0.01	1.359 ± 0.01	4.98 ± 0.02
	Mature Stage	3.58 ± 0.05	0.527 ± 0.03	2.900 ± 0.04	5.60 ± 0.03
	Over Mature	3.61 ± 0.01	0.534 ± 0.01	2.922 ± 0.03	5.36 ± 0.01
Dudhswar Pan	Premature	1.29 ± 0.02	00 ± 0.03	1.226 ± 0.02	5.69 ± 0.01
	Mature Stage	3.22 ± 0.04	0.497 ± 0.04	2.587 ± 0.01	6.48 ± 0.04
	Over Mature	3.25 ± 0.03	0.523 ± 0.02	2.591 ± 0.02	6.21 ± 0.02
Kal Bangla Pan	Premature	1.38 ± 0.02	00 ± 0.02	1.311 ± 0.03	6.28 ± 0.03
	Mature Stage	3.46 ± 0.05	0.576 ± 0.03	2.740 ± 0.04	7.05 ± 0.05
	Over Mature	3.50 ± 0.04	0.583 ± 0.01	2.771 ± 0.01	6.83 ± 0.01

R.2.10 Non-reducing sugar (Sucrose) content of betel leaves

Sucrose is an early product of photosynthetic reaction and is the most abundant transport form of sugar and is the major free sugar in many fruits. Sucrose serves as an important reserve carbohydrate in plants, especially in such storage organs as tuber, fruit and seed. During germination, sucrose is readily degradable source of energy.

Non-reducing sugar (Sucrose) contents of different varieties of betel leaves are presented in the Table-6. It was found that Doga variety contained the highest amount of sucrose (2.922 gm%) while Dudhswar variety contained the lowest amount (2.591 gm%). Non-reducing sugar content in betel leaves varied between 1.226-1.359 gm% in premature stage, 2.587-2.9 gm% in mature stage and 2.591-2.922 gm% in over mature stage. From the experimental results, it was observed that non-reducing sugar content of the different varieties of betel leaves increased significantly upto mature stage.

R.2.11 Starch content of betel leaves

Starch, the principal storage carbohydrate of chlorophyll containing plants, is made up of amylose and amylopectin. Starch contents of four varieties of betel leaves at different maturity stages are given in the Table-6.

Starch contents of different varieties of betel leaves were found to be varied between 4.98-6.28 gm% in premature stage, 5.60-7.05 gm% in mature stage and 5.36-6.83 gm% in over mature stage. Doga variety contained the highest and Kal Bangla variety contained the lowest amount of starch in all the stages.

R.2.12 Vitamin-C (ascorbic acid) content of betel leaves

Ascorbic acid plays an important role in the metabolism of plants. It occurs as L-ascorbic acid and in its oxidized form, dehydroascorbic acid in nearly all the plants. Ascorbic acid reduced quinones to phenols and this reaction has received much attention in defense mechanism.

Ascorbic acid contents of four varieties of betel leaves at mature stage are given in the Table-7. Of the four varieties examined, the Kal Bangla variety contained the maximum amount of vitamin-C (341 mg%) whereas Shail, Doga and Dudhswar varieties contained 327 mg%, 308 mg% and 297 mg% of ascorbic acid respectively.

R.2.13 β-Carotene content of betel leaves

β-carotenes are precursors of vitamin-A. Animal cannot synthesize it but can convert it to vitamin-A. In plants, it is very necessary for growth and development of soft tissue through its effect upon protein synthesis. Vitamin-A also plays a role in the maintenance of normal epithelial structure.

β-Carotene contents of four varieties of betel leaves in mature stage are given in the Table-7. Among the four varities, β-carotene content was highest in Doga variety (131 mg%) and lowest in Dudhswar variety (120 mg%).

Table-7: Vitamins Content of Betel Leaf at Mature Stage

Varieties	Amount in mg% of			
	Vitamin-C	β-Carotene	Vitamin-B_1	Vitamin-B_2
Shail Pan	327 ± 0.04	128 ± 0.01	34.40 ± 0.04	0.71 ± 0.01
Doga Pan	308 ± 0.06	131 ± 0.03	36.02 ± 0.02	0.78 ± 0.02
Dudhswar Pan	297 ± 0.07	120 ± 0.02	32.24 ± 0.03	0.63 ± 0.04
Kal Bangla Pan	341 ± 0.05	122 ± 0.04	35.16 ± 0.02	0.74 ± 0.03

R.2.14 Vitamin-B_1 content of betel leaves

Thiamine exixts in tissues mostly in the form of thiamine pyrophosphate (TPP) known as co-carboxylase. TPP serves as co-enzyme in the metabolism of carbohydrate, fat and protein. Beside the metabolic role, thiamine has a specific role in neurophysiology, dependent of its co-enzyme function. In plants and herbs, it is very essential. Thiamine is also essential for growth, normal appetite, digestion and healthy nerves.

Vitamin-B_1 contents of four varieties of betel leaves are given in the Table-7. As found in the present study betel leaves are good sources of vitamin-B_1. Of the four varieties examined, Doga variety contained highest amount of vitamin-B_1 (36.02 mg%) and Dudhswar variety contained the lowest amount (32.24 mg%).

R.2.15 Vitamin-B_2 content of betel leaves

Riboflavin combines in the tissue phosphoric acid to become part of the structure of two flavin co-enzymes, FMN and FAD, acts on the activity of the enzymes. It is essential for normal growth and tissue maintenance.

Vitamin-B_2 contents of four varieties of betel leaves in mature stage are given in the Table-7. It can be suggested from the present finding that betel leaves are not good sources of Vitamin-B_2, and its content was found to be varied between 0.63 mg% to 0.78 mg%.

R.2.16 Calcium content of betel leaves

In betel leaves, calcium is a constituent of cell walls, being needed in large amount for cell division of the growing part of the plant. In deficiency of calcium, plant root tend to be short and stubby, as a result leading to the death of their tissues (Rangaswami, 1976). Calcium is also required for many essential enzyme activities in leaves.

Calcium contents of four varieties of betel leaves are given in the Table-8. From the experiments it can be assumed that betel leaves are quite a good source of calcium. Dudhswar variety contained the maximum amount of calcium (231 mg%) followed by Kal Bangla variety (227 mg%), Doga variety (225%) and Shail variety (216 mg%).

R.2.17 Phosphorus content of betel leaves

Phosphorus is one of the major nutrients for betel leaf growth. It is the structural constituent of nucleotide (ATP), which is an energy carrier for all the metabolic activities. It is also essential for constituent of cell nucleus, cell division and

development of meristematic tissues in the growing regions of leaves (A.F. Chandrnath *et al.*, 1991).

Phosphorus contents of four varieties of betel leaves are given in the Table-8. The phosphorus content varies between 145 to 157 mg%. Of the four varieties, Kal Bangla variety contained the highest (157 mg%) and Dudhswar variety contained the lowest (145 mg%) amount of phosphorus.

R.2.18 Iron contents of betel leaves

Iron plays important role in maintaining cell permeability, in cell division and in metabolic activities of the leaves. All the four varieties contained almost the same amount of iron. As given in the Table-8, the amount of iron in different varieties of betel leaves were found to be varied between 9.15 mg% to 9.78 mg%. According to the results, betel leaves may be considered as good source of iron.

Table-8: Minerals Content of Betel Leaf at Mature Stage

Varieties	Amount in mg% of		
	Calcium	Iron	Phosphorus
Shail Pan	216 ± 0.01	9.78 ± 1.05	150 ± 0.02
Doga Pan	225 ± 0.04	9.26 ± 1.08	153 ± 0.02
Dudhswar Pan	231 ±0.03	9.64 ± 1.06	145 ± 0.03
Kal Bangla Pan	227 ± 0.02	9.15 ± 1.03	157 ± 0.04

CHAPTER THREE

ENZYMATIC ACTIVITIES OF DIFFERENT VARIETIES OF BETEL LEAVES AT DIFFERENT MATURE STAGES

INTRODUCTION

Betel leaf is one of the most important and widely cultivated herb in Bangladesh. It plays a vital role in providing a substantial quantity of vitamin C and other nutrients. There are many varieties of betel leaves in our country but their nutritive values are not known clearly. It was found that the nutritional quality of betel leaf extract is greatly affected with the changes of maturity. Enzymes present in the betel leaf may play some role on the nutrient changes in betel leaf at different maturity stages. Proteolytic and hydrolytic enzymes may play important physiological and biological roles during maturation and senescence of fruits (Hasinaga *et. al.*, 1983; Desai and Despande, 1978; Mahadevan, 1970), and also found that proteolytic enzymes play important role in disease development. P.K. Kharanath (1996) found that hydrolytic and proteolytic enzymes have great physiological roles during maturation and senescence of betel leaves. Dilley (1970) suggested that the dramatic physical and chemical changes attending ripening occur as a result of catabolic and anabolic processes.

In this study, the activities of invertase, cellulase, amylase, catalase, peroxidase, polyphenoloxidase, protease and β - galactosidase were measured and correlated with the content of nutrients such as total sugar, reducing sugar, non-reducing sugar (sucrose), and starch in different maturity stages of four varieties of betel leaves.

MATERIALS AND METHODS

M.3.1 Preparation of crude enzyme extract:

At first 8-10 gm of betel leaves were cut into small pieces and grinded in a mortar with pestle, and then homogenized well with cold buffer of respective pHs (for amylase, invertase and cellulase: 0.1M sodium acetate-acetic acid buffer, pH6, pH5 and pH5.2 respectively; for protease: 0.1M phosphate buffer, pH 7.0; for polyphenoloxidase and peroxidase: 0.1M phosphate buffer, pH 6.0; for catalase: 0.1M phosphate buffer, pH 6.4; and for β-galactosidase: 0.1M sodium-citrate buffer of pH 4.1). The homogenate was filtered through a double layer of muslin cloth and further clarified by centrifugation at 6000 rpm. for 15 minutes at 4^0C. The supernatant was then collected and used as crude enzyme extract.

M.3.1.1 Measurement of amylase activity:

Amylase activity was assayed following the method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). One-percent of starch solution was used as substrate (1gm in 100ml of 0.1M acetic acid-sodium acetate buffer, pH 6). The amylase activity was measured by estimating the amount of maltose released by it. The amount of maltose released was calculated from the standard curve (Fig-9), prepared with maltose. One unit of amylase activity was defined as the amount required for liberating 1µg of maltose from starch per minute at 37°C.

Reagents:

a) 0.1M acetic acid-sodium acetate buffer, pH 6
b) 1% starch solution in 0.1M acetic acid-sodium acetate buffer, pH 6
c) 1% NaOH solution
d) Crystalline phenol
e) Sodium sulphite
f) Dinitrosalicyclic acid (DNS)
g) 40% sodium potassium tartrate

Preparation of DNS reagent:

Simultaneously, 1gm of DNS powder, 200mg of crystalline phenol and 50mg of sodium sulphite were placed in a beaker and mixed with 100ml of 1% NaOH solution by stirring. Sodium sulphite was always added just prior to use.

Procedure:

Three sets of experiments (Blank, control and sample) were performed for the measurement of amylase activity. 5 ml of crude enzyme extract was taken in a 100 ml flask, 10 ml of 0.1M acetic acid-sodium acetate buffer, pH 6 and 5 ml of 1% soluble starch solution were added to it. The mixture was incubated at 37°C for 15 minutes. Aliquot of 3 ml of the extract was pipetted into a test tube and 3 ml of DNS reagent was added to it. The mixture was heated for 5 minutes in boiling water bath. After the color had developed, 1 ml of 40% sodium potassium tartrate solution was added to the warm tubes. A control tube was also prepared by adding heat-denatured enzyme. The absorbance of solution was measured at 520 nm and the amount of maltose was calculated from standard curve of maltose (Fig. 9).

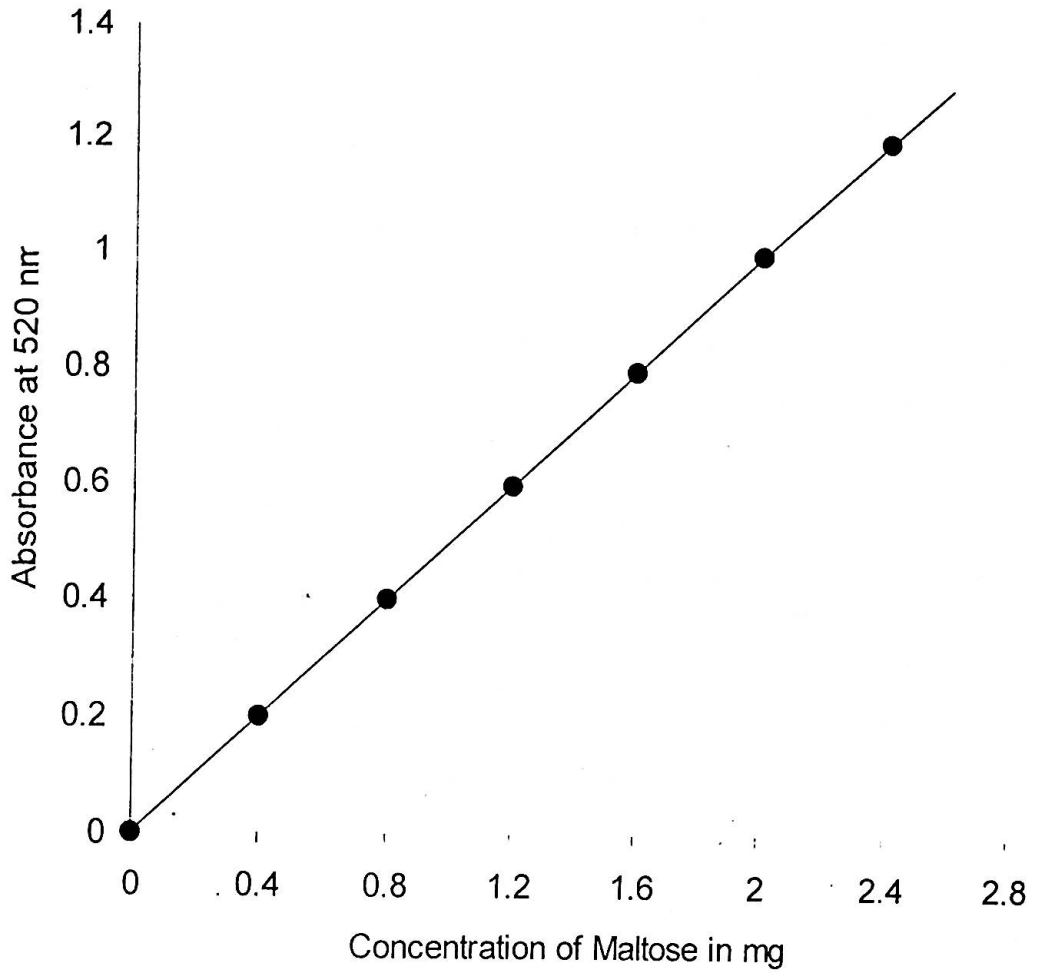


Fig. 9: Standard curve of maltose for the determination of amylase activity.

M.3.1.2 Measurement of invertase activity:

Invertase activity was assayed following the method of Physiological Plant Pathology (Mahadevan and Sridhar, 1982). 2.5% sucrose solution was used as a substrate. The invertase activity was measured by estimating the amount of glucose released. One unit of invertase activity was defined as the amount required for liberating 1 µg of glucose and fructose from the breakdown of sucrose per minute at 37^0C.

Reagents:

a) 0.1M acetic acid-sodium acetate buffer, pH 5
b) 2.5% sucrose solution in water
c) 1% NaOH solution
d) Crystalline phenol
e) Sodium sulphite
f) Dinitrosalicylic acid (DNS)
g) 40% sodium potassium tartrate

Preparation of DNS reagent: Same as described before.

Method:

Three sets of experiments (Blank, control and sample) were performed for the measurement of invertase activity. 5 ml of crude enzyme extract was taken in a 100 ml flask, 10 ml of 0.1M acetic acid-sodium acetate buffer, pH 5 and 5 ml of 2.5% sucrose solution were added to it. The mixture was incubated at 37°C for 15 minutes. Aliquot of 3 ml of the extract was pipetted into a test tube and 3 ml of DNS reagent was added to it. The mixture was heated for 5 minutes in a boiling water bath. After the color had developed, 1 ml of 40% sodium potassium tartrate solution was added to the warm tubes. A control tube was also prepared in which the enzyme was denatured by heating before the addition of substrateThe absorbance of solution was measured at 575 nm and the amount of glucose was calculated from the standard curve of glucose (Fig. 10)

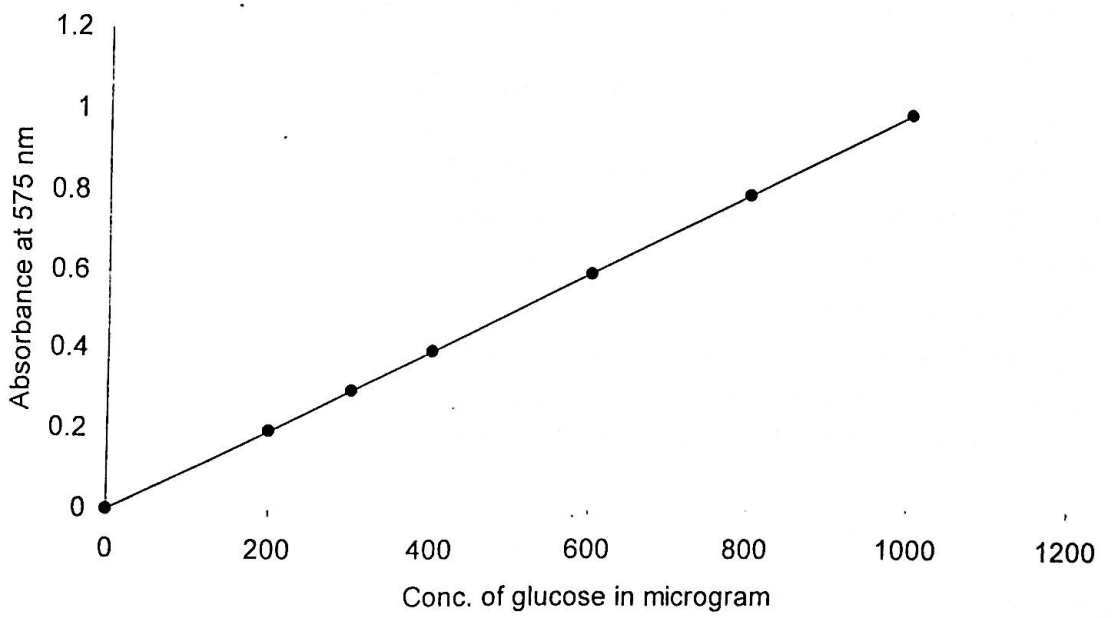


Fig-10: Standard curve of glucose for the determination of invertase and cellulase activity.

M.3.1.3 Measurement of cellulase activity:

The cellulase activity was measured following the method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). Carboxymethyl cellulose (CMC) was used as substrate. Cellulose activity was measured by estimating the reducing sugar released from the substrate using DNS method (Miller, 1972). One unit of cellulase activity was defined as the amount of enzyme required for liberating 1µg of reducing sugar from CMC per minute at 37°C.

Reagents:

a) Acetic acid – sodium acetate buffer, pH 5.2

b) 0.5% Carboxymethyl cellulose (CMC) solution: 0.5 gm of CMC was dissolved in 100ml of acetic acid-sodium acetate buffer, pH 5.2 at 50-60°C. It was taken in a blender and homogenized it for 3 to 5 minutes at low speed. The content was stirred with a glass rod and again homogenized at high speed for 3 to 5 minutes. Then it was filtered through a filter paper Whatman No 1. The clear supernatant was used as substrate.

Procedure:

4ml of 0.5% CMC solution, 1ml of buffer and 2ml of the crude enzyme solution were taken in a test tube. The content of the tube was mixed uniformly and incubated at 37°C for 30 minutes in a boiling water bath. Aliquot of 2ml from each tube was withdrawn at prefixed time intervals (3minutes) and the amount of reducing sugar released was determined by the DNS method (Miller, 1972). A blank was prepared by taking 2ml of buffer in place of crude enzyme extract.

M.3.1.4 Measurement of β-galactosidase enzyme activity:

β-galactosidase activity was assayed following the modified method as described by Lazan *et al.*, (1993), using Methyl-β-D-galactopyranoside as substrate. Amount of reducing sugar released was estimated by dinitrosalicyclic acid method (Miller, 1972). The β-galactosidase activity was measured by estimating the amount of reducing sugar (galactose) released from β-D-galactopyranoside (Fig-11). One unit of β-galactosidase activity was defined as the amount of enzyme that calalyzed the liberation of 1µg of galactose per minute at 37°C and the amount of galactose released was calculated from the standard curve prepared with galactose.

Reagents:

a) 0.1M Sodium-citrate buffer, pH 4.1

b) 13 mM Methyl β-D-galactopyranoside solution in sodium citrate buffer, pH 4.1

c) 0.2M Sodium carbonate

d) 40% Rochelle salt (sodium potassium tartrate)

e) Dinitrosalicyclic acid (DNS).

Preparation of DNS reagent: Same as described before.

Procedure:

Three sets of experiments (Blank, control and sample) were performed for the measurement of β-galactosidase activity. The following different solutions were taken in different test tubes.

Table: Measurement of β-galactosidase activity

Substances	Blank (ml)	Control (ml)	Sample (ml)
0.1M sodium citrate buffer, pH 4.1	0.5	0.5	0.5
13mM Methyl β-D-galactopyranoside	0.5	0.5	0.5

The contents in the test tubes were mixed uniformly and the tubes were incubated in a water bath at 37°C for 10 minutes. Then 0.5 ml of crude enzyme extract was added to the sample and control test tubes, while 0.5ml of distilled water was added to the blank test tube. Immediately, after the addition of crude enzyme extract, 1.5ml of 0.2M sodium carbonate solution was added to the control tube.

The rest of the tubes were incubated at 37°C for 15 minutes and the reaction was then stopped by adding 1.5ml of 0.2M sodium carbonate. Then, 3ml of DNS reagent were mixed to all the test tubes. The tubes were heated in a boiling water bath for 5-7 minutes and 1ml of 40% Rechelle salt was added, while the tubes were still warm. The tubes were cooled under running tap water at room temperature. Finally, absorbance of all the tubes was measured at 575nm. The amount of galactose was calculated from the standard curve of galactose.

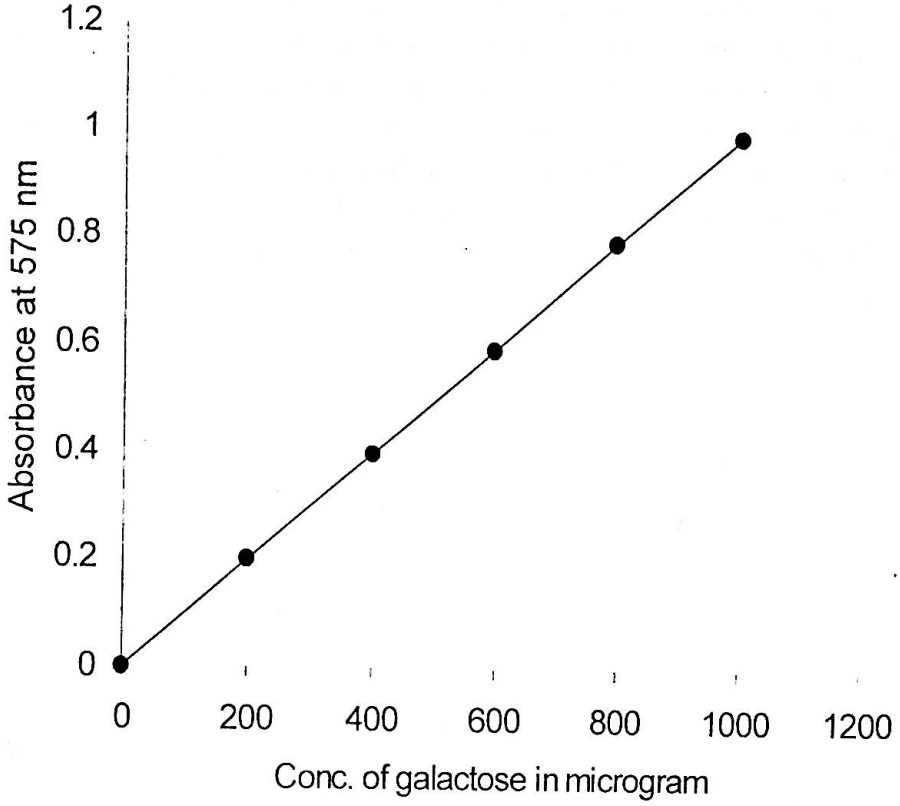


Fig-11: Standard curve of galactose for the determination of β-galactosidase activity.

M.3.1.5 Measurement of protease activity:

The protease activity was measured following the method of Kunitz (1947). The milk protein, casein was used as substrate. The activity is determined by detecting the release of amino acid (tyrosine). The amount of tyrosine released was calculated from the standard curve (Fig-12) constructed with tyrosine. One unit of protease activity was defined as the amount required for liberating 1 μg of tyrosine per minute at 45°C.

Reagents:

a) 1.2% casein solution: 1.2 gm of casein was dissolved in 0.1M phosphate buffer, pH 7.0. Since casein is sparingly soluble in water, it was dissolved in a minimal quanitity of 0.1M NaOH and the volume was raised to 100 ml with the buffer.

b) 0.4M Trichloro acetic acid (TCA).

Procedure:

2.5ml of 1.2% casein solution was taken in different test tubes (a) for control-1 no (b) for blank-1no, and (c) for experiment-2 nos. Then 0.5ml of crude enzyme extract was added to the control and experimental test tubes, whereas 0.5ml of buffer was added to the blank test tube. Immediately, after the addition of the crude enzyme extract, 2.5ml of TCA was added to the control test tube to stop the reaction. The rest of the tubes were incubated at 45°C for an hour and the reaction was stopped by the addition of 2.5ml of TCA into the test tube. After cooling, the reaction mixture was centrifuged at 5000 rpm for 7 minutes. The supernatant was collected and absorbance was taken at 275nm against the reagent blank.

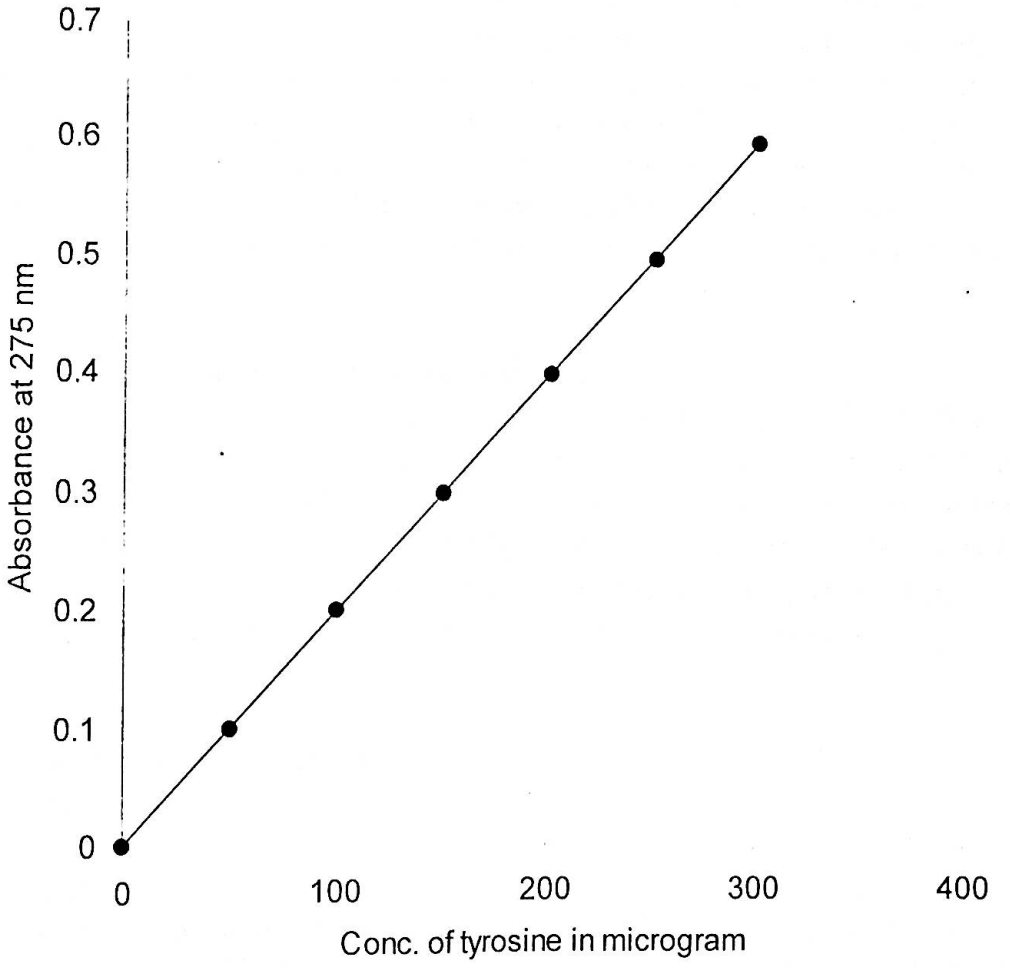


Fig-12: Standard curve of tyrosine for the determination of protease activity.

M.3.1.6 Measurement of polyphenol oxidase activity:

The polyphenoloxidase activity was measured following the method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method, catechol was used as substrate. One unit of enzyme activity was defined as a change in absorbancy of 0.001, per minute per ml of enzyme extract.

Reagents:

a) 0. 1M phosphate buffer, pH 6.0
b) 0.01M catechol in 0.1M phosphate buffer, pH 6.0

Procedure:

Aliquot of 2ml of the crude enzyme extract and 3ml of 0.1M phosphate buffer, pH 6.0, were pipetted into the cuvette or different test tubes. The contents were mixed by inverting, placed in a spectrophotometer, set at 495nm and the absorbancy was adjusted to zero. The test tubes were removed and 1ml of 0.01M catechol was added separately and quickly mixed by inversion. The tubes were placed in the spectrophotometer and the changes in absorbancy at 495 nm was measured for 3 minutes. A blank was prepared by taking 2ml boiled enzyme, 3ml of 0.1M phosphate buffer, pH6.0 and 1ml of 0.01M catechol, and treated similarly.

M.3.1.7 Measurement of catalase activity:

The catalase activity was measured following the method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method, hydrogen peroxide (H_2O_2) was used as substrate. The enzyme activity was measured by estimating the residual hydrogen peroxide in the reaction mixture. One unit of catalase is defined as that amount of enzyme, which breakdown 1μ mole of hydrogen peroxide per minute under the assay condition.

Reagents:

a) 0.1M phosphate buffer, pH 6.4
b) 1% H_2O_2

Procedure:

Aliquot of 2.5 ml of 0.1M phosphate buffer, pH 6.4 was pipetted into test tube. Then 0.1ml of 1% H_2O_2 and 0.2ml of the crude enzyme extract were added. The reaction was followed by recording changes in absorbancy at 230nm for 1.25 minutes at 15 seconds interval. The enzyme extract and buffer were used to adjust the absorbancy to zero. A control was prepared by taking 2.5ml of 0.1M phosphate buffer, pH 6.4; 0.2 ml of boiled enzyme extract and 0.1ml of 1% H_2O_2 and treated similarly.

M.3.1.8 Measurement of peroxidase activity:

The peroxidase activity was measured following the method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). In this method, pyrogallol was used as substrate. In the presence of hydrogen peroxide, the amount of purpurogallin formed during the reaction can be followed in a spectrophotometer. One unit of peroxidase is defined as the amount of purpurogallin formed, per minute, under the assay condition.

Reagents:

a) 0.05M pyrogallol dissolved in 0.1M phosphate buffer, pH 6.0
b) 1% H_2O_2

Procedure:

Aliquot of 3ml of 0.05M pyrogallol solution prepared in 0.1M phosphate buffer, pH 6.0 and 0.1ml of the crude enzyme extract were pipetted in a test tube. The contents were mixed well, placed in a spectrophotometer, set at 420nm and the absorbancy was adjusted to zero. The test tube was removed and 1ml of 1% H_2O_2 was mixed quickly by inversion. The test tube was placed in the spectrophotometer, and the absorbancy changes were measured at 20 seconds interval for 3 minutes.

RESULTS AND DISCUSSIONS

R.3.1.1 Amylase activities and its relation to sugar compositions in betel leaves at different maturity stages

Amylase having physiological, commercial and historical significance also called diastase. It is found in both plants and animals. Payen and Persoz (1833) were the first to become aware of enzymatic starch hydrolysis, they found that malt extract converted starch to sugar. Starch is the principal storage carbohydrate in plant cells, which is made up of amylose and amylopectin. Amylase is a hydrolytic enzyme, which hydrolyzes starch to produce monomeric carbohydrate. Starch breaks down during the germination of cereals seeds result from the action of hydrolytic enzymes and it is generally accepted that phosphorylases are not involved in this process, while α-amylase play a major role during the degradation of native starch granules (Beck *et.al.*, 1989; Pereta *et. al.*,1992; Walker *et. al.* 1963). The concerted action of α-amylase, β-amylase, a debranching enzyme and α-glycosidase are essential for the complete hydrolysis of starch (Sunet. *et.al.*, 1991; Guglielminetti *et.al.*, 1995). The activities of amylase and its relation to the sugar compositions in four different varieties of betel leaf extracts at different maturity stages are cited in Table-9.

Among the four varieties, the activities of amylase in all the stages were found to be highest in Kal Bangla variety and lowest in Dudhswar variety. Activities were varied between 32.49-60.23 Units/gm in premature stage, between 58.70-75.78 Units/gm in mature stage and 53.35-70.51 Units/gm in over premature stage. Amylase activities in all varieties were found to be increased significantly upto mature stage and decreased abruptly from mature to over mature stage. Similar trends were reported by Desai and Despande (1978), Mao and Kinsella (1981) and Garica *et al.* (1988) in banana.

Total soluble sugar and sucrose content in all the varieties were increased from premature to over mature stage (Table-9). Amylase activities, total soluble sugar and sucrose content were found to be increased upto mature stage. But the amount of amylase decreased abruptly from mature to over mature stage while total soluble sugar and sucrose content increased further. These observations indicated that the increased amount of total sugar and sucrose upto mature stage were directly proportional to amylase activities and from mature to over mature stage the amount of amylase were inversely proportional to total soluble sugar and sucrose content. Recent evidences associating amylase with sugar content and sucrose transport implies that amylase control is a potential means of sugar regulation (Alexander, 1967; Alexander, 1968). Many scientists (Alexander, 1964;

Alexander, 1965) observed that amylase level was frequently found in inverse proportion to sucrose forming activity. Reducing sugar was absent in all varieties of betel leaf extracts in premature stage but it was increased thereafter upto over mature stage, which were consistent with the increased amount of total sugar and sucrose from premature to over mature stage.

R.3.1.2 Invertase activities and its relation to sugar compositions in betel leaves at different maturity stages

Invertase (β-fructofuraniside; EC 3.2.1.26) was first isolated from yeast more than a century ago (Berthelot, 1860). The enzyme occurs widely in plant, microbial and animals sources (Nakawa *et.al.* (1971); Krishan *et. al* (1985); Hirayama *et. al.* (1989)). Invertase plays an important role in the hydrolysis of sucrose to glucose and fructose in higher plants, especially in the storage organs. Sucrose is an early product of photosynthetic reaction and is the most abundant transportable free carbohydrate in the plant kingdom. Sucrose serves as an important reserve carbohydrate in plants, especially in such storage organs as tuber, root and seed. During germination, sucrose is a readily degradation source of energy (Bracho *et. al.*, 1990). The activities of invertase and sugar compositions in different varieties of betel leaves at different maturity stages are shown in Table-9.

Among the varieties, invertase activities were varied between 26.67-64.00 Units/gm in premature stage, between 35.56-72.12 Units/gm in mature stage and 40.32-82.32 Units/gm in over premature stage. The activities of invertase in all the stages were found to be highest in Doga variety and lowest in Kal Bangla variety. The activities of invertase were lowest in all varieties at premature stage then increased remarkably upto over mature stage which were in good agreement with the increasing amount of total soluble sugar and sucrose from premature to over mature stage. Reducing sugar was absent in all varieties of betel leaf extracts at premature stage but it was increased thereafter upto over mature stage.

Sarah *et al.* (2001) reported that invertase activities were much lower in green tomato than in red. In L. *esculentum* species, a marked increase in invertase activity was found during fruit development while a concomitant increase in sucrose concentration was observed (Iwatsubo *et. al.*,1976; Miron *et.al.*, 1991). High sucrose levels could become of potential importance in improving tomato yield and flavour in cultivated tomato variety (Yelle *et.al.*, 1991).

R.3.1.3 Cellulase activities and its relation to sugar compositions in betel leaves at different maturity stages

Cellulolytic enzymes, a group of hydrolytic enzymes including cellulase, capable of hydrolyzing cellulose to glucose. There are at least three major types of cellulolytic enzymes produced by fungi; endoglucanases, cellobiohydrolases and cellobiases (Klyosov, 1990). These are produced by a large number of microorganisms like fungi and bacteria (Enari, 1983; Wood, 1960). Many plant pathogens are also known to produce either adaptively or non-adaptively proteolytic, cellulolytic and various polysaccharides (Wood, 1960). They are used to perform various functions including removing cell walls or crude fiber to release valuable components (flavours, enzymes, polysaccharides and other proteins) from plant cells to improve nutritional value of animal feeds or to prepare plant protoplasts for genetic research (Mandels, 1985). The cellulase activities and sugar compositions of four different varieties of betel leaf extracts at different maturity stages are presented in the Table-9.

Among the varieties, the activity of cellulase was highest in Shail variety (8.52 Units in premature stage, 17.15 Units in mature stage and 9.23 Units in over mature stage per gm of leaf) and lowest in Kal Bangla variety (4.4 Units in premature stage, 9.35 Units in mature stage and 5.03 Units in over mature stage per gm of leaf) in all maturity stages. Cellulase activities were found to be increased remarkably upto mature stage and decreased abruptly from mature to over mature stage. During quantitative estimation of cellulase enzyme in *Botryodiplodia theobromae* in culture, the activity of enzyme was found to be increased upto mature stage as reported by Chakrabarti and Nandi (1976). Nehemiah and Despande (1976) found that cellulose production and decomposition of cotton fabric was increased by *Alternania brasicae.*

The present finding clearly demonstrated that the activities of cellulase and the amount of total sugar and sucrose were found to be increased upto mature stage. After that the amount of cellulase reduced drastically but total sugar content and sucrose content increased.

Table-9: Activities of amylase, invertase and cellulase and their relations to sugar compositions in betel leaves at different maturity stages.

Varieties	Stages	Activity of Enzymes (Units/gm)			Compositions (gm%)			
		Amylase	Invertase	Cellulase	Total sugar	Reducing sugar	Sucrose	Starch
Shail Pan	Premature	55.20 ± 0.02	40.54 ± 0.02	8.52± 0.020	1.31 ± 0.01	00 ± 0.02	1.245 ± 0.02	6.17 ± 0.03
	Mature	72.13 ± 0.02	50.22 ± 0.12	17.15± 0.013	3.27 ± 0.03	0.545 ± 0.05	2.589 ± 0.05	6.92 ± 0.04
	Over Mature	68.54 ± 0.02	62.25 ± 0.02	9.23± 0.021	3.33 ± 0.02	0.555 ± 0.04	2.636 ± 0.03	6.74 ± 0.02
Doga Pan	Premature	35.56± 0.004	64.00 ± 0.05	8.32± 0.051	1.43 ± 0.02	00 ± 0.01	1.359 ± 0.01	4.98 ± 0.02
	Mature	69.33± 0.024	72.12 ± 0.08	16.63± 0.022	3.58 ± 0.05	0.527 ± 0.03	2.900 ± 0.04	5.60 ± 0.03
	Over Mature	54.87± 0.021	82.32 ± 0.07	9.10± 0.031	3.61 ± 0.01	0.534 ± 0.01	2.922 ± 0.03	5.36 ± 0.01
Dudhswar Pan	Premature	32.49± 0.032	39.44 ± 0.03	4.88± 0.024	1.29 ± 0.02	00 ± 0.03	1.226 ± 0.02	5.69 ± 0.01
	Mature	58.70± 0.025	47.56 ± 0.07	10.05± 0.015	3.22 ± 0.04	0.497 ± 0.04	2.587 ± 0.01	6.48 ± 0.04
	Over Mature	53.35± 0.015	56.42 ± 0.03	5.11± 0.019	3.25 ± 0.03	0.523 ± 0.02	2.591 ± 0.02	6.21 ± 0.02
Kal Bangla Pan	Premature	60.23± 0.011	26.67 ± 0.02	4.40± 0.032	1.38 ± 0.02	00 ± 0.02	1.311 ± 0.03	6.28 ± 0.03
	Mature	75.78± 0.015	35.56 ± 0.02	9.35± 0.024	3.46 ± 0.05	0.576 ± 0.03	2.740 ± 0.04	7.05 ± 0.05
	Over Mature	70.51± 0.031	40.32 ± 0.01	5.03± 0.027	3.50 ± 0.04	0.583 ± 0.01	2.771 ± 0.01	6.83 ± 0.01

R.3.1.4 β-galactosidase activities in betel leaves at different maturity stages

β-galactosidase (EC 3.2.1.23) is widely distributed in plants, animals and microorganisms. β-galactosidase, a carbohydrate splitting enzyme, plays a significant role in plant tissues specially after maturation of fruits. Wallenfels and Weil (1972) have reviewed the extensive literature on the enzymology of β-galactosidase. The activities of β-galactosidase in the extracts of different varieties of betel leaf at different maturity stages are shown in Table-10.

Of the different varieties, Doga variety contained the highest and Dudhswar variety contained the lowest β-galactosidase activity. β-galactosidase activities were ranged from 29.99 to 40.25 Units in premature stage, from 16.27 to 22.33 Units in mature stage and from 6.66 to 9.21 Units in over mature stage per gm of fresh

betel leaves. All the varieties contained highest β-galactosidase activities at premature stage, but lowest β-galactosidase activities at over mature stage (Table-10). It was clear from the results that the activities of β-galactosidase decreased rapidly with the advancement of maturity.

R.3.1.5 Protease activities in betel leaves at different maturity stages

Protease is a hydrolytic enzyme, which, acts on proteinaceous substances releasing amino acids and amides serve as nitrogen and carbon sources to the pathogen, as many microorganisms utilize amino acids as carbon and nitrogen sources. The activities of protease in the extracts of different varieties of betel leaf at different maturity stages are illustrated in the Table -10.

Of the above mentioned varieties, the activity of protease was highest in Doga variety (2.25 Units in premature stage, 4.43 Units in mature stage and 7.50 Units in over mature stage per gm of leaf) and lowest in Dudhswar variety (1.87 Units in premature stage, 3.90 Units in mature stage and 6.01 Units in over mature stage per gm of leaf) in all maturity stages (Table-10). The activities of protease were lowest in all varieties at premature stage and then increased sharply upto over mature stage.

Hashinaga, *et al* (1983) reported that protease activity (KFP-1) increased in Kiwifruit flesh during ripening. Increased protease activity was also observed in passion fruit juice during maturation (1978). High protease activity during ripening stage may be attributed to protein catabolism, which is related to leaf senescene (Dilley, 1970).

Table-10: Activities of β-Galactosidase and Protease in betel leaves.

Varieties	Stages	Activity of Enzymes (Units/gm)	
		β-galactosidase	Protease
Shail Pan	Premature	32.31± 0.05	1.90± 0.041
	Mature	19.63± 0.043	4.01± 0.021
	Over Mature	7.33± 0.045	6.05± 0.044
Doga Pan	Premature	40.25± 0.033	2.25± 0.045
	Mature	22.33± 0.045	4.43± 0.023
	Over Mature	9.21± 0.035	7.50± 0.033
Dudhswar Pan	Premature	29.99± 0.022	1.87± 0.042
	Mature	16.27± 0.032	3.90± 0021
	Over Mature	6.66± 0.041	6.01± 0.031
Kal Bangla Pan	Premature	35.33± 0.012	2.10± 0.011
	Mature	20.15± 0.022	4.33± 0.023
	Over Mature	8.27± 0.016	7.05± 0.014

R.3.1.6 Activity of polyphenoloxidase in betel leaves

Polyphenoloxidase is also known as phenoloxidase, tyrosinase, dopaoxidase, catechol oxidase and potato oxidase. It is a bifunctional, copper-containing oxidase having catecholase and cresolase activity. The enzyme catalyzes the oxidation of monophenols and orthodiphenols. Monophenols, particularly tyrosine and p-cresol and orthophenols such as adrenaline, pyrogallol and substituted catechols are important substrates of the enzyme.

The activities of polyphenoloxidase in the extracts of different varieties of betel leaf at mature stage are illustrated in Table-11. Among the four varieties, the activity of polyphenoloxidase was highest in Doga variety (22.4 Units/gm) and lowest in Dudhswar variety (20.9 Units/gm).

R.3.1.7 Activity of catalase in betel leaves

Catalase catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen.

$$H_2O_2 \rightarrow H_2O + {}^1/_2O_2$$

The activities of catalase enzyme in all four varieties of betel leaves are given in the Table-11. The results indicated that the betel leaves are significantly very rich sources of catalase. All the varieties contained almost same amount of catalase activities and the catalase activities ranged between 49.50 Units/gm (in Dudhswar variety) and 57.08 Units/gm (in Shail variety).

R.3.1.8 Activity of peroxidase in betel leaves

Peroxidases are widely distributed in the plant kingdom. Peroxidase catalyzes the oxidation of various "Hydrogen donors", like p-cresol, benzidine, ascorbic acid, nitrate and cytochromes in the presence of hydrogen peroxide (H_2O_2). The reaction may be represented as.

$$H_2O_2 \rightarrow 2AH + 2H_2O$$

The activities of peroxidase enzyme in four varieties of betel leaves are given in the Table-11. The peroxidase acivity was highest in Shail variety (37.45 Units/gm) and lowest in Doga variety (28.24 Units/gm).

Table-11: Activities of polyphenol oxidase, catalase and peroxidase enzymes

Varieties (at mature stage)	Activities in Units/gm		
	Polyphenol oxidase	Catalase	Peroxidase
Shail Pan	21.5 ± 1.04	57.08 ± 1.84	37.45 ± 1.01
Doga Pan	22.4 ± 1.07	51.02 ± 1.68	28.24 ± 1.02
Dudhswar Pan	20.9 ± 1.13	49.50 ± 1.74	29.61 ± 1.04
Kal Bangla Pan	21.3 ± 1.06	56.32 ± 1.62	32.48 ± 1.03

CHAPTER FOUR

PURIFICATION AND CHARACTERIZATION OF INVERTASES AND POLYPHENOL OXICDASE FROM BETEL LEAF OF DOGA VARIETY

INTRODUCTION

Sucrose is the most abundant transprotable free carbohydrate in the plant kingdom. It is formed by plants but not by higher animals. Sucrose is a major intermediate product of photosynthesis. In many plants, it is the principal form in which sugar is transported from the leaves to the portions of plants via their vascular systems. Sucrose serves as an important preserve carbohydrate in plants, especially in such storage organs as tuber, root, fruit and seed. During germination, sucrose is a readily degradable source of energy. In storage organs, invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) hydrolyzes sucrose to yield glucose and fructose.

Invertase is one of the enzyme isolated from yeast more than a century ago (Berthelot, 1860). The enzyme occurs widely in many plants, microorganisms and animal sources (Nakagawa *et. al.*, 1971; Krishnan *et. al.*, 1985; Hirayama *et. al.*, 1989). Grape invertase isolated from certain white grapes had been shown to be present in both soluble and bound forms (Arnold, 1965). Soluble invertase purified from Semillon (Nakanishi *et. al.*, 1990) was stable under acidic conditions. Sugarcane invertase was found in a number of isoforms, namely: neutral invertase, vascular acid invertase, cell wall bound acid invertase and apoplastic soluble acid invertase (Moore, 1995).

Polyphenoloxidase is also known as phenoloxidase, tyrosinase, dopaoxidase, catechol oxidase and potato oxidase. It is a bifunctional, copper-containing oxidase having catecholase and cresolase activity. The enzyme catalyzes the oxidation of monophenols and orthodiphenols. Monophenols, particularly tyrosine and p-cresol and orthophenols such as adrenaline, pyrogallol and substituted catechols are important substrates of the enzyme. The present study describes the purification and characterization of invetrase and polyphenol oxidase from "Doga" variety of betel leaf at mature stage.

M.4.1 COLLECTION OF BETEL LEAVES

Betel leaves were collected from the area, Mohonpur in Rajshahi district. After collection, the leaves were cleaned and used for purification procedures.

M.4.2 METHODS

M.4.2.1 Choice of extraction media

Proteins from betel leaves were extracted under identical conditions using five different extracting solvents. After extraction, the total concentration of the protein was determined by measuring the absorbance at 280 nm. The suitable extracting

solvent was selected from their ratio of absorbance at 280 nm and 260 nm as reported by Clark and Switzer (1977).

Table 12. Preparation of crude enzyme extract from betel leaves in different extracting solvents.

Extracting Media	Amount of leaf (gm)	O.D. at 280 nm	O.D. at 260 nm	Ratio of O.D. 280 & 260 nm
20 mM aceteic acid-sodium acetate buffer, pH 5.0	0.5gm	1.525	1.525	1
Distilled water	0.5gm	1.53	1.55	0.987
Phosphate buffer, pH 7.6	0.5gm	1.53	1.54	0.993
Tris-HCl buffer, pH 8.4	0.5gm	1.52	1.545	0.983
M NaCl pH 5.4	0.5gm	1.54	1.542	0.998

20 mM aceteic acid-sodium acetate buffer, pH 5.0 was used as extracting solvent for extraction of crude enzyme from betel leaves as the highest ratio of absorbance (at 280 nm & 260 nm) was found in this medium.

M.4.2.2 PREPARATION OF CRUDE ENZYME EXTRACT

The betel leaves (250 gm) were weighed, cut into small pieces and grinded in a mortar and pestle with 20 mM acetic acid-sodium acetate buffer, pH 5.0 and finally crushed into paste using homogenizer. The temperature was maintained at 4^0C by putting ice in the outer chamber of homogenizer. The suspension was then filtered through double layers of muslin cloth in the cold chamber. The filtrate was collected and further clarified by centrifugation at 10000 r.p.m. for 15 minutes at 4^0C. The clear supernatant was concentrated about 1/8th of the original volume by using commercial sucrose at 4^0C. Then it was dialysed against 20 mM acetic acid-sodium acetate buffer, pH 5.0 for 24 hours at 4^0C. It was again centrifuged at 7000 r.p.m. for 10 minutes to remove insoluble materials and the clear supernatant was used as crude enzyme extract.

M.4.3 PURIFICATION OF ENZYMES

M.4.3.1 DEAE-Cellulose Column Chromatography

A. Procedure

i) Activation of DEAE-cellulose Powder: The DEAE-cellulose powder was suspended in 0.5M NaOH in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several

times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.5M HCl and left for few hours. It was again washed with distilled water to neutralize its pH.

ii) Packing of the column: This is very critical step in all types of column chromatographic experiment. If the column is not packed properly, accurate results never be expected. Because a poorly packed column gives rise to uneven flow rates. The activated DEAE-cellulose suspension was taken in a filtering flask and deaerated by vacuum pump. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

iii) Equilibration of the column: After packing, the column was equilibrated with 20 mM acetic acid-sodium acetate buffer, pH 5.0.

iv) Application of sample: Before loading of the sample, the outlet tube of the column was opened and the eluant buffer from the top of the column bed was allowed to diffuse into the cellulose. The crude extract was then loaded on the top of bed. After diffusion of the sample, about 1 ml of eluant buffer was poured on the top of the bed and was allowed to diffuse. Then an additional amount of buffer was added, so that the space about 3-4 cm above the bed was filled with eluant. The buffer was then allowed to flow continuously through the column at a flow rate of about 24 ml per hour and 3 ml fractions of the eluant were collected by automatic fraction collector. Absorbance of each fraction was measured at 280 nm.

M.4.3.2 CM-Cellulose Column Chromatography

A. Materials: i) CM-Cellulose powder (20 gm)
ii) 0.2M HCl
iii) 0.2M NaOH solution

B. Procedure:

i) Activation of CM-Cellulose powder: The CM-Cellulose powder was suspended in 0.5M HCl in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.5M NaOH solution and left for few hours. It was again washed with distilled water to neutralize its pH.

ii) Packing of the column: The activated CM-Cellulose suspension was taken in a filtering flask and deaerated by vacuum pump. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

iii) Equilibration of the column: After packing, the column was equilibrated with 20 mM acetic acid-sodium acetate buffer, pH 5.0.

iv) Application of sample: The active fractions obtained by DEAE-Cellulose chromatography was dialyzed against distilled H_2O for 12 hours and against 20 mM acetic acid-sodium acetate buffer, pH 5.0 for 12 hours at 4^0C. The dialyzed sample was loaded onto CM-Cellulose column at 4^0C. The proteins were eluted from the column with the same buffer containing NaCl by stepwise elution.

M.4.3.3 Gel Filtration on Sephadex G-75

i) Activation of gel powder: Sephadex G-75 powder was suspended in 10% acetic acid containing 1M sodium chloride (1 mole of NaCl was dissolved in one liter of 10% acetic acid) in a beaker and left it to swell for overnight. It was stirred by glass rod after short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water for several times until its pH reached near to neutrality.

ii) Packing of the column: The gel suspension was taken in a filtering flask and deaerated by vacuum pump; otherwise it would affect the flow rate of the column after packing. The gel suspension was adjusted so that it was fairly thick slurry, but not thick enough to retain bubbles. The column was mounted on a stable laboratory stands and its narrow end was fitted with an outlet tube. It was ensured that there was no air bubble in the dead space of the bed support. This was easily achieved by filling approximately 1/4 th of the column, including the outlet tube with distilled water. When dead space was properly filled, the outlet tube was closed with pinch cork and the gel suspension from a gel reservoir was added gently to the column. In order to avoid trapping of any bubble, this was performed by pouring the gel in inner wall of the column. In this way, a column of desired length was packed uniformly with the gel suspension.

iii) Equilibration of the column: After completion of the column packing it was equilibrated with the eluant buffer (20 mM acetic acid-sodium acetate buffer, pH 5.0). The buffer was continued to run through the column until the pH of the eluate became same as the pH of the eluant buffer.

iv) Application of sample: The active fractions obtained by CM-Cellulose chromatography was dialyzed against distilled H_2O for 12 hours and against 20 mM acetic acid-sodium acetate buffer, pH 5.0 for 12 hours at 4^0C. The dialyzed sample was loaded onto Sephadex G-75 column at 4^0C. The proteins were eluted from the column with the same buffer containing NaCl by linear and stepwise elution. Absorbance of each fraction was measured at 280 nm.

M.4.4 TEST OF PURITY: Sodium dodecyl sulfate polyacrylamide slab gel electrophoriesis (SDS-PAGE) Method

Principle: Polyacrylamide gel electrophoresis method is commonly used for checking the purity of proteins and their molecular weight determination. Sodium dodecyl sulfate (SDS) is an anionic detergent which binds to most proteins in amounts roughly proportional to molecular weight of the protein, about one molecule of SDS for every two molecules of amino acid residues. The bound SDS contributes large net negative charge, rendering the intrinsic charge of the protein insignificant. In addition, native conformation of the protein is altered when SDS is bound and most protein assumes similar shape and thus similar ratio change to mass. Slab gel electrophoresis in presence of SDS therefore separates proteins almost exclusively on the basis of mass, with smaller polypeptides migrating more rapidly. Protein-SDS complexes will therefore all move towards the anode during electrophoresis and their movements are inversely proportional to their molecular weights. If standard proteins of known molecular weights are also run, the molecular weights of the sample proteins can be determined by comparing them with proteins of known molecular weights. The protein pattern of the selected fractions was determined by 10% SDS-PAGE according to the method of Laemmli (1970) as modified by Smith (1955).

M.4.5 CHARACTERIZATION OF THE ENZYMES

M.4.5.1 Molecular Weight Determination

M.4.5.1.1 By gel filtration method

The molecular weight of the purified proteins were estimated from the data of gel filtration on Sephadex G-150 (0.75×100 cm) with trypsin inhibitor (20,000), egg albumin (45,000), bovine serum albumin (67,000) and β-galactosidase (116000) as reference proteins following the procedures as described by Andrews (1962).

M.4.5.1.2 By Sodium dodecyl sulfate polyacrylamide gel electroforesis (SDS PAGE) method:

The molecular weights of the purified proteins were determined by this method that was described before for purity test of sample. {M.4.4 Sodium dodecyl sulfate polyacrylamide gel electroforesis (SDS-PAGE) method}

M.4.5.2 Determination of Km values of invertases and polyphenol oxidase

M.4.5.2.1 Determination of Km values of invertases from betel leaf

The initial value is equal to the amount of product formed per minute. The initial velocity (V_0) was determined by measuring the amount of one of the products (glucose or fructose) at various time intervals (Robyt *et. al.*, 1990).

Reagents:

i) Acetic acid-sodium acetate buffer, pH 5.0.
ii) 0.005M, 0.01M, 0.02M, 0.04M and 0.08M of sucrose solution in acetic acid-sodium acetate buffer, pH 5.0.
iii) 1% NaOH solution
iv) Dinitrosalicylic acid (DNS).
v) 40% sodium potassium tartrate solution.

Method:

One ml of 0.005M, 0.01M, 0.02M, 0.04M and 0.08M of sucrose solution were taken in different test tubes: a) for control-1 no. b) for blank-1 no. and c) for experiment-2 nos. Then 2 ml of acetic acid-sodium acetate buffer, pH 5.0 was added to each tube and mixed uniformly. One ml of enzyme was added to the control tubes and experimental tubes. Immediately after the addition of enzyme 0.5 ml of 1% NaOH was added to the control tubes.

All the tubes were incubated at 37°C for various times (10 min., 20 min., 30 min., 40 min. and 50 min.). The tubes were removed from the water bath at appropriate time intervals and the reaction was stopped by the addition of 0.5 ml of 1% NaOH solution. 4.5 ml of DNS reagents was mixed to all the tubes. The tubes were heated in a boiling water bath for 5 minutes and 1.33 ml of 40% sodium potassium tartrate solution was added to each warm tube. After cooling in running tap water at room temperature, the absorbance was measured at 575 nm.

M.4.5.2.2 Determination of Km values of polyphenol oxidase from betel leaf

Michaelis constant (Km) was determined by Lineweaver-Burk's double reciprocal plot. The initial value is equal to the amount of product formed per minute. The initial velocity (V_0) was determined quantitatively by measuring the amount of one of the products at various time intervals.

Reagents:

i) 0.1 M phosphate buffer, pH 6.0.
ii) 0.005M, 0.01M, 0.02M, 0.04M and 0.08M catechol dissolved in 0.1 M phosphate buffer, pH6.0.

Procedure:

Aliquot of 2ml of the crude enzyme extract and 3ml of 0.1M phosphate buffer, pH 6.0, were pipetted into the cuvette or different test tubes. The contents were mixed by inverting, placed in a spectrophotometer, set at 495nm and the absorbancy was adjusted to zero. The test tubes were removed and 1ml catechol of different concentrations was added separately and quickly mixed by inversion. The tubes were placed in the spectrophotometer and the changes in absorbancy at 495 nm was measured for 3 minutes. A blank was prepared by taking 2ml boiled enzyme, 3ml of 0.1M phosphate buffer and 1ml of 0.01M catechol, and treated similarly.

M.4.5.3 Test for glycoprotein and Estimation of Sugar

Phenol in the presence of sulfuric acid can be used for quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharide and polysaccharides as described by Dubois *et al.* (1956). This method was also employed for detecting the presence of sugar in proteins and enzymes.

A. Materials

i) 5% phenol (in water)
ii) Concentrated sulfuric acid
iii) Protein solution

B. Procedure

The protein solution (0.1 ml from protein solution of 0 .075 - 0.097mg / ml) was taken in a test tube and made upto 2 ml by distilled water. Then 1 ml of 5% phenol was added to it and finally 5 ml of conc. H_2SO_4 was added rapidly. To obtain good mixing the stream of acid being directed against the liquid surface rather than against the side of the test tube. The tube was allowed to stand for 10 minutes. Then it was shaken and kept in the dark at 25 to 30°C for 20 minutes. It was taken out and the absorbance of the solution was measured at 490 nm.

C. Preparation of standard curve

A standard glucose solution (0.1mg/ml) was prepared. Then 0.0, 0.1, 0.2, 0.3, 0.4, 0.6, & 0.8 ml of this solution containing 0.0, 0.01, 0.02, 0.03, 0.04, 0.06, & 0.08 mg of glucose, respectively, were taken in different test tubes and made upto 2 ml with distilled water. The solution was treated similarly as described above. A standard graph of glucose was constructed by plotting the concentration of glucose against their absorbance. From the graph, the concentration of sugar in protein was calculated.

M.4.5.4 Determination of Protein concentration by the Folin-Lowry Method (Lowry *et al.* 1951)

Procedure: Same as described before in chapter two.

RESULTS AND DISCUSSIONS

R.4.1 PURIFICATION OF BETEL LEAF INVERTASE

R.4.1.1 DEAE-Cellulose chromatography of crude enzyme extract

The crude enzyme extract after dialysis against 20mM acetic acid-sodium acetate buffer, pH5.0 was applied on the DEAE-Cellulose column previously equilibrated with acetic acid – sodium acetate buffer of the same pH and concentration at 4°C. The crude enzyme extract was first eluted with the buffer only and then with the increasing concentration of NaCl. The elution profiles are shown in Fig-13. The proteins of crude enzyme extract were eluted from the column in five fractions. The major fraction, F-1 was eluted from the column by the buffer only, while the other four fractions namely F-2, F-3, F-4 and F-5 were eluted from the column by the buffer containing 0.1M, 0.2M, 0.25M and 0.3M NaCl respectively. It was found that the fractions F-1 and F-4 contained invertase activity. The areas as indicated by solid bars were pooled separately and purified further by ion-exchange chromatography on CM-Cellulose.

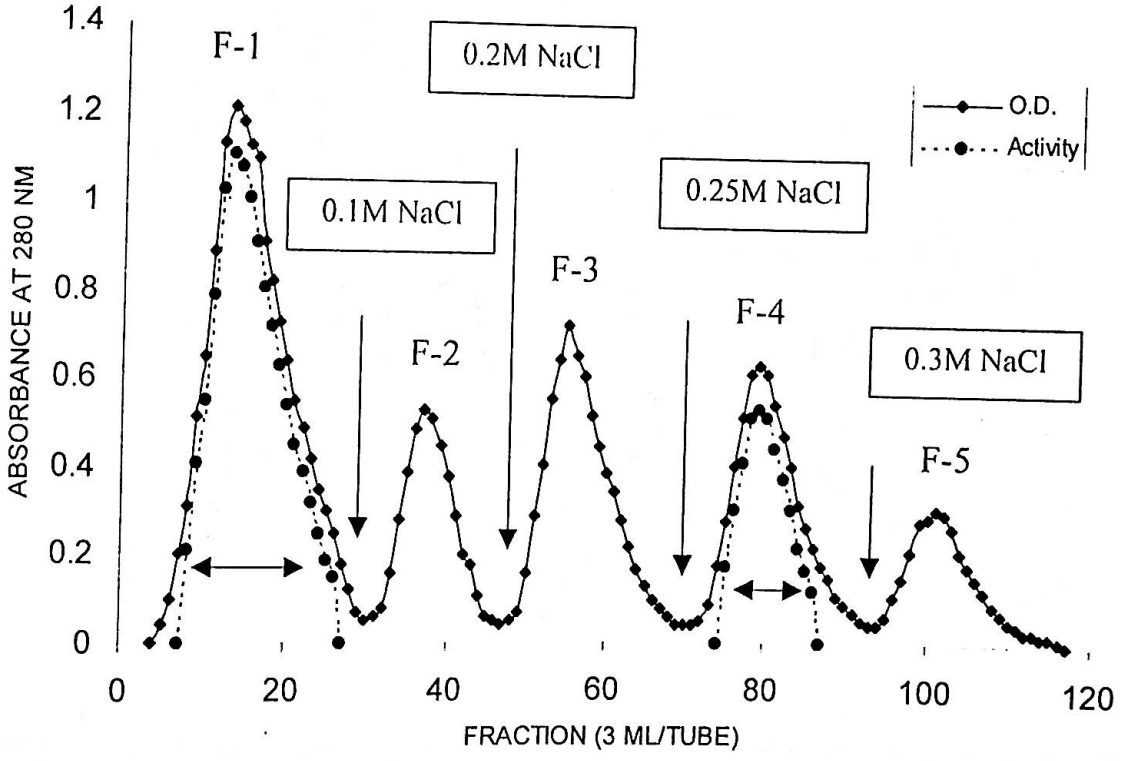


Fig-13: Ion-exchange chromatography of crude enzyme extract on DEAE-cellulose. Crude enzyme extract (170 mg) was applied to the column (2.0x24 cm) prewashed with 20 mM acetic acid-sodium acetate buffer, pH5.0 at 4°C and eluted by a stepwise increases of NaCl in the same buffer.

R.4.1.2 CM-Cellulose Chromatography of F-1 fraction

The enzyme active fraction F-1, obtained from DEAE-Cellulose chromatography, was applied to CM-Cellulose column previously equilibrated with the same buffer at 4°C. As shown in Fig-14, the components of the fraction F-1 were separated from the column in four fractions. Of these fractions, F-1a was eluted from the column by the buffer only while the other fractions, i.e. F-1b, F-1c and F-1d were eluted from the column by the buffer containing 0.15M, 0.25M and 0.35M NaCl respectively. It was found that the fractions F-1a contained only the invertase activity. The homogeneity of this fraction was checked by SDS-polyacrylamide gel electrophoresis. As shown in the Fig-14, F-1a fraction gave more than one band indicating that it contained impure enzyme.

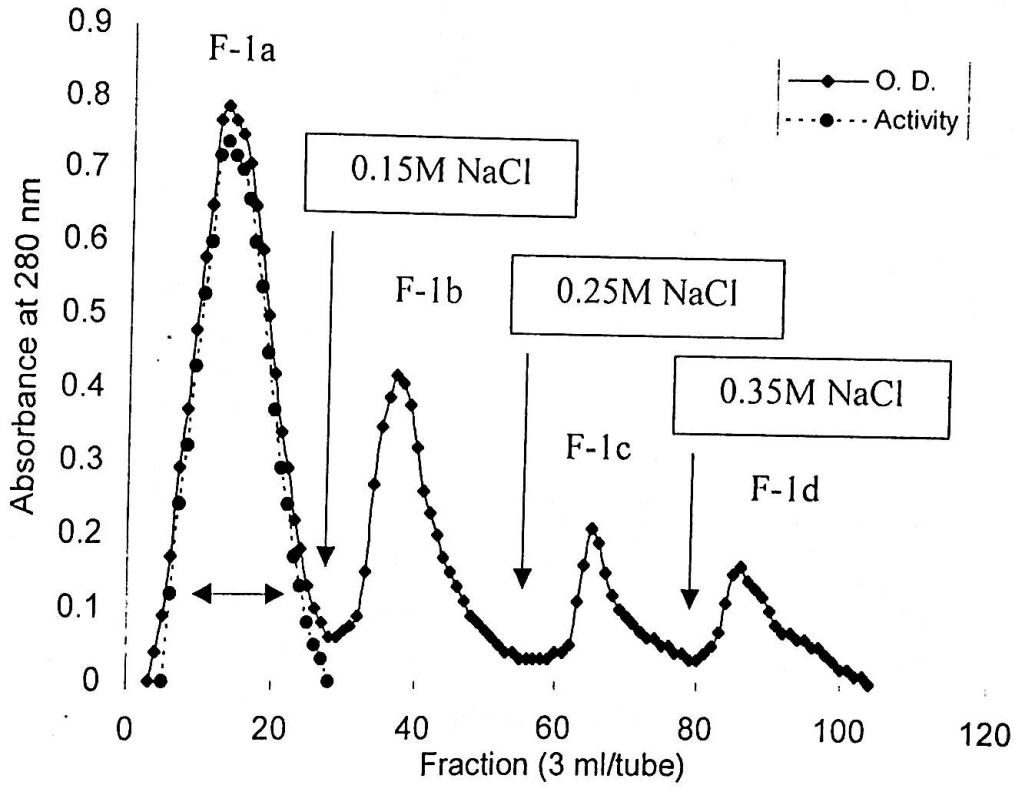


Fig-14: Ion-exchange chromatography of F-1 fraction on CM-cellulose. Fraction, F-1 (21 mg) obtained from DEAE-cellulose chromatography was applied to the column (1.25x16 cm) prewashed with 20 mM acetic acid-sodium acetate buffer, pH5.0 at 4°C and eluted by a stepwise increases of NaCl in the same buffer.

R.4.1.3 Gel filtration chromatography of F-1a fraction

The active fraction F-1a from CM-cellulose column chromatography was concentrated with commercial sucrose and applied to Sephadex G-75 column at 4^0C previously equilibrated with the same buffer. As shown in the Fig-4C, the contents of F-1a fraction were separated into three fractions, F-1a′, F-1a″ and F-1a‴ by gel filtration and it was found that only the fraction F-1a″ contained invertase activity. The area as indicated by solid bar of F-1a″ was pooled separately and its homogeneity was determined by SDS-polyacrylamide gel electrophoresis. The SDS-polyacrylamide gel electrophoresis of Sephadex G-75 fraction might be contained pure invertase enzyme as it gave a single band (Fig-15).

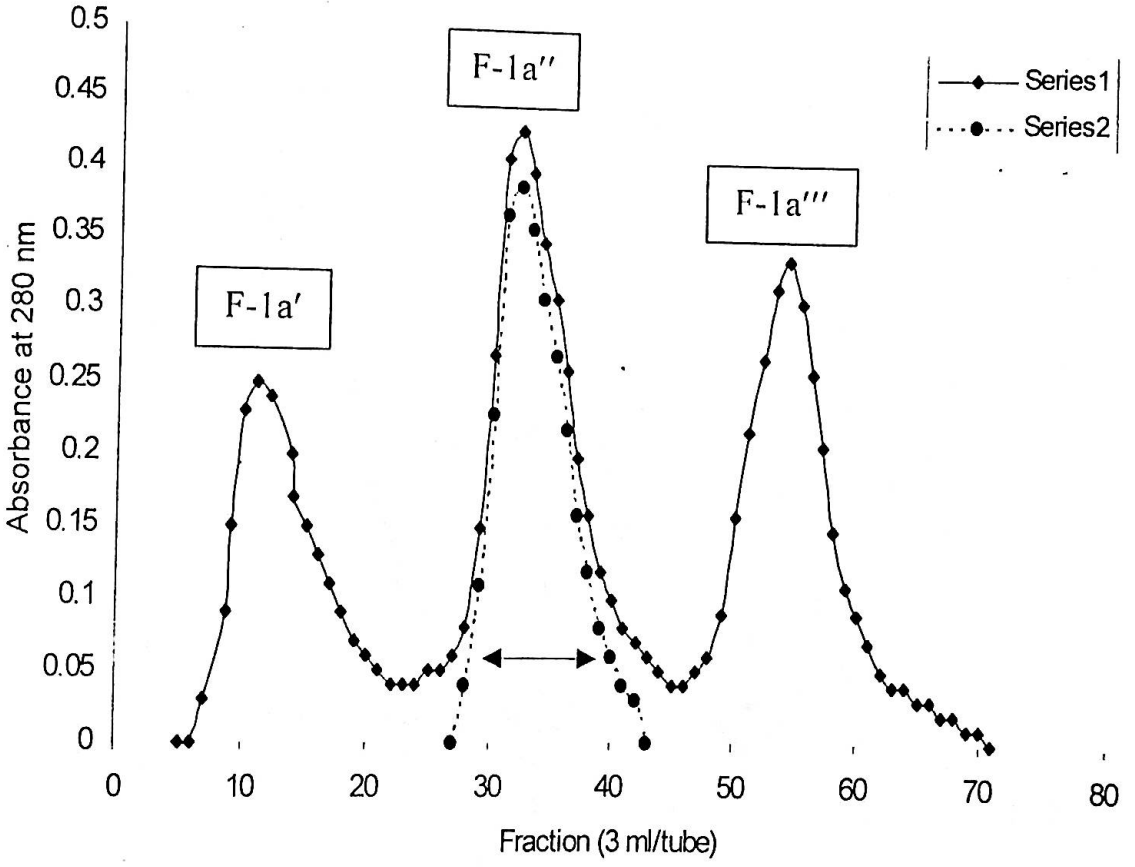


Fig-15: Gel filtration of F-1a fraction on Sephadex G-75. Fraction, F-1a (4.01 mg) obtained from CM-cellulose chromatography was applied to the column (3.0x100 cm) pre-equilibrated with 20 mM acetic acid-sodium acetate buffer, pH5.0 at 4^0C and developed with the same buffer.

R.4.1.4 CM-Cellulose Chromatography of F-4 fraction

The enzyme active fraction F-4, obtained from DEAE-Cellulose chromatography, was dialyzed against 20mM acetic acid-sodium acetate buffer, pH 5.0 for 24 hours and applied to CM-Cellulose column previously equilibrated with the same buffer at 4°C. As shown in Fig-16, the components of the fraction F-4 were separated from the column in three fractions. Of these fractions, F-4a was eluted from the column by the buffer only while the other fractions, i.e. F-4b and F-4c were eluted from the column by the buffer containing 0.15M and 0.30M NaCl respectively. It was found that the fraction F-4a contained only the invertase activity. The homogeneity of this fraction was checked by SDS-polyacrylamide gel electrophoresis. As shown in the Fig-16, F-4a fraction gave single band indicating that it contained pure invertase enzyme.

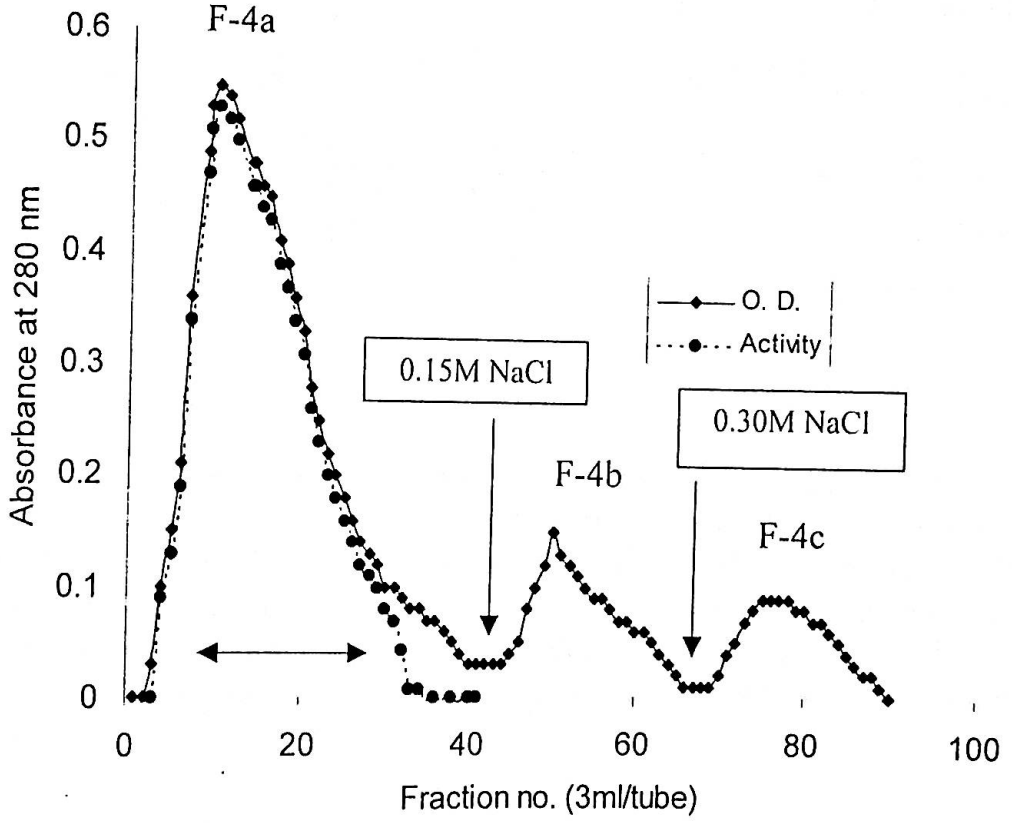


Fig-16: Ion-exchange chromatography of F-4 fraction on CM-cellulose. Fraction, F-4 (4.6 mg) obtained from DEAE-cellulose chromatography was applied to the column (1.25x16 cm) prewashed with 20 mM acetic acid-sodium acetate buffer, pH5.0 at 4°C and eluted by a stepwise increase of NaCl in the same buffer.

Table-12: Summary of the purification of betel leaf invertases

Fraction		Total protein (mg)	Total activity (Units)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Homogenate		660	1419	2.15	100	1
Crude extract		170	907.8	5.34	63.97	2.48
DEAE-Cellulose Chromatography	F-1	21	506.64	24.13	35.70	11.22
	F-4	4.6	127.96	27.82	9.02	12.94
CM-Cellulose Chromatography	F-1a	4.01	268.39	66.93	18.91	31.13
	F-4a	1.26	82.96	65.84	5.85	30.62
Sephadex G-75 gel filtration	F-1a''	1.24	92.65	74.42	6.53	34.61

The Table-12 summarizes the data pertaining to the purificatioin of betel leaf invertases. As presented in the table, the specific activities of invertases were increased at each purification steps. Although the yield of enzyme activity was decreased during the purification steps, but the purification fold of the invertases were increased to about 65 fold. The decrease in yield might be due to denaturation of enzymes during the lengthy purification procedures or some other reasons.

R.4.2 PURIFICATRION OF POLYPHENOL OXIDASE FROM BETEL LEAF

R.4.2.1 DEAE-Cellulose chromatography of crude enzyme extract

The crude enzyme extract after dialysis against 0.1M phosphate buffer, pH6.0 was applied on the DEAE-Cellulose column previously equilibrated with phosphate buffer of the same pH and concentration at 4°C. The crude enzyme extract was first eluted with the buffer only and then with the increasing concentration of NaCl. The elution profiles are shown in Fig-17. The proteins of crude enzyme extract were eluted from the column in five fractions. The major fraction, F-1 was eluted from the column by the buffer only, while the other four fractions namely F-2, F-3, F-4 and F-5 were eluted from the column by the buffer containing 0.1M, 0.2M, 0.25M and 0.3M respectively. It was found that the fractions F-1 contained polyphenoloxidase activity. The area as indicated by solid bar was pooled separately and the purity was checked by SDS-polyacrylamide gel electrophoresis. As shown in the Fig-19, F-1 fraction gave more than one band on gel indicating that it contained some other proteins and it was further purified by ion-exchange chromatography on CM-Cellulose.

R.4.2.2 CM-Cellulose Chromatography of F-1 fraction

The enzyme active fraction F-1, obtained from DEAE-Cellulose chromatography, was dialyzed against 0.1M phosphate buffer, pH6.0 for 24 hours and applied to CM-Cellulose column previously equilibrated with the same buffer at 4°C. As shown in Fig-18, the components of the fraction F-1 were separated from the column in four fractions. Of these fractions, F-1a was eluted from the column by the buffer only while the other fractions, i.e. F-1b, F-1c and F-1d were eluted from the column by the buffer containing 0.15M, 0.25M and 0.35M NaCl respectively. It was found that the fractions F-1b contained only the polyphenoloxidase activity. The homogeneity of this fraction was checked by SDS-polyacrylamide gel electrophoresis. As shown in the Fig-19, F-1b fraction gave single band indicating that it contained pure protein.

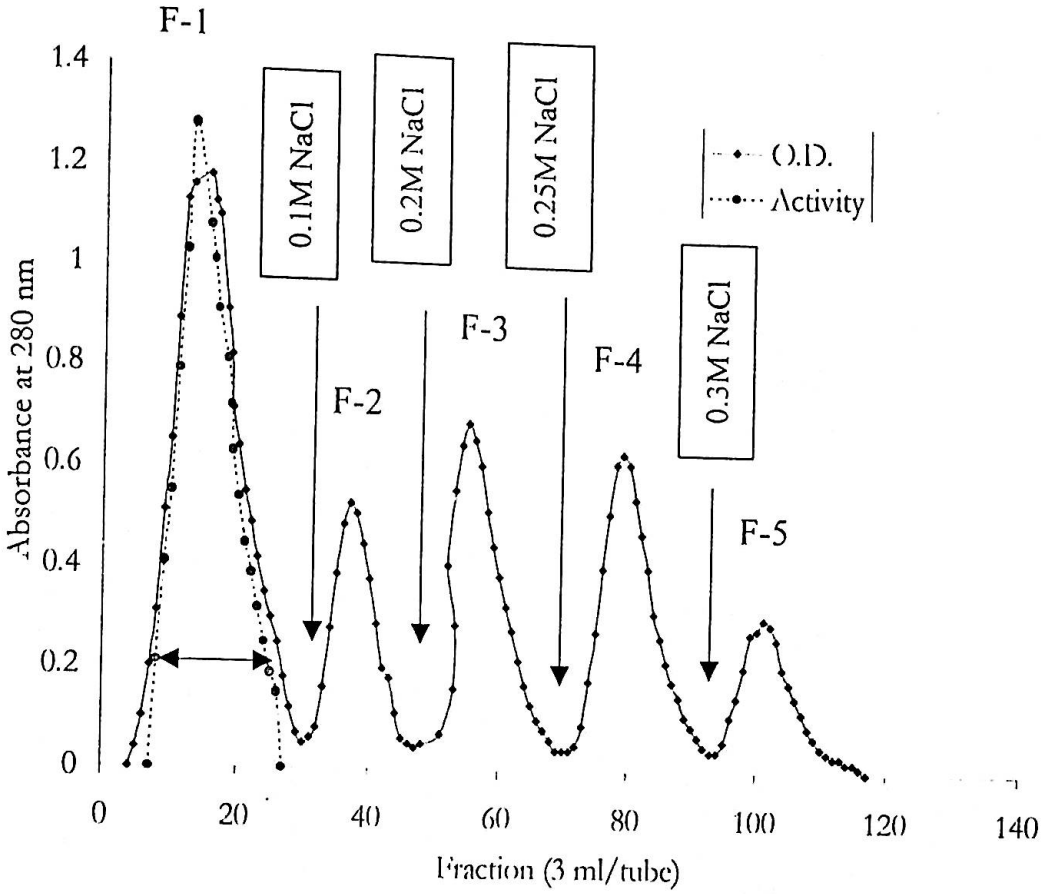


Fig-17: Ion-exchange chromatography of crude enzyme extract on DEAE-cellulose. Crude enzyme extract (116 mg) was applied to the column (2.0x24 cm) prewashed with 0.1M phosphate buffer, pH6.0 at 4^0C and eluted by a stepwise increases of NaCl in the same buffer.

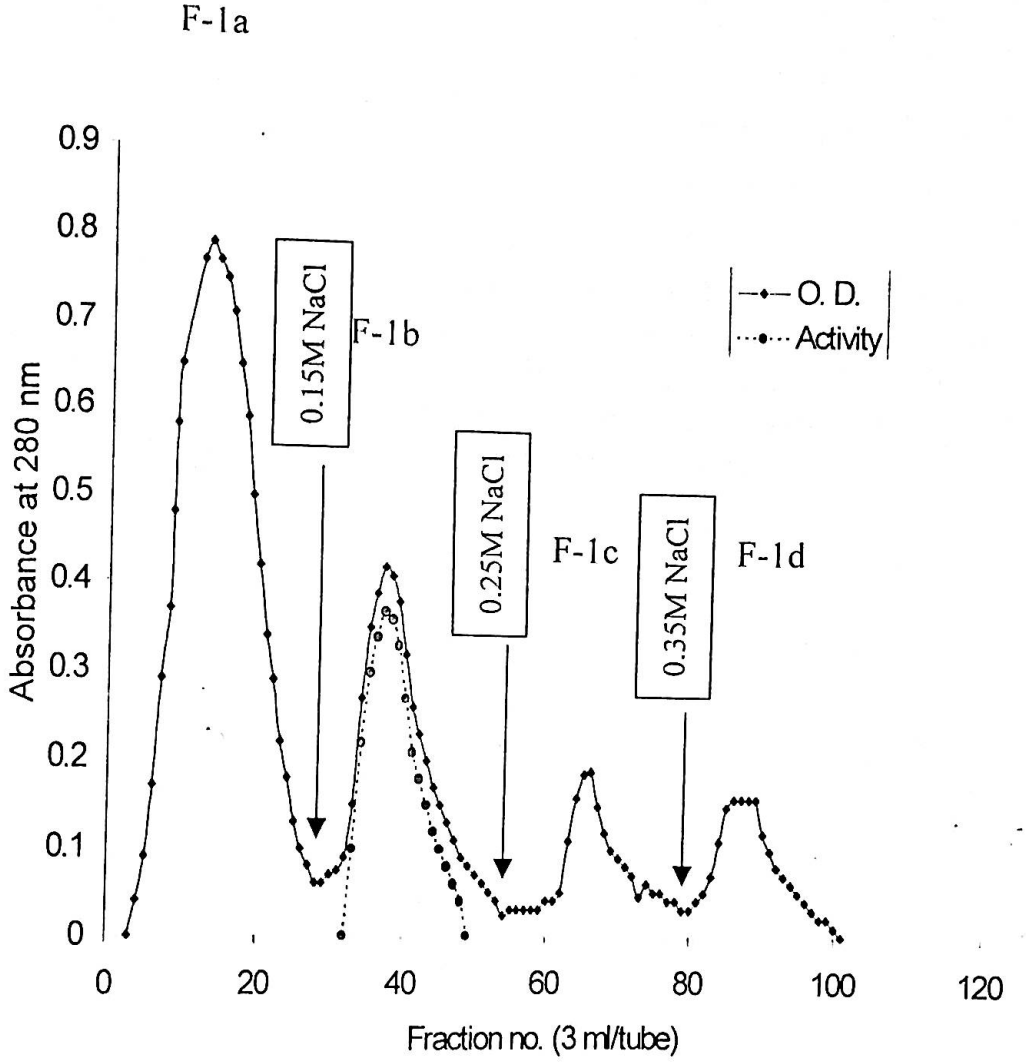


Fig-18: Ion-exchange chromatography of F-1 fraction on CM-cellulose. Fraction, F-1 (4.15 mg) obtained from DEAE-cellulose chromatography was applied to the column (1.25x16 cm) prewashed with 0.1M phosphate buffer, pH6.0 at 4^0C and eluted by a stepwise increases of NaCl in the same buffer.

Table- 13: Summary of the purification of betel leaf Polyphenol oxidase

Fraction		Total protein (mg)	Total activity (Units)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Homogenate		450	540	1.20	100	1
Crude extract		116	295	2.54	54.63	2.12
DEAE-Cellulose Chromatography	F-1	4.15	137.4	33.11	25.44	27.59
CM-Cellulose Chromatography	F-1b	1.24	55.7	44.92	10.31	37.43

The Table-13 summarizes the data pertaining to the purificatioin of betel leaf polyphenoloxidase. As presented in the table, the specific activity of polyphenoloxidase was increased at each purification step. Although the yield of enzyme activity was decreased during the purification steps, but the purification fold of the polyphenoloxidase was increased to about 37 fold.

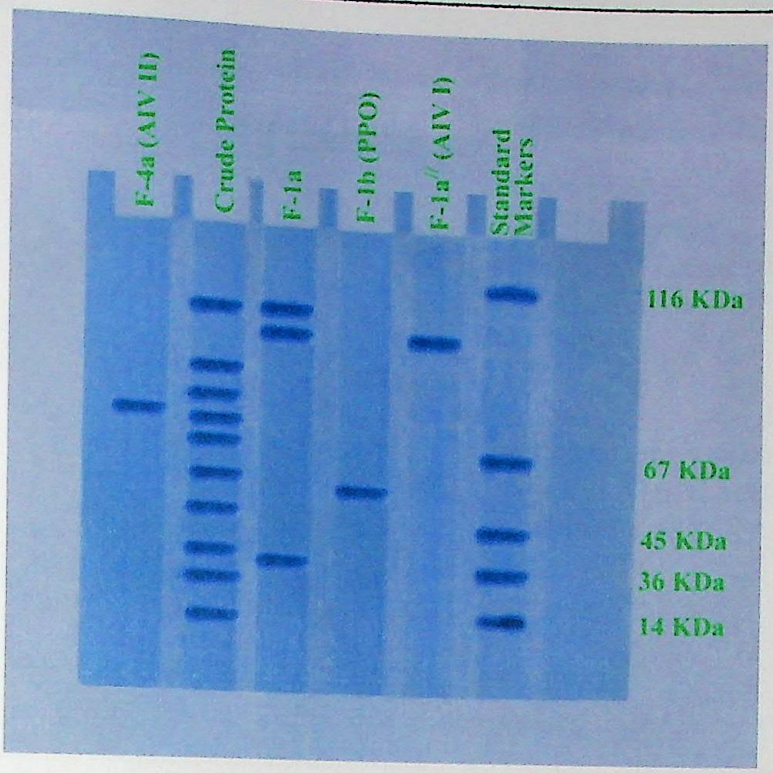


Fig-19: SDS-Polyacrylamide gel electrophoretic pattern of proteins/enzymes.

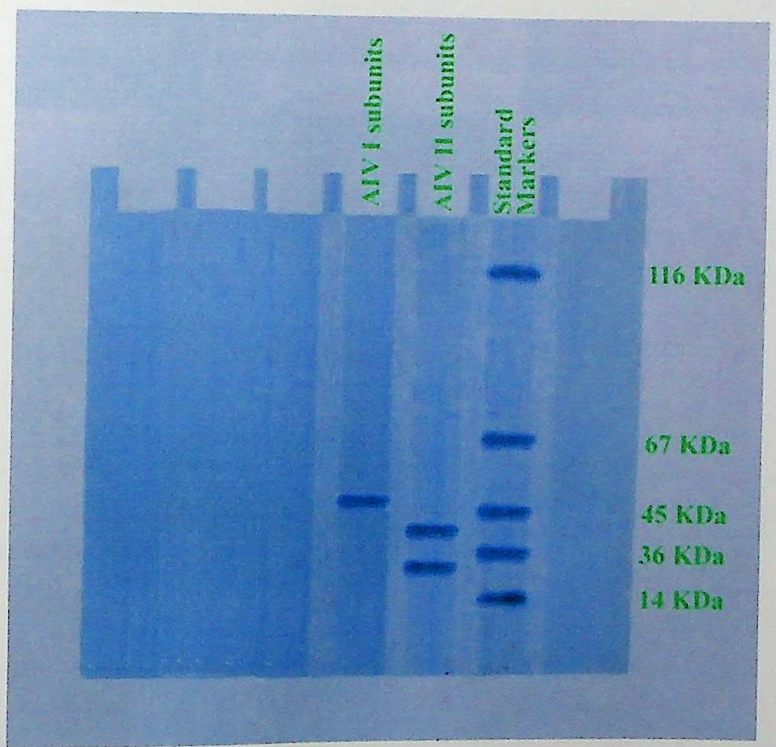
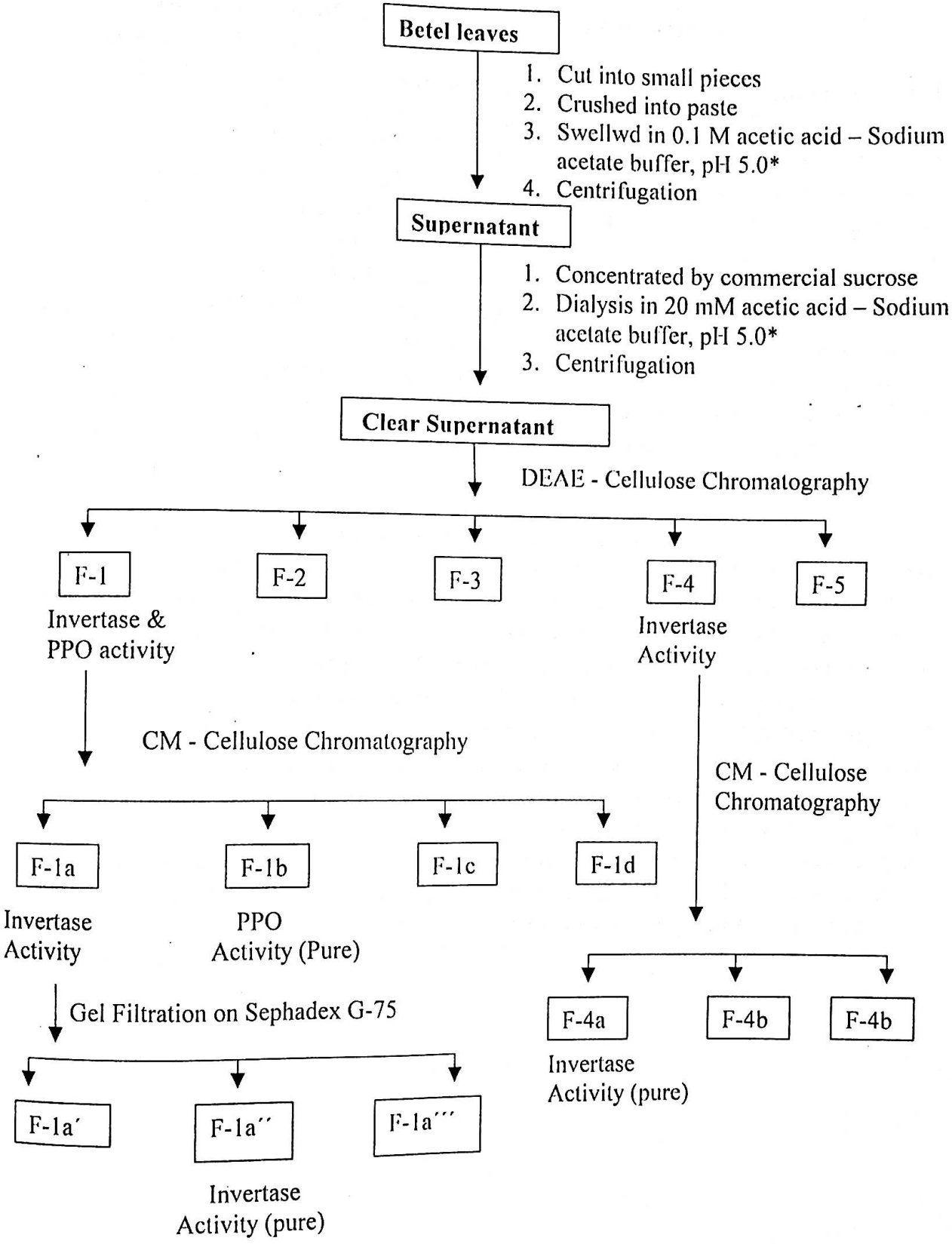


Fig-20: SDS-Polyacrylamide gel electrophoretic pattern of purified enzymes in the presence of 2-mercaptoethanol.

Fig-21: Schematic representation of the purification steps of betel leaf invertase and polyphenoloxidase.



* For polyphenol oxidase purification: 0.1M phosphate buffer, pH6.0 was used.

R.4.3 CHARACTERIZATION OF INVERTASES OF BETEL LEAVES

R.4.3.1 Determination of molecular weight of invertases

R.4.3.1.1 By gel filtration

The molecular weight of purified betel leaf invertases were determined by gel filtration on a Sephadex G-150 according to Andrews (1965). Trypsin inhibitor (MW-20kDa), Egg Albumin (MW-45kDa), Bovine Serum Albumin (MW-67kDa) and β-galactosidase (MW-116kDa) were used as molecular weight markers. The molecular weight was calculated from the standard curve of references proteins which was constructed by plotting the molecular weight of the proteins against elution volumes and the molecular weight (Mr) was estimated to be 94 kDa for acid invertase I (AIV I/F-1a'') and 72 kDa for acid invertase II (AIV II/F-4a). Very similar Mr (98 kDa) determined by gel filtration was reported for invertase from *oryza sativa* (Maria Ines Isla *et al.*, 1995). Darren. J. *et al* (1998) reported 60 kDa Mr invertase from sugarcane and Rahman. M.H. *et. al.* (2001) reported 68 kDa Mr invertase from mango fruit while Mr 84 kDa was estimated by Quiroga EN *et. al.* (1995) from *Pycnoporus sanguineus.*

R.4.3.1.2 By SDS-polyacrylamide gel electrophoresis

The molecular weight of purified betel leaf invertases were determined by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The molecular weight markers were the same as used in gel filtration. The standard curve of these proteins was constructed by plotting the molecular weight of the proteins against their mobility on the gel (Fig-23). The molecular weight (Mr) was estimated to be 93.5 kDa for acid invertase I (AIV I) and 71.5 kDa for acid invertase II (AIV II). In the presence of 2-marcaptoethanol, SDS-polyacrylamide gel electrophoresis pattern of acid invertase I (AIV I) showed single band on the gel with an Mr of 46 kDa indicating that the enzyme was a dimer consisting of two identical subunits. On the other hand, acid invertase II (AIV II) gave two distinct bands on the gel in the presence of 2-mercaptoethanol during SDS-polyacrylamide gel electrophoresis with Mr 40.5 kDa and 31 kDa indicating that the enzyme was a hetero dimer.

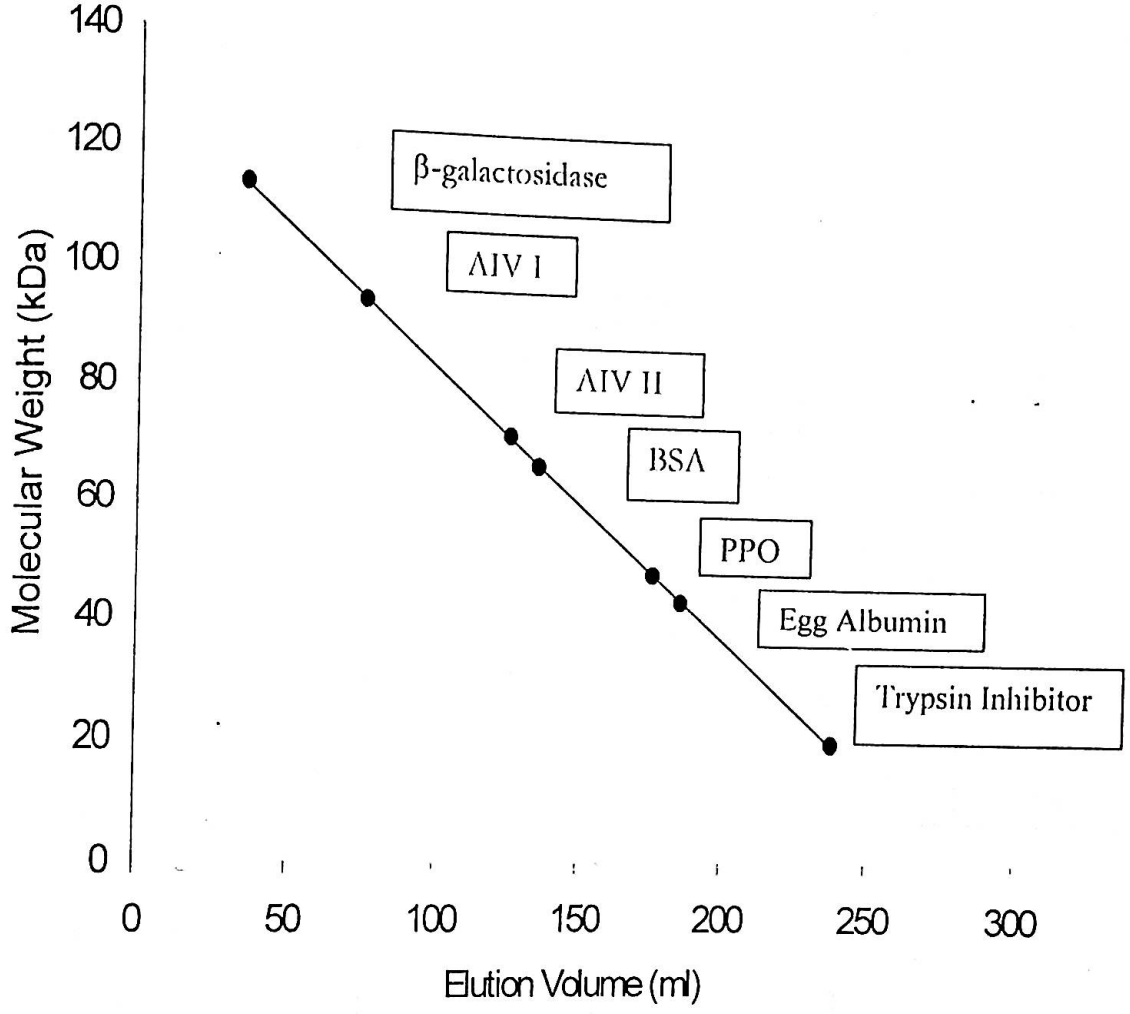


Fig-22: Standard curve for the determination of molecular weight of betel leaf invertases and polyphenol oxidase by gel filtration method.

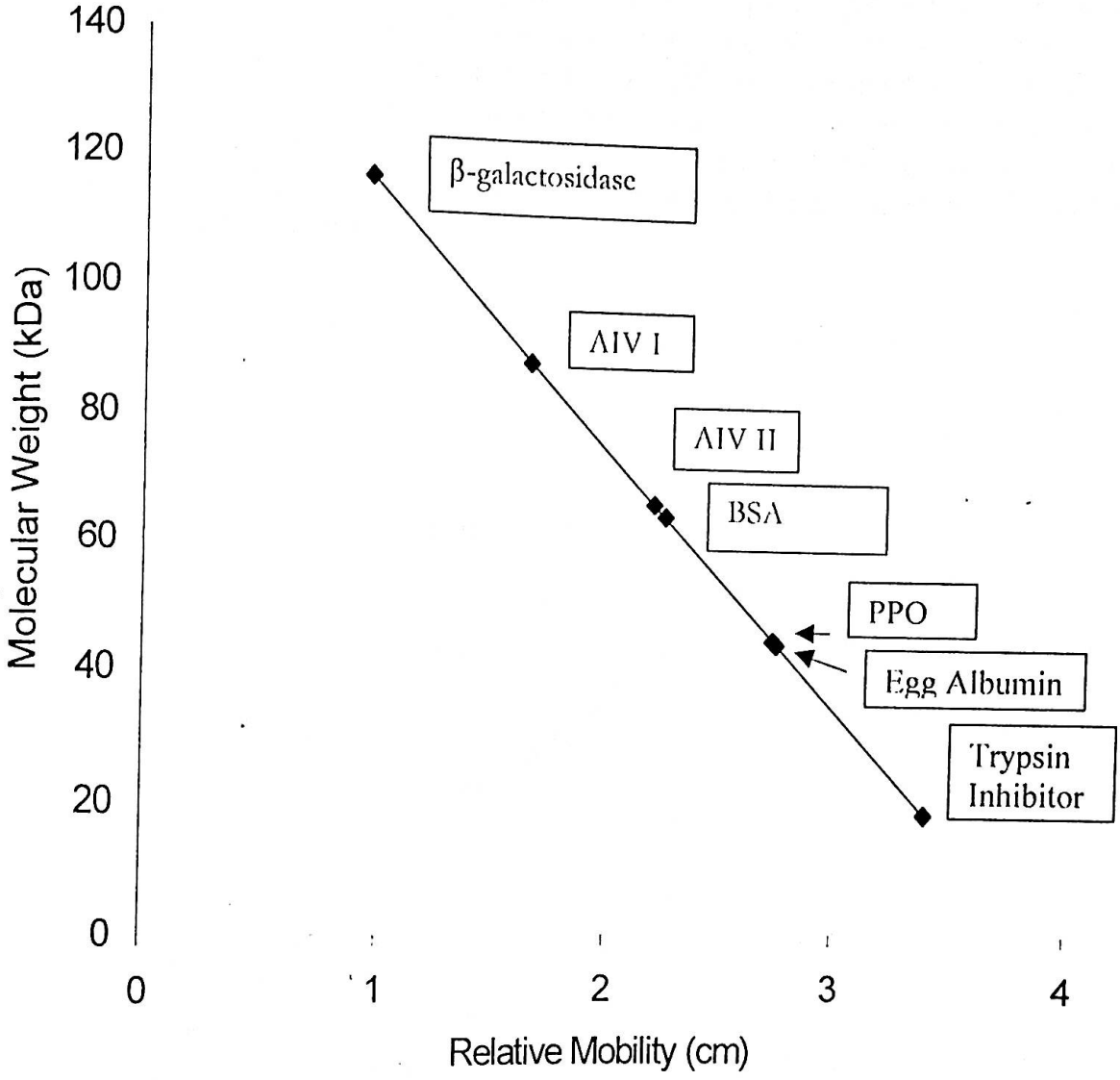


Fig-23: Standard curve for the determination of molecular weight of betel leaf invertases and polyphenol oxidase by SDS-PAGE method.

R.4.3.2 Km value of the purified invertases

The Km value of the purified invertases were determined by the Lineweaver-Burk double reciprocal plot (Fig.-24 and Fig.-25). The Km values of AIV I and AIV II against sucrose as substrate were found to be 3.86 mM and 4.81 mM at pH 5.0 in acetic acid-sodium acetate buffer. Hashizume *et. al* (2003) reported the Km values for sucrose of AIV I and AIV II from Japanese pear fruit using sucrose as substrate were 3.33 and 4.58 mM, respectively. Konno *et. al.* (1993) reported 4.35 mM Km value for tomato invertase. Isla *et. al.* (1995) observed Km value of 6.6 mM for oryza sativa invertase using sucrose as substrate. Km values of invertase were reported to be 3mM for rose potato and 5.3 mM for kennebee potato invertase (Pressy, R., 1967). Lee *et. al.* (1996) reported higher Km value (20 mM at pH 7.5) for carrot invertase.

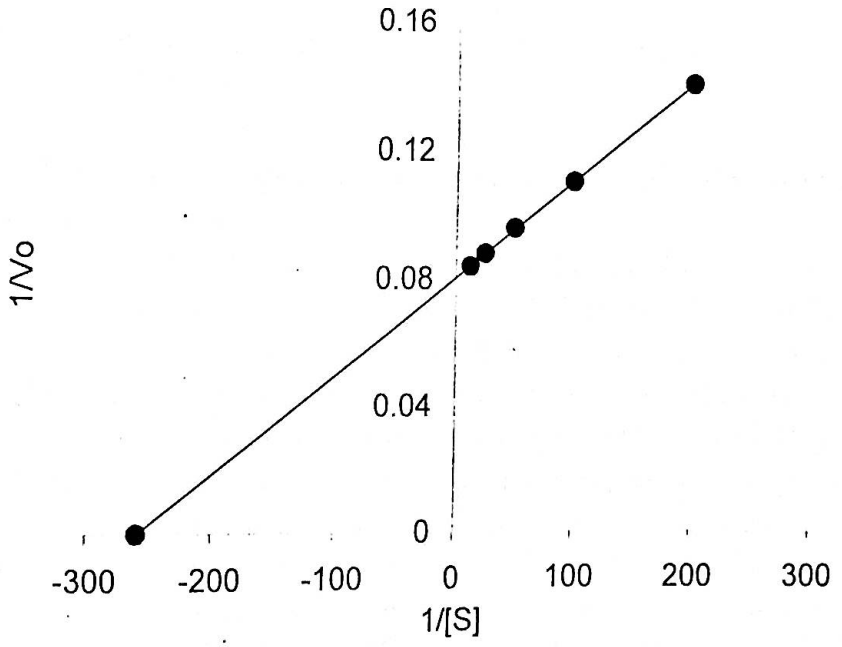


Fig-24: Linweaver-Burk double reciprocal plot for the determination of Km value of the purified betel leaf AIV I.

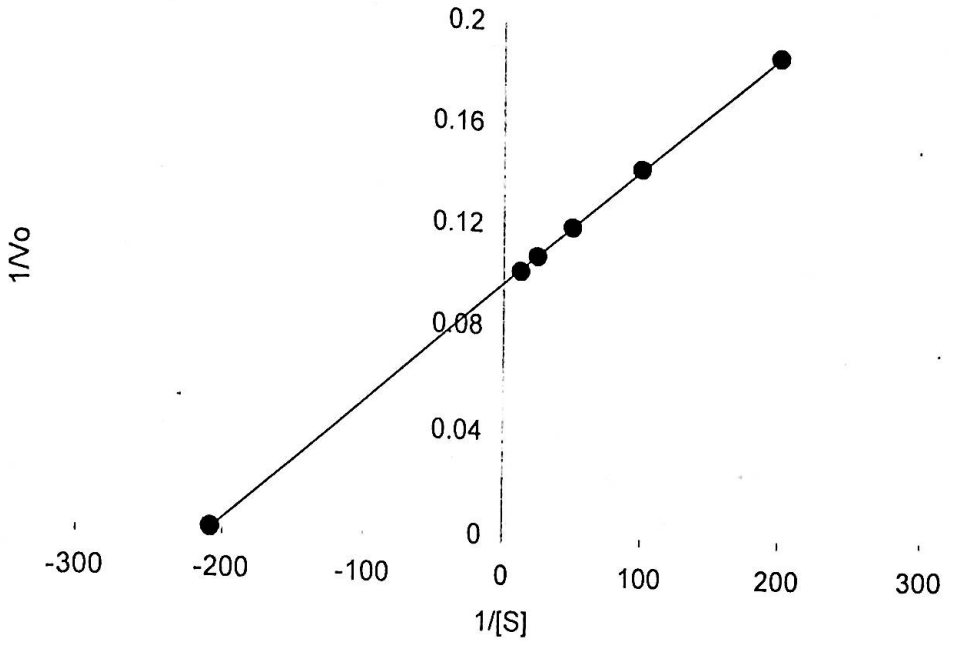


Fig- 25: Linweaver-Burk double reciprocal plot for the determination of Km value of the purified betel leaf AIV II.

R.4.3.3 Test for glycoprotein and estimation of the percentage of sugar in betel leaf invertases

The purified betel leaf invertases were glycoprotein in nature as they gave yellow-orange color with the phenol-sulfuric acid (Crueger, W. and Crueger, A., 1990). The percentage of sugar present in the glycoprotein was calculated from the standard curve of glucose (Fig-26) and it was found that the AIV I and AIV II contained 14.6 % and 19.4% of neutral sugar. Peter *et. al.*(1994) and Nakagawa *et. al.*(1971) also showed purified tomato invertase as a glycoprotein. Like tomato invertase, many plant invertases have been reported as glycoproteins (Rahman, M.H. *et. al.*, 2001; Anderson, R.S. *et. al.*, 1978; Krishan, H.B. *et. al.*, 1988; Singh, M.B. *et. al.*, 1984). Grape berries invertase has been reported to contain about 25% carbohydrate (Arnold, W.N., 1965) while Geracimo *et al.* (1990) showed the purified potato imvertase as a glycoprotein with 10.9% carbohydrate. Carbohydrate content in grape juice invertase was found to be 330 microgram as glucose per mg protein (Nakanishi *et. al.*, 1990). Rodriguez, J. *et. al.*(1995) noticed that invertase from *Pichia anomala* contaimed carbohydrate moiety accounts for approx. 30% of the total mass of the molecule.

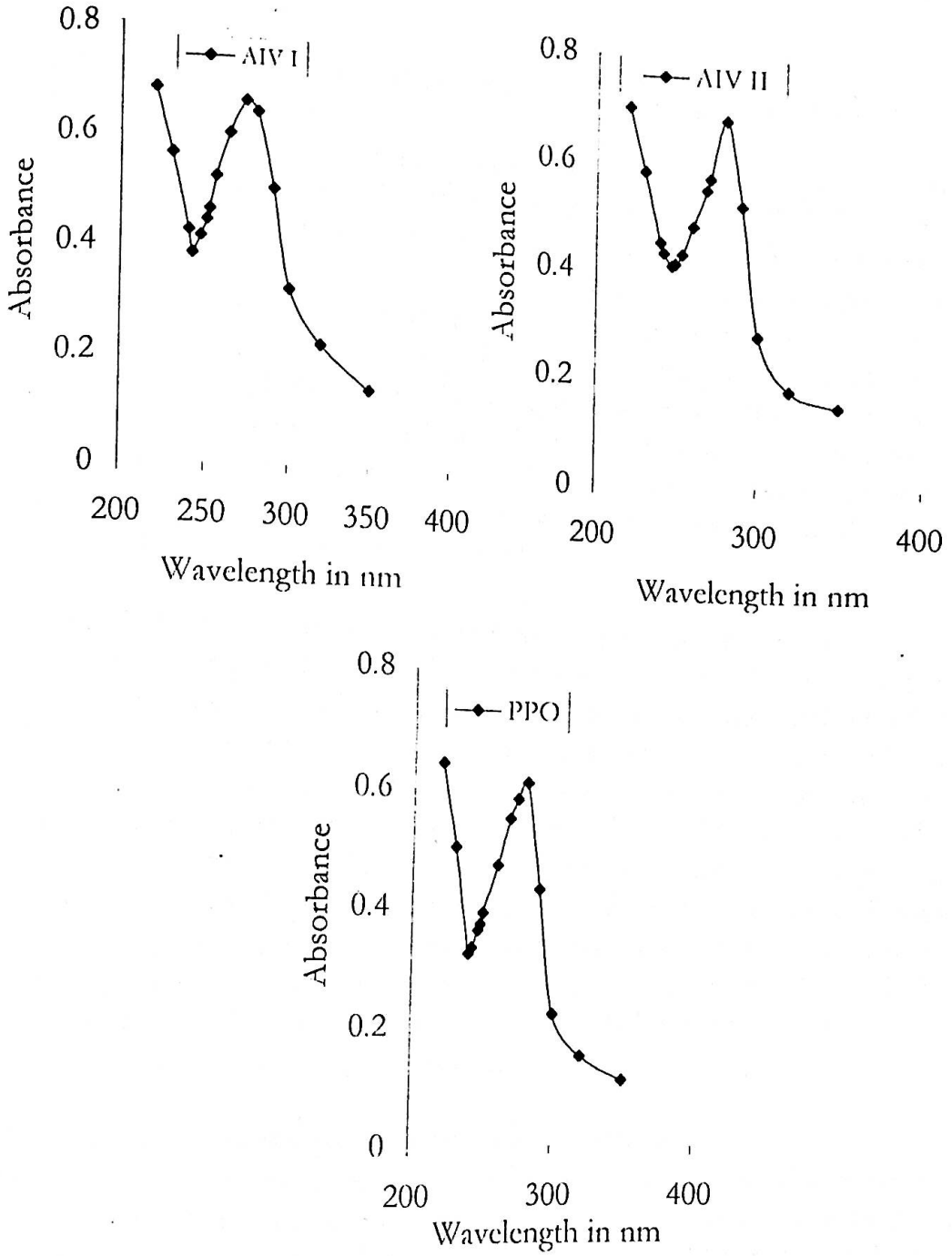


Fig- 27: UV Absorption Spectra of invertases and polyphenol oxidase enzyme.

R.4.3.5 Protein concentration-Absorbance relationship of betel leaf invertases

The absorbance of 1.0 for AIV I and AIV II at 280 nm were found to be equal to 0.53 mg/ml and 0.42 mg/ml of protein by Lowry method.

R.4.4 CHARACTERIZATION OF POLYPHENOLOXIDASE OF BETEL LEAVES

R.4.4.1 Determination of molecular weight of polyphenol oxidase

R.4.4.1.1 By gel filtration

The molecular weight of purified betel leaf polyphenol oxidase was determined by gel filtration on a Sephadex G-150 according to Andrews (1965). Trypsin inhibitor (MW-20kDa), Egg Albumin (MW-45kDa), Bovine Serum Albumin (MW-67kDa) and β-galactosidase (MW-116kDa) were used as molecular weight markers. The molecular weight was calculated from the standard curve of reference proteins which was constructed by plotting the molecular weight of the proteins against elution volumes and the molecular weight (Mr) was estimated to be 48 kDa. Nearly the same Mr (47 kDa) of polyphenol oxidase was reported from garland chrysanthemum by gel filtration (Nkya, E. *et. al.*, 2003). Yang, C.P. *et. al.* (2001) reported 41 kDa Mr for polyphenol oxidase from banana peel while they reported 41 kDa Mr for polyphenol oxidase from banana pulp.

R.4.4.1.2 By SDS-polyacrylamide gel electrophoresis method

The molecular weight of purified betel leaf polyphenol oxidase was determined by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The molecular weight markers were the same as used in gel filtration. The standard curve of these proteins was constructed by plotting the molecular weight of the proteins against their mobility on the gel (Fig.-23). The molecular weight (Mr) was estimated to be 45.5 kDa for polyphenol oxidase. In the presence of 2-marcaptoethanol, SDS-polyacrylamide gel electrophoresis pattern showed single band on the gel with the same Mr indicating that the enzyme was a monomer. This study is a good agreement with Nkya, E. *et. al.*, (2003); Yang, C.P. *et. al.* (2000) and Shi, C. *et. al.*, (2002) who showed monomeric polyphenol oxidase from garland chrysanthemum, banana pulp and tobacco respectively.

R.4.4.2 Km value of the purified polyphenol oxidase

The Km value of the purified polyphenol oxidase was determined by the Lineweaver-Burk double reciprocal plot (Fig.-28). The Km value of polyphenol oxidase enzyme against catechol as substrate was found to be 3.54 mM. Shi, C. *et. al.* (2002) reported the Km value of 1.2 mM for polyphenol oxidase using catechol as substrate. Km value of polyphenol oxidase was reported to be 2.8 mM from banana pulp (Yang, C.P. *et. al.*, 2000) and 3.9 mM from banana peel (Yang, C.P. *et. al.*, 2002) using dopamine as substrate. Wititsuwannakul, D. et. al. (2002) showed Km values of 9.09 mM and 7.14 mM for two polyphenol oxidase from latex of *Hevea brasiliensis* using catechol as substrate.

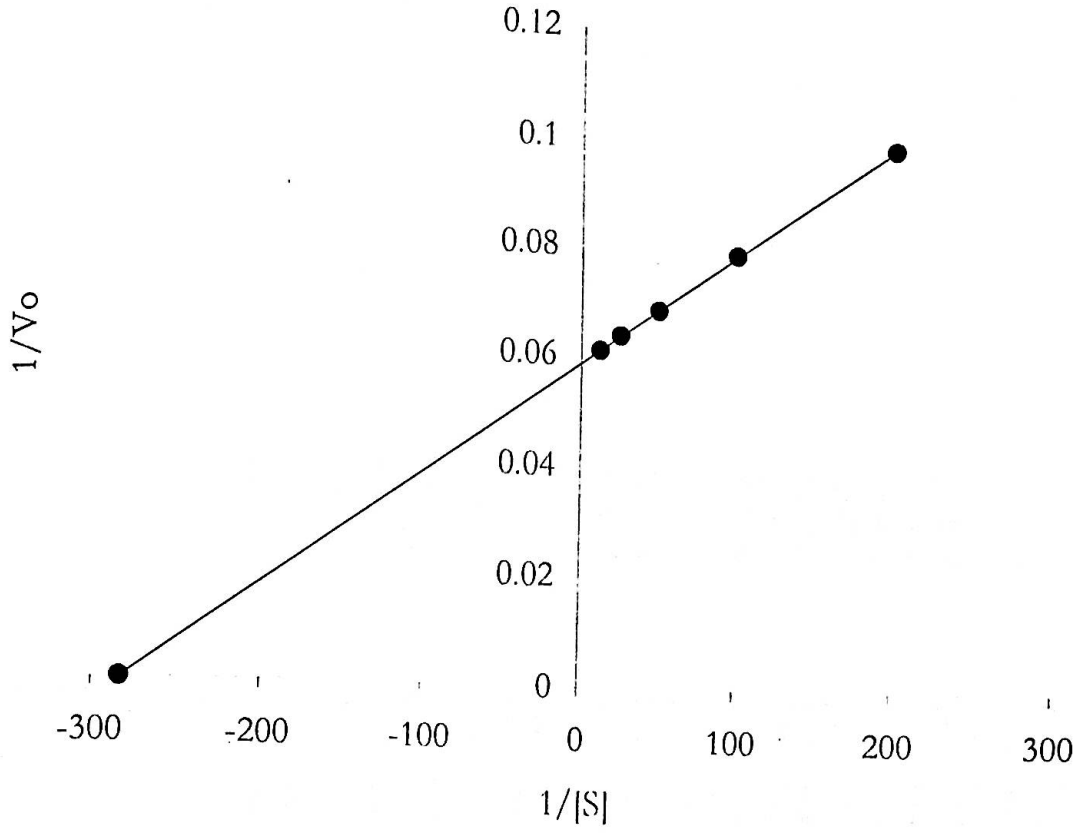


Fig- 28: Linweaver-Burk double reciprocal plot for the determination of Km value of the purified betel leaf polyphenol oxidase.

R.4.4.3 Ultraviolet absorption spectrum of betel leaf polyphenol oxidase

The ultraviolet absorption spectrum of the purified enzyme was recorded in aqueous solution with Shimadzu model UV-180 double beam spectrophotometer at room temperature between 350-220 nm. The purified polyphenol oxidase in aqueous solution gave absorption maxima around 278 nm and minima around 240 nm (Fig-27).

R.4.4.4 Protein concentration-Absorbance relationship of betel leaf polyphenol oxidase

The absorbance of 1.0 for polyphenol oxidase at 280 nm was found to be equal to 0.52 mg/ml of protein by Lowry method.

CHAPTER FIVE

EFFECTS OF PHYSICO-CHEMICAL AGENTS ON THE ACTIVITY OF INVERTASES AND POLYPHENOL OXIDASE

Introduction

The three dimensional structure of a protein or enzyme is governed by its primary structure and its environment. The organized native structure (conformation) of a protein is known to be affected from the effect of external environmental changes such as temperature, acidity, urea or other denaturing agents and a number of other chemicals.

In structural studies of proteins it is often necessary to establish conditions for reversible denaturation. The choice of denaturating conditions depends on the stability of the protein or enzyme of interest. Among the techniques used for reversible denaturation are lowering of the pH (Itano *et al.* 1958), freezing and thawing in concentrated salt (Market, C.L. 1963), and adding denaturants such as urea and acetic acid (Chilson *et al.* 1964: and 1965: Meighen and Schachman 1970).

In the present study, invertases and polyphnol oxidase have been subjected to various physical and chemical treatments, and their effects on the activities have been observed .The study is expected to provide important informations regarding some of the physico-chemical properties such as pH stability, thermal stability, the stability of the enzymes towards denaturing agents, the structural configuration of the active sites of the enzymes, the nature of the inhibitors of the enzymes, conception of positive and negative modifier of the enzymes etc. The experimental results also give indications in establishing conditions for chemical modification which in turn, are expected to be helpful in understanding the relationship between structure and function.

Effects of Physical and chemical agents on the activity of enzymes

M.5.1 Effects of pH and determination of optimum pH of betel leaf invertases and polyphenol oxidase

The invertases and polyphenol oxidase activities at various pHs (2-10) were measured at 37°C following the procedure according to method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). Sucrose solution (2.5%) for invertases and 0.01 M catechol solution for polyphenol oxidase were made in the above mentioned buffer of different pHs and were used as substrates for the determination of enzyme activities.

M.5.2 Effects of temperature and determination of optimum temperature of betel leaf invertases and polyphenol oxidase

The activities of invertases and polyphenol oxidase at different temperatures (0-80°C) were measured according to method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). To examine the temperature effect and optimum temperature of the enzymes, the enzyme solutions were pre-incubated at various temperatures (10-80°C) for 30 minutes and cooled at 0°C. The residual activities of the treated enzymes were assayed under the standard assay conditions.

M.5.3 Effects of various chemicals and metallic salts on the activity of betel leaf invertases and polyphenol oxidase

Enzyme activities were determined in the presence of various concentrations of chemicals following the procedure according to method as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982). The chemicals/metallic salts of different concentrations were added to enzyme solution and incubated for 30 minutes at room temperature. Then the enzymatic activities of the samples were assayed as described earlier.

M.5.4 Effects of various proteins and lectins on the activity of betel leaf invertases

Enzyme activities were determined in the presence of various concentrations of different proteins and lectins following the procedure as described in Physiological Plant Pathology (Mahadevan and Sridhar, 1982).

Results and Discussions

R.5.1 Effects of pH on the activities of betel leaf invertases and polyphenol oxidase

The activities of betel leaf invertases against sucrose as substrate and activities of betel leaf polyphenol oxidase against catechol as substrate were assayed using different buffers of various pHs ranging from 2-10 (pH2 chloride buffer, pHs3-6 acetic acid-sodium acetate buffer, pHs7-8 phosphate buffer and pHs 9-10 sodium borate-HCl buffer). As shown in the figure-29, the activities of all the enzymes are affected remarkably with the changes of pH. Both the invertases AIV I and AIV II as well as polyphenol oxidase are more active at the acidic pH than the neutral or basic pH values. Again all the enzymes are inactive at the extreme acidic pH values and more than 80% activities were destroyed at or below pH 2.0. The optimum pH for the enzymes, AIV I, AIV II and polyphenol oxidase were found at pH4.5, pH5.5 and pH6.2 respectively. Very similar pattern of pH profiles were reported for cherry invertase (Hari B. *et. al.*, 1990), invertase of *Oryza sativa* (Isla *et. al.*, 1995), Grape berries (Ishikawa, *et. al.*, 1989) and for tomato invertase (Peter, B. and Stephanie, D., 1994). Nunez-Delicado E. *et. al* (2003) reported optimum pH5.5 for latent persimmon fruit polyphenol oxidase. Wititsuwannakul, D. *et. al.* (2002) reported optimum pH7.0, Yang, C.P. *et. al.* (2001) reported optimum pH6.5, Shi. C. *et. al.* (2002) reported optimum pH 6.0 and Nagai, T. *et. al.* (2001) reported optimum pH5.0 for polyphenol oxidase activity.

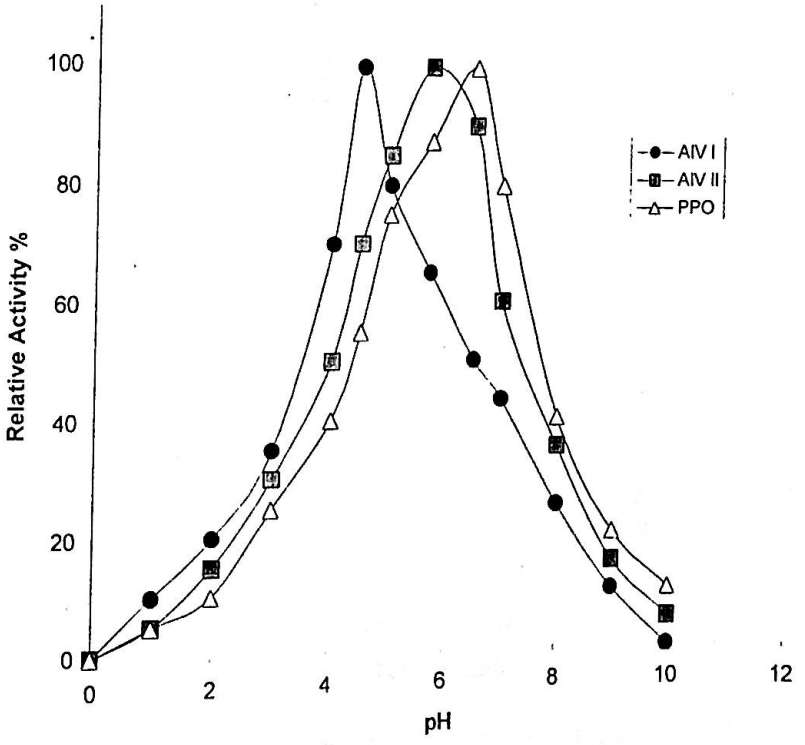


Figure-29: Effect of pH on the activities of the enzymes.

R.5.2 Effects of temperature on the activities of betel leaf invertases and polyphenol oxidase

The activities of purified invertases and polyphenol oxidase were found to be affected profoundly by temperature. As shown in the figure-30, the activities of invertases and polyphenol oxidase were increased gradually with increase in temperature and the maximum activity was observed at 37°C for AIV I, 35°C for AIV II and 32°C for PPO. Further, the activities were gradually decreased with further rise of temperature and there was a drastic drop in the activity above 50°C. Finger, *et. al.* (2000) reported the optimum temperature of 35°C for acid invertase from Beta vulgaris roots. They also noted that the rapid inactivation of that enzyme occurred at 40°C and complete inactivation was observed at 55°C. Dauvrin *et. al.* (1989) reported the optimum temperature of 50°C for invertase from the rumen holotrich ciliate *Isotricha prostoma.* The PPO enzyme showed maximum activity at 32°C. At 60°C, the PPO enzyme showed only 10% activity and became completely inactive at 80°C. Wititsuwannakul, D. *et. al.* (2002) reported optimum temperature for PPO at 35-45° C from latex of *Hevea brasiliensis* while Yang, C.P. *et. al.* (2001) reported optimum temperature at 30°C from banana pulp and Shi. C. *et. al.* (2002) reported optimum temperature at 40°C from tobacco.

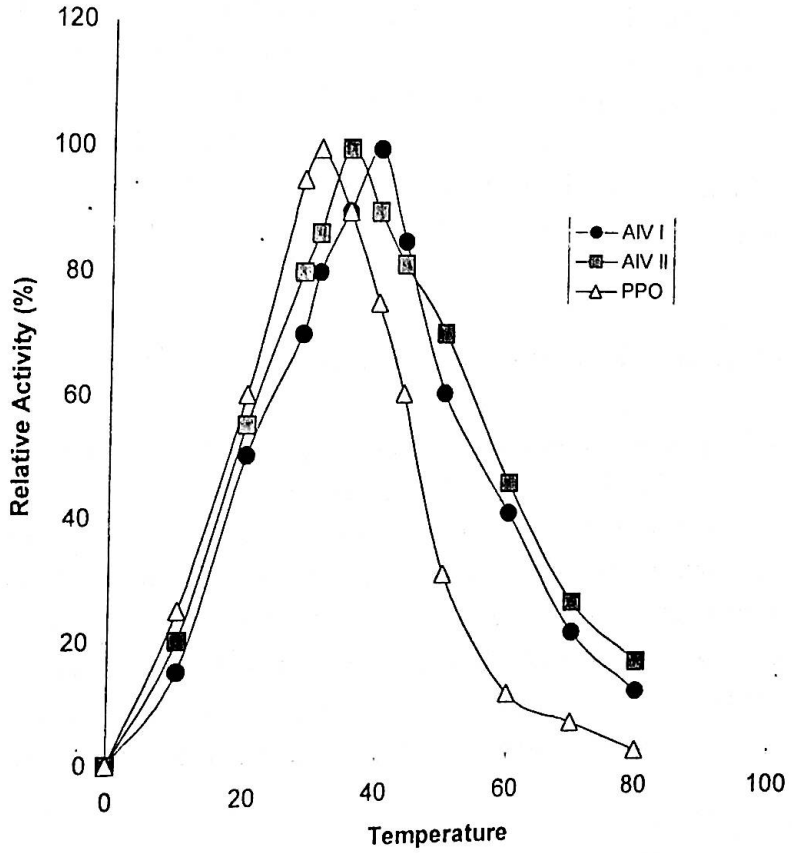


Figure-30: Effect of Temperature on the activities of the enzymes

R.5.3 Effects of various chemicals on the activities of betel leaf invertases and polyphenol oxidase

R.5.3.1 Effects of various chemicals on the activities of betel leaf invertases

The effects of various chemicals (viz. Tris, Glucose, Fructose, EDTA and Acetic acid) on the activity of betel leaf invertases were cited in the Table-14. It was found that the activity of invertase was gradually decreased with the increased concentration of all of these chemicals. Activities of acid invertase I (AIV I) were inhibited poorly by Tris (3.69%-17.26%), glucose (2.54% - 11.82%) and fructose (5.68% - 20.55%) while Acid invertase II (AIV II) activity was inhibited 1.36% - 14.63% by Tris, 1.28% - 13.17% by glucose and 4% - 17.60% by fructose respectively. Alexander (1969) found that acid invertase was 25% inhibited by tris of 0.05M concentration. Product inhibition of invertase has been demonstrated in many species (Van den *et. al.*, 1995; Lee *et. al.*, 1996; Ross *et. al.*, 1996). Betel leaf invertases were also inhibited by their products glucose and fructose. Fructose is slightly stronger inhibitor than glucose and invertase activities decreased with the increase in concentration of glucose and fructose. From this study, it is clear that glucose and fructose are competitive inhibitors of invertase. Darren *et. al.* (1998) found that sugarcane neutral invertase was inhibited about 27% and 37% by its products glucose and fructose respectively. On the other hand, moderate to significant inhibition effects of the invertases were found by EDTA and acetic acid. The activities of acid invertases, AIV I and AIV II were inhibited sequentially with increase in concentration of EDTA and acetic acid, and at 5mM concentration the activities were found to be about 50% by EDTA and about 45 – 49% by acetic acid.

R.5.3.2 Effects of various chemicals on the activities of betel leaf polyphenol oxidase

The effects of various chemicals (viz. Tris, EDTA and Acetic acid) on the activities of betel leaf polyphenol oxidase were cited in the Table-15. It was found that the activity of polyphenol oxidase was gradually decreased with the increased concentration of all of these chemicals. Moderate to significant inhibition effects of the polyphenol oxidase were found by Tris, EDTA and acetic acid with the increase in concentration. The activity of polyphenol oxidase was inhibited by Tris (37.66% - 69.26%), by EDTA (48.68% - 82.38%) and by acetic acid (45.35% - 63.49%) at different concentrations of these chemicals.

Table-14: Effect of various chemicals on the activities of Invertases from betel leaf.

Name of Chemicals	Concentration (mM)	Relative Activities (%)	
		AIV I	AIV II
None	0	100	100
Tris	1	96.31 ± 0.74	98.64 ± 0.68
	3	91.25 ± 0.82	91.42 ± 0.64
	5	82.74 ± 0.76	85.37 ± 0.72
Glucose	1	97.46 ± 0.54	98.72 ± 0.56
	3	92.54 ± 0.62	93.81 ± 0.51
	5	88.18 ± 0.64	86.83 ± 0.49
Fructose	1	94.32 ± 0.67	96.60 ± 0.64
	3	87.86 ± 0.61	90.32 ± 0.61
	5	79.45 ± 0.58	82.40 ± 0.69
EDTA	1	85.60 ± 0.87	89.63 ± 0.69
	3	72.70 ± 0.91	75.34 ± 0.78
	5	48.56 ± 0.95	49.68 ± 0.73
Acetic Acid	1	69.85 ± 0.32	73.54 ± 0.41
	3	57.32 ± 0.44	61.23 ± 0.45
	5	44.94 ± 0.51	49.15 ± 0.49

Table-15: Effect of various chemicals on the activities of betel leaf Polyphenoloxidase activity

Name of Chemicals	Concentration (mM)	Relative Activities (%)
None	0	100
Tris	1	62.34 ± 0.59
	3	45.67 ± 0.55
	5	30.74 ± 0.51
EDTA	1	51.32 ± 0.61
	3	35.54 ± 0.68
	5	17.62 ± 0.63
Acetic Acid	1	54.65 ± 0.36
	3	46.74 ± 0.41
	5	36.51 ± 0.46

R.5.4 Effects of various metallic salts on the activities of betel leaf invertases and polyphenol oxidase

R.5.4.1 Effects of various metallic salts on the activities of betel leaf invertases

The effects of various metallic salts on the activities of betel leaf invertase were given in Table-16. Plant invertases were characteristically inhibited by heavy metals and reagents that react with sulfhydryl groups (Krishnan *et. al.*, 1985; Arnold, 1965; Singh *et. al.*, 1984; Morell *et. al.*, 1984; Pressy *et. al.*, 1980). The activity of invertases were decreased drastically with the increased concentration of $HgCl_2$ and the activities were almost completely inhibited by 5 mM $HgCl_2$. Similar types of inhibition were reported for sugarcane neutral invertase (Darren *et. al*, 1998) and mango invertase (Rahman, M. H. *et. al.*, 2001). Hari, *et. al.* (1990) reported that cherry fruit invertase was inhibited by more than 80% with 2 μM $HgCl_2$. Complete inhibition of invertases by $HgCl_2$ was consistent with the reports of Schwimmer, *et. al.* (1961) and Presy (1967). It can be concluded from these findings that –SH groups containing amino acids are located at or near the active sites of betel leaf invertases. $ZnSO_4$ reduced considerably the activity of betel leaf acid invertase I by 9.86% - 39.75% and acid invertase II by 15.81% - 45.33% at different concentrations. Darren *et. al.* (1998) observed almost complete inhiobition with $ZnCl_2$ in sugarcane neutral invertase. The activities of AIV I and AIV II were decreased by 11.02% - 23.68% and 7.69% - 14.36% respectively at different concentrations of $AgNO_3$. Calcium and Manganese salts had almost no effects on betel leaf invertases. Hari, *et. al.* (1990) also found that Ca^{2+} had no effect on cherry fruit invertase at 10mM concentrations. On the other hand, invertase activities were increased moderately by $MgCl_2$, KCl and $CuCl_2$. The activities of AIV I were increased by 10.35% - 34.36%, 7.68% - 34.25% and 18.46% - 37.36% while that of AIV II were increased by 12.54% - 32.45%, 4.65% - 20.81% and 24.37% - 39.43% at different concentrations of $MgCl_2$, KCl and $CuCl_2$ respectively.

R.5.4.2 Effects of various metallic salts on the activities of betel leaf polyphenol oxidase

The effects of various metallic salts on the activities of betel leaf polyphenol oxidase were given in Table-17. Plant polyphenol oxidases were characteristically inhibited by heavy metals and reagents. The activities of polyphenol oxidase was decreased drastically with the increased concentrations of $HgCl_2$ and the activities was almost completely inhibited by 5mM $HgCl_2$. So, like invertases, -SH group containing amino acids are also located at the active sites of betel leaf polyphenol oxidase. Polyphenol oxidase activities were also decreased by increasing

concentrations of $FeCl_2$ and $MgCl_2$. The activities were decreased about 35% by 5mM $FeCl_2$ while that was inhibited about 54% by 5mM $MgCl_2$. On the other hand, increasing concentrations of $CaCl_2$ and $CuCl_2$ significantly increased the polyphenol oxidase activities and the activities were increased about 34.54% by 5mM $CaCl_2$ and about 42.39% by 5mM $CuCl_2$. Shahanaz *et. al.* (2001) reported the similar type of metallic salts effects on the activity of sajna leaves polyphenol oxidase.

Table-16: Effect of various metallic salts on the activities of invertases

Name of Chemicals	Concentration (mM)	Relative Activities (%)	
		AIV I	AIV II
None	0	100	100
$HgCl_2$	1	38.15 ± 1.38	36.47 ± 1.26
	3	19.34 ± 1.24	16.27 ± 1.21
	5	4.62 ± 1.05	3.21 ± 2.93
$ZnSO_4$	1	90.14 ± 3.21	84.19 ± 3.11
	3	78.32 ± 2.86	73.45 ± 2.82
	5	60.25 ± 1.67	54.67 ± 1.61
$AgNO_3$	1	88.98 ± 2.95	92.31 ± 3.02
	3	81.65 ± 2.14	88.47 ± 2.84
	5	76.32 ± 1.87	85.64 ± 2.37
$CaCl_2$	1	100.12 ± 3.64	100.0 ± 3.50
	3	99.34 ± 3.32	98.35 ± 3.16
	5	98.87 ± 3.29	98.42 ± 3.14
$MnCl_2$	1	99.64 ± 3.27	100.14 ± 3.33
	3	100.78 ± 3.34	102.27 ± 3.45
	5	102.20 ± 3.38	104.62 ± 3.46
$MgCl_2$	1	110.35 ± 3.25	112.54 ± 3.27
	3	120.54 ± 4.36	121.38 ± 3.78
	5	134.36 ± 4.83	132.45 ± 3.97
KCl	1	107.68 ± 3.35	104.65 ± 3.28
	3	123.47 ± 3.76	115.24 ± 3.64
	5	134.25 ± 4.12	120.81 ± 3.73
$CuCl_2$	1	118.46 ± 3.27	124.37 ± 4.31
	3	124.71 ± 3.48	131.68 ± 4.12
	5	137.36 ± 4.21	139.43 ± 4.62

Table-17: Effect of various metallic salts on the activities of Polyphenol oxidase.

Name of Chemicals	Concentration (mM)	Relative Activities (%)
None	0	100
$HgCl_2$	1	39.15 ± 1.14
	3	17.67 ± 0.98
	5	4.85 ± 1.25
$FeCl_2$	1	82.34 ± 3.27
	3	72.28 ± 2.98
	5	65.46 ± 1.18
$MgCl_2$	1	78.37 ± 3.08
	3	66.18 ± 1.29
	5	45.25 ± 1.06
$CaCl_2$	1	108.32 ± 3.54
	3	122.65 ± 3.97
	5	134.54 ± 4.25
$CuCl_2$	1	112.48 ± 2.63
	3	135.73 ± 2.32
	5	142.39 ± 2.86

R.5.5 Effects of various proteins and lectins on the activities of betel leaf invertases

The activities of betel leaf invertases against sucrose as substrate were assayed using 1×10^{-4} M, 2×10^{-4} M, 3×10^{-4} M, 4×10^{-4} M and 5×10^{-4} M concentrations of different proteins and lectins. The point of optimal activation (POA: minimal concentration of effector that produces the maximal enzyme activation) of AIV I and AIV II were represented in Table-18 using bovine serum albumin (BSA), *Potamogeton nodosus* lectin (PNL), concanavalin A, mulberry seed lectin (MSL), β-Galactosidase, alkaline phosphatase and urease. In case of acid invertase I (AIV I), the POA was 3×10^{-4} M for BSA, 2×10^{-4} M for PNL, 1×10^{-4} M for concanavaline A, 3×10^{-4} M for MSL, 5×10^{-4} M for β-Galactosidase, 4×10^{-4} M for alkaline phosphatase and 3×10^{-4} M for urease that caused 20-25%, 55-62%, 80-92%, 46-55%, 40-45%, 33-40% and 45-57% activation of the enzyme respectively. In case of AIV II, the POA was 2×10^{-4} M for BSA, 3×10^{-4} M for PNL, 1×10^{-4} M for concanavalin A, 4×10^{-4} M for MSL, 5×10^{-4} M for β-Galactosidase, 5×10^{-4} M for alkaline phosphatase and 5×10^{-4} M for urease that

caused 25-30%, 45-56%, 75-88%, 35-45%, 30-42%, 35-44% and 42-50% activation of the enzyme respectively. Isla *et. al.* (1995) reported similar type of activation effect on *Oryza sativa* invertase.

Table-18: Protein and Lectin effect on invertase activity

Protein/Lectin	Acid invertase I		Acid invertase II	
	POA* (Molar)	Apprx. Activation (%)	POA (Molar)	Apprx Activatin (%)
Control	- - -	- - -	- - -	- - -
Bovine Serum Albumin (BSA)	3×10^{-4}	20-25	2×10^{-4}	25-30
Potamogeton nodosus Lectin (PNL)	2×10^{-4}	55-62	3×10^{-4}	45-56
Concanavaline A	1×10^{-4}	80-92	1×10^{-4}	75-88
Mulberry Seed Lectin (MSL)	3×10^{-4}	46-55	4×10^{-4}	35-45
β-Galactosidase	5×10^{-4}	40-45	5×10^{-4}	30-42
Alkaline Phosphatase	4×10^{-4}	33-40	5×10^{-4}	35-44
Urease	3×10^{-4}	45-57	5×10^{-4}	42-50

POA*= Point of Optimal Activation

CHAPTER SIX

BIOACTIVITY STUDIES OF DIFFERENT VARIETIES OF BETEL LEAF EXTRACTS AT MATURE STAGE

6.1 ANTIBACTERIAL SCREENING

6.1.1 Introduction:

Antimicrobial screening is used to perform the primary selection of the compounds as therapeutic agent. The antibacterial screening is done by primary assay, a qualitative assay to determine whether the compounds are antibacterially active or not. In general, antimicrobial screening is undertaken in two phases which is described as follows:

i) Primary assay:
It is a qualitative assay to detect the presence or absence of the activity. The primary assay can be performed in vitro by disc diffusion assay (Beur *et. al.*, 1966) method, which includes -
a. Plate Diffusion test and
b. Streak test.
The streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and this test is suitable for the determination of the spectrum of the activity. But the plate diffusion test is commonly used.

ii) Secondary assay:
It quantifies the relative potency such as minimum inhibitory concentration. The minimum concentration of antimicrobial agent required to inhibit the growth of the organism *in vivo* is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique.

Current study includes only the disc diffusion technique.

6.1.2 Materials

6.1.2.1 Apparatus and reagents:

i) Blank filter paper discs (sterilized)
ii) Standard kanamycin disc (K-30)
iii) Petridishes
iv) Inoculating loop
v) Test tubes
vi) Sterile forceps
vii) Micro-pipette
viii) Bunsen burner

ix) Laminar air flow unit (BIOCRAFT & SCIENTIFIC INDUSTRIES, INDIA)
x) Methanol
xi) Rectified spirit
xii) Nutrient agar (DIFCO)
xiii) Incubator (OSK, 9639A, JAPAN)
xiv) Refrigerator (ARISTON, ITALY)
xv) Autoclave (ALP Co. Ltd. KT-30L, TOKYO)

6.1.2.2 Test materials used for the study

i) Betel Leaf extracts
ii) Standard Kanamycin disc (K-30)

6.1.2.3 Test organisms used for the study

Five pathogenic bacteria were selected for the test, three of them were gram negative and others were gram positive. The test organisms were collected from the Department of Biochemistry and Department of Pharmacy, University of Rajshahi, Bangladesh.

Table 19: List of the pathogenic bacteria

Test Organisms	Type	Srain Number
Shigella dysenteriae	Gram negative	AL-35587
Escherichia coli	Gram negative	FPFC- 1407
Klebsiella Spp.	Gram negative	------
Staphylococcus β-hemolyticus	Gram positive	CRL
Staphylococcus aureus	Gram positive	ATCC- 259233

6.1.3 Disc diffusion method

6.1.3.1 Principle of Disc diffusion method:

In the diffusion assay, the surface of a nutrient agar medium contained in a petridish, was uniformly inoculated with the test bacterial culture. The test solution of compounds were added to such a plate by pipetting them either into circular holes cut into the agar or into previously applied glass or metal cylinders or they were absorbed on to filter paper discs, which were put on the surface of the agar. The test substances diffused into the agar with decreasing concentration towards the periphery. In the case of positive reaction, an inhibitory zone was observed after incubation for several hours where the concentration exceeds the MIC for

that particular organism. The diameter of zone of inhibition was proportional to the logarithm of the concentration of the antibiotic. The diameter of zone of inhibition produced under constant experimental conditions were dependent on the following factors:

a. Thickness of the agar medium.
b. Diffusion rate of the test compound.
c. Inoculum size.
d. Incubation time.
e. Temperature of cultivation.
f. Culture medium composition.
g. Growth rate of the test organism.

6.1.3.2 Mechanism by which Disc diffusion technique acts:

A number of events occurred simultaneously during this process:

i) Initially the dried disc absorbed water from the surrounding test medium and the drug (test sample) became dissolved in it.
ii) The drug (test sample) migrated through the adjacent test medium due to concentration gradient.
iii) This results in a gradual change of the drug (test sample) concentration in the agar surrounding each disc.

The plates seeded with test organism disc containing antibiotics were kept at low temperature (4^0C) for 4 hours and then incubated at 37^0C for 16 hours in an incubator. A clear zone was observed where the drug was present higher than the minimum inhibitory concentration.

6.1.3.3 Preparation of Nutrient agar media

10 gm Bacto-trypton, 5gm Bacto yeast extract, 10 gm sodium chloride were dissolved in 800 ml distilled water and pH of the medium was adjusted to 7.5 by .5N sodium hydroxide. Then 15gm agar was added and the volume was made 1000 ml with distilled water and sterilized by autoclaving at 15lbs/sq. inch, 121°C for 20 minutes and stored at 4°C.

6.1.3.4 Preparation of fresh culture of the test pathogenic bacteria:

i. The nutrient agar medium was dispensed in number of clean test tubes to prepare slants (5ml in each test tube).
ii. The test tubes were then plugged with cotton and sterilized in an autoclave at 15 lbs/sq. inch and 121°C for 15 minutes.
iii. Finally, different pure strains of the test organisms were streaked on these slants with the help of sterile inoculating loops under the laminar airflow unit and incubated upto 24 hours at 37°C.

6.1.3.5 Preparation of test plates:

i. A number of petridishes were washed and sterilized by dry heat (2 hours at 120^0C).
ii. Some test tubes were washed, 15ml nutrient agar medium was poured and plugged with cotton.
iii. The test tubes were sterilized and allowed to cool to about 40^0C .
iv. By means of a sterile inoculating loop, the culture of the bacterial spores were inoculated in the medium to the test tube and was agitated to ensure uniform dispersion of the organism into the medium.
v. Finally, the medium was poured into the sterile petridish and agitated clock wise, anti-clockwise, left to right and right to left for uniform dispersion.

Thus the plates were ready for sensitivity test.

6.1.3.6 Preparation of the discs containing sample:

For the preparation of discs containing samples the following procedures were utilized.

a. Sample discs

i) Filter paper discs were taken in a petridishes and sterilized in oven at 110°C for 1 hour.
ii) The leaf extracts were concentrated upto dryness. The stock solution was prepared by dissolving the extract in DMSO solution to make the concentration of one µg/µl. By means of a micropipette 50µl and 100µl of the leaf extracts were placed on the filter paper disc
iii) These discs were then air dried.

b. Standard discs

Prepared kanamycin discs containing 30 µg/disc were used as standard discs.

6.1.3.7 Placement of disc, diffusion and incubation:

Procedure:

i. By means of sterile forceps the sample impregnated filter paper discs and standard antibiotic disc were placed gently on the solidified agar plates seeded with test organisms to ensure contact with the medium.
ii. The plates were then kept in a refrigerator for 12-16 hours at 4°C in order to provide sufficient time to diffuse into the medium.
iii. They were finally incubated at 37°C for 24 hours in an incubator.

Precaution:

The discs were placed in such that the discs were no closer than 15mm to the edge of the place and for enough apart to prevent over lapping the zones of inhibition.

6.1.3.8 Measurement of the zone of inhibition:

After incubation, the antibacterial activity of the test sample was determined by measuring the diameter of inhibitory clear zone produced in term of mm with a transparent scale.

6.1.4 Results of antibacterial Screening

The antibacterial activities of the betel leaf extracts were determined at a concentration of 50 µg/disc and 100 µg/disc against the tested organisms and the results are shown in Table-20. It was found that the entire test sample exhibit positive response against all the tested bacteria. The standard kanamycin disc (30 µg/disc) showed 28 mm inhibition zone against *Staphylococcus aureus* where as 50 µg/disc and 100 µg/disc of Doga pan showed 15 mm and 22 mm zone of inhibition. Doga leaf extract of 50 µg/disc showed 14, 13, 16 and 12 mm zone of inhibition and 100 µg/disc showed 24, 18, 22 and 19 mm against *S. β-hemolyticus, E. Coli, S. Dysenteriae* and *Klebsiella Spp.* respectively. Shail pan showed 14 and 21 mm, 13 and 23 mm, 12 and 17 mm, 15 and 21 mm, 13 and 16 mm zone of inhibition against *S. Aureus, S. β-hemolyticus, E. Coli, S. Dysenteriae* and *Klebsiella Spp.* bacteria respectively for 50 µg/disc and 100 µg/disc. Dudhswar pan showed 14 and 20 mm, 13 and 22 mm, 14 and 19 mm, 15 and 21 mm, 11 and 18 mm zone of inhibition against *S. Aureus, S. β-hemolyticus, E. Coli, S. Dysenteriae* and *Klebsiella Spp.* bacteria respectively for 50 µg/disc and 100 µg/disc whereas 13 and 19 mm, 14 and 19 mm, 14 and 21 mm, 13 and 22 mm, 12 and 17 mm zone of inhibition were observed against them for Kal Bangla leaf extract of 50 µg/disc and 100 µg/disc. The standard Kanamycin (30 µg/disc) gave inhibition zone of 28 mm, 29 mm, 27 mm, 28 mm and 26 mm against *S. Aureus, S. β-hemolyticus, E. Coli, S. Dysenteriae* and *Klebsiella Spp.* bacteria respectively.

Table-20: Antibacterial Activity of betel leaves extracts:

Varieties	Zone of Inhibition (in mm)									
	S. Aureus		*S. β-hemolyticus*		*E. Coli*		*S. Dysenteriae*		*Klebsiella Spp.*	
	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc	50 µg/ disc	100 µg/ disc
Shail Pan	14	21	13	23	12	17	15	21	13	16
Doga Pan	15	22	14	24	13	18	16	22	12	19
Dudhswar Pan	14	20	13	22	14	19	15	21	11	18
K.Bangla Pan	13	19	14	19	14	21	13	22	12	17
Kanamycin (30 µg/disc)	28		29		27		28		26	

6.2 Determination of minimum inhibitory concentration (MIC) of different varieties of betel leaves

6.2.1 Introduction

The lowest concentration of antimicrobial agent requires to inhibit the growth of the organism *in vitro* is referred as the minimum inhibitory concentration (MIC). MIC may be defined as the lowest concentration of antimicrobial drug required to inhibit the growth of organism. The data derived from the test can be corrected with the knowledge of expected or measured antibiotic level *in vivo* to predict the efficacy of antibiotic as compared with standard antibiotics. The serial dilution technique (Reiner, 1982) was followed using nutrient broth medium to determine the MIC value of the chemotherapeutic agent.

There are two methods for determining the MIC. They are as follows-

i) Serial tube dilution technique or Turbidimetric assay

ii) Paper disc plate technique or agar diffusion assay

Here "Serial tube dilution technique" was followed using nutrient broth medium to determine the MIC values against the *Shigella dysenteriae*, *Klebsiella Spp.*, *Escherichia coli*, *Staphylococcus β-hemolyticus* and *Staphylococcus aureus.*

6.2.2 Serial tube dilution technique:

The tubes of broth medium, containing graded doses of compounds are inoculated with the test organisms. After suitable incubation, the growth of organisms will occur in those inhibitory tubes where the concentration of antibiotic is below the inhibitory level and the culture will become turbid (cloudy). Therefore, growth of microorganisms will not occur when the concentrations of antibiotic and compounds are above the inhibitory level and the tube will remain clear.

6.2.3 Preparation of inoculum

Fresh cultures of the test organisms were grown at 37.5°C for overnight on nutrient agar medium. Bacterial suspensions were then prepared in sterile nutrient broth medium in such a manner so that the suspension contains 10^7 cells/ml. This suspension was used as inoculums.

6.2.4 Preparation of the sample solution

The leaf extracts were concentrated upto dryness. Then the stock solution was prepared by dissolving 2.048 mg of dried extract in 2 ml of DMSO solution. Thus solutions with a concentration of 1.024 mg/ml were obtained.

6.2.5 Procedure of serial tube dilution technique

For the determination of MIC of 'Doga' variety leaf extract against *Shigella dysenteriae.*

i) 12 Cleaned test tubes were taken. 9(nine) of which were marked as 1-9 and rest 3 (three) were assigned as C_M (Medium), C_S (medium + betel leaf dried extract) and C_I (medium + inoculum).

ii) According to the specification (2.3% w/v) nutrient broth medium was prepared and 1 ml was taken in each of the 12 test tubes.

iii) All the test tubes were cotton plugged and sterilized in an autoclave at 121°C and 1 atms. pressure for 15 minutes.

iv) After cooling, 1 ml of the extract solution was added to the first test tube and mixed well. Then 1 ml of this content was transferred to the second test tube.

v) The content of the second test tube was mixed well and again 1 ml of this mixture was transferred to the third test tube. This process of serial dilution was continued upto the ninth test tube and 1 ml sample solution was discarded from the ninth test tube.

vi) 10µl of properly diluted inoculum of *Shigella dysenteriae* was added to each of the nine test tubes and mixed well.

vii) To the control test tube, 1 ml of the sample solution was added, mixed well and 1 ml of this mixed content was discarded. This was done to check the clarity of the medium in presence of diluted solution of the extract.

viii) 10µl of the inoculum was added to the control test tube C_I to observe the growth of the organism in the medium used.

ix) The control test tube C_M containing medium only was used to confirm the sterility of the medium.

x) All the test tubes were incubated at 37.5°C for 24 hours. Step iv-vii were done in the laminar flow unit to overcome contamination.

xi) The same procedure was also applied to determine the MIC of all the other test samples (Shail leaf extract, Dudhswarl leaf extract and Kal Bangla leaf extract,) against *Shigella dysenteriae*, *Escherichia coli*, *Staphylococcus β-hemolyticus* and *Staphylococcus aureus*).

6.2.6 Results of MIC tests

The MIC values of the Doga leaf extract at mature stage were found to be 64 µg/ml against three pathogenic bacteria e.g. *Shigella dysenteriae*, *Staphylococcus β-hemolyticus* and *Staphylococcus aureus* and 128 µg/ml against the pathogen, *Escherichia coli.*

The MIC values of the Shail variety were 256 µg/ml against *Escherichia coli*, 128 µg/ml against two pathogenic bacteria e.g. *Staphylococcus aureus* and *Shigella dysenteriae*, and 64 µg/ml against *Staphylococcus β-hemolyticus*.

The MIC values of the Dudhswar variety leaf extract were 128 µg/ml against all four tested pathogenic bacteria while the MIC values of Kal Bangla pan were 128 µg/ml against *Staphylococcus aureus* and *Staphylococcus β-hemolyticus* and 64 µg/ml against the rest of two tested pathogens e.g. *Shigella dysenteriae* and *Escherichia coli*.

Table-21: MIC of the betel leaf extracts against five pathogenic bacteria.

Marked no. of the test tubes	Nutrient broth medium added (ml)	Conc. of dried extract (µg/ml)	Inoculum added (µl)	Bacterial growth observed against															
				Staphylococcus aureus.				*Escherichia coli,*				*Shigella dysenteriae*				*Staphylococcus β-hemolyticus*			
				Doga Pan	Shail Pan	Dudhswar Pan	Kal Bangla Pan	Doga Pan	Shail Pan	Dudhswar Pan	Kal Bangla Pan	Doga Pan	Shail Pan	Dudhswar Pan	Kal Bangla Pan	Doga Pan	Shail Pan	Dudhswar Pan	Kal Bangla Pan
1	1	512	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
4	1	64	10	-	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+
5	1	32	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	1	16	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	1	8	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C_M	1	0	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C_S	1	512	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C_I	1	0	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' indicates 'growth' and '-' indicates 'no growth'

6.3 Antifungal Screening

6.3.1 Introduction

Antifungal screening is also used to perform the primary selection of the compound as therapeutic agent. To determine antifungal activity, the following two methods are generally used:

i) Agar well diffusion method and
ii) Disc diffusion method

The antifungal activity of the betel leaf extracts were performed by using the disc diffusion assay (Beur *et. al.*, 1966) method and the whole testing procedure was the same as the antibacterial activity test. The only difference was that the period of incubation was 48 hours at room temperature.

6.3.2 Materials

6.3.2.1 Test organisms used for the study

Four identified fungi collected from the Department of Botany, University of Rajshahi were used for the test of antifungal activity. The fungi are listed below.

i) Aspergillus flavus
ii) Aspergillus niger
iii) Aspergillus fumigatus
iv) Candida albicans

6.3.2.2 Culture medium used for the study

Potato dextrose agar (PDA) medium was used to perform the antifungal activity and for subculture of the test organisms.

6.3.3 Methods

6.3.3.1 Preparation of the medium

The constituents of the medium was accurately weighed and dispersed in a conical flask with distilled water. It was heated in water bath to dissolve the ingredients until a transparent solution was obtained. The pH of the medium was adjusted to 5.6. The volume was adjusted by adding distilled water and sterilized in an autoclave.

Table-22: Ingredients of Potato dextrose agar media (PDA).

Ingredient	Amount
Potato	20.0 gm
Dextrose	2.0 gm
Agar	2.0 gm
Distilled water q. s. to	100 ml

6.3.3.2 Preparation of the test plates, preparation of the discs, preparation of the test samples, placement of the discs, diffusion and incubation

Preparation of the test plates, discs, test samples, placement of the discs, diffusion and incubation processes were almost same of the antibacterial screening. Here only the incubation period was replaced by 48 hours at room temperature.

6.3.3.3 Measurement of the zone of inhibition

After incubation, the antifungal activity of the test sample was determined by measuring the diameter of inhibitory clear zone produced in term of mm with a transparent scale.

6.3.3.4 Results of antifungal test

An interesting result was exhibited by the betel leaf extracts. Although the leaf extracts exhibit significant antibacterial activity but it showed no antifungal activity against any test fungus. It is yet to determine the cause behind these phenomena.

6.4 Cytotoxicity Studies using brine shrimp (*Artemia salina*)

6.4.1 Introduction

Brine shrimp lethality bioassay (Persoone *et al,* 1980; Mayer,1982; Mclaughlin *et al,* 1988; Mclaughlin, 1990) is a recent development in the assay procedure of bioactive compounds. Natural product extracts and pure compounds can be tested for their bioactivity by this method. Here in vivo lethality, a simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening and fractionation in the discovery of new bioactive natural products. Generally, the median effective dose (ED_{50}) values for cytotoxicities are one tenth of median lethal dose, LD_{50} values in the brine shrimp test. The bioassay indicates cytotoxicity as well as a wide range of pharmacological activities (e.g. anticancer, antiviral, insecticidal, pesticidal, AIDS etc) of the compounds.

6.4.2 Materials

1) *Artemia salina* Leach (brine shrimp eggs)
2) Sea salt (NaCl)
3) Pipettes (5 ml & 1 ml)
4) Micro pipette (10-100 µl adjustable)
5) Two drum vials
6) Magnifying glass
7) Small tank with perforated dividing dam to grow shrimp, cover and lamp to attract shrimp.

6.4.3 Procedures

A) Preparation of sea water: 38 grams of NaCl was weighed, dissolved in one liter of distilled water and then filtered off.

B) Hatching of brine shrimp eggs: Sea water was taken in small tank and shrimp eggs were added to one side of the divided tank which was covered. The shrimps were allowed for two days to hatch and matured as nauplii (larvae). The hatched shrimps were attracted to the lamp on the other side of the divided through the perforations in the dam. These nauplii were taken for bioassay.

C) Preparation of sample solution: The leaf extracts were concentrated upto dryness. Then the stock solution was prepared by dissolving 2 mg of dried extract in 2 ml of DMSO solution. Thus solutions with a concentration of 1 mg/ml were obtained. 2ml of each solution were dialyzed separately against distilled water for 3 hours at 4°C.

D) Application of test solution and nauplii in the vials: At room temperature the test samples were dissolved in dimethyl sulphoxide (DMSO) and five grade doses 1.5, 3.0, 6.0, 12.0 and 24.0 µg/ml, respectively were used for 5ml seawater containing 100 brine shrimp nauplii in each group. Three vials were used for each concentration and control was used containing 100 nauplii in 5 ml of sea water. A magnifying glass was used for convenience of counting of the nauplii.

E) Counting of nauplii: After 24h incubation the vials were observed and the number of survivors in each vial were counted using magnifying glass and noted. From these data, the percentage of mortality of the nauplii was calculated for each concentration and the LD_{50} values were determined from the log dose response curve (Goldstein & Kalkan, 1974).

6.4.4 Results of Cytotoxicity Studies

The results of brine shrimp lethality bioassay are shown in Table-23. Test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp nauplii was found to be increased with the incresased concentration of the sample and concentration of logarithm versus percent mortality (Goldstein, 1974) was plotted and a best fitted line was drawn which showed an almost linear correlation as shown in the figure-31. Among the four varieties, the Dudhswar pan showed the highest cytotoxicity. The LD_{50} values were found to be 7.02 µg/ml for Dudhswar variety, 10.75 µg/ml for Shail pan, 17.25 µg/ml for Doga pan and 10.52 µg/ml for Kal Bangla pan extract. This experiment showed that LD_{50} values of the four betel leaf extracts were around the toxic level. From the present

investigation, it can be concluded that the Dudhswar, Shail and Kal Bangla variety may have important role in anticancer and antitumour activity as they showed more cytotoxic activity and further investigations are needed to confirm their bioactivity by applying on human cancer cell and bacteria to establish these as anticancer and antitumour agents which may explore as potent chemotherapeutic agent(s) in modern clinical trials. But it is not clear from this experiment which of the compound(s) is responsible for the cytotoxic activity of betel leaves.

Table-23: Brine shrimp lethality bioassay of betel leaf extracts at mature stage using *Artemia salina*.

Varieties	Stages	LD_{50} (µg/ml)
Shail Pan	Mature Stage	10.75
Doga Pan	Mature Stage	17.25
Dudhswar Pan	Mature Stage	7.02
Kal Bangla Pan	Mature Stage	10.52

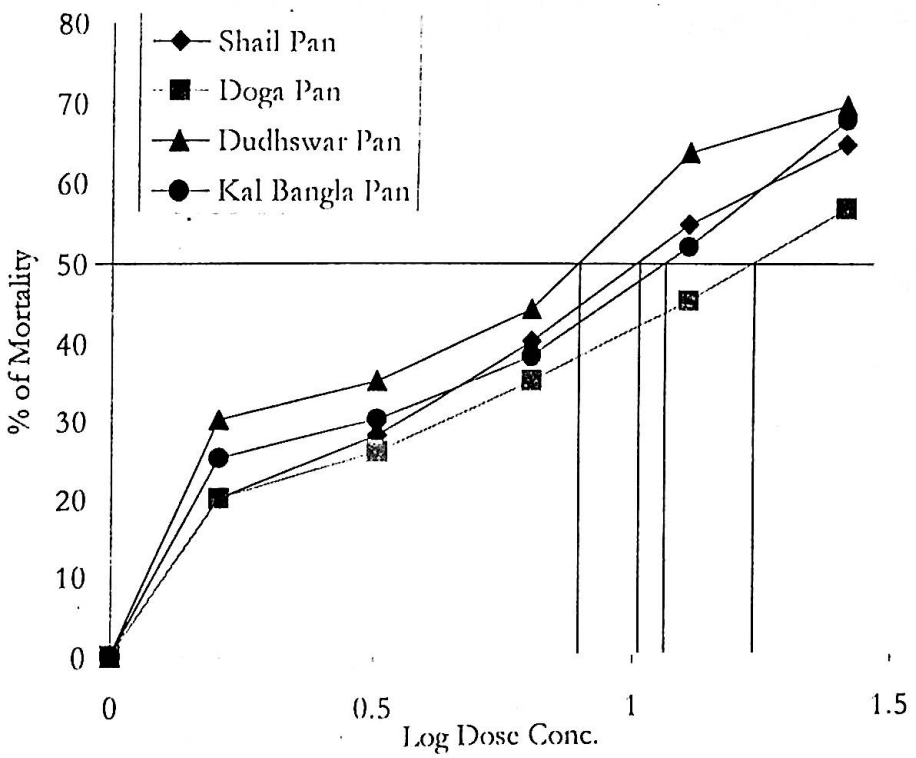


Figure-31: Determination of LD_{50} from Log Dose response curve.

CHEMICALS AND EQUIPMENTS

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CHEMICALS:

The important chemicals used in this study are mentioned below with their manufacturers:

Acetone
BDH Chemical Ltd., Poole England.

Acrylamide
Sigma Chemicals Co., U.S.A.

Albumin Bovine (BSA)
Sigma Chemicals Co., U.S.A.

Ammonium persulfate
Bio-Rad Laboratories, Richmond, U.S.A.

Ammonium Sulfate
Merck, Germany.

Aniline
BDH Chemical Ltd., Poole England.

Arabinose
Sigma Chemicals Co., U.S.A.

Borate (Natrium tetraborate)
Sigma Chemicals Co., U.S.A.

Bromophenol blue
Bio-Rad Laboratories, Richmond, U.S.A.

Butanol
BDH Chemical Ltd., Poole England.

β-Galactosidase
BDH Chemical Ltd., Poole England.

Coomassie brilliant blue R-250
Bio-Rad Laboratories, Richmond, U.S.A.

Copper Sulfate
BDH Chemical Ltd., Poole England.

DEAE-cellulose
Pharmacia fine Chemical Co. Ltd, Sweden.

Di Sodium hydrogen orthophosphate dihydrate
BDH Chemical Ltd., Poole England.

Galactose
Sigma Chemicals Co., U.S.A.

Glacial Acetic Acid
BDH Chemical Ltd., Poole England.

Glucose

Sigma Chemicals Co., U.S.A.

Glycerol

Bio-Rad Laboratories, Richmond, U.S.A.

Glycine

Bio-Rad Laboratories, Richmond, U.S.A.

Hydrochloric Acid

BDH Chemical Ltd., Poole England.

Isopropanol

Merck, Germany.

Lauryl Sulfate (SDS)

Sigma Chemicals Co. St. Louis, U.S.A.

Lysozyme

Sigma Chemicals Co. St Louis, U.S.A.

α-Amylase

Sigma Chemicals Co., U.S.A.

Mannose

Sigma Chemicals Co., U.S.A.

β-marcaptoethanol

Sigma Chemicals Co., U.S.A.

N,N Methylene-bis-Acrylamide

Sigma Chemicals Co., U.S.A.

Orthophosphoric Acid

BDH Chemical Co Ltd., England.

Phenol

Aldrich Chemical Company, Inc. U.S.A.

Potassium Sodium Tartrate

BDH Chemical Ltd., Poole England.

Phthalic Acid

BDH Chemical Ltd., Poole England.

Riboflavin

BDH Chemical Co Ltd., Germany.

Ribose

Sigma Chemicals Co., U.S.A.

Sephadex G-75

Sigma Chemicals Co., U.S.A.

Silica Gel G

Merck, India Ltd.

Sodium Dihydrogen orthophosphate

BDH Chemical Ltd., Poole England.

Sodium Chloride
Merck, India Ltd.
Sodium Hydroxide
Merck, Germany.
Sodium Azide
Sigma Chemicals Co. St. Louis, U.S.A.
Sodium Carbonate
Hopkin & Williams, Essex, England.
Sulfuric Acid
BDH Chemical Ltd., Poole England.
TEMED (N, N, N, N-tetramethylene diamine)
Sigma Chemicals Co., U.S.A.
Trichloro acetic acid (TCA)
BDH Chemical Ltd., Poole England.
Urease
Fluka Bio Chemika, Switzerland.
Standard Kanamycin disc (K-30)

EQUIPMENTS:

The important equipments used throughout this study are listed below:

1) Centrifuge (refrigerated) Model-H 502
2) Electric balance - Mettler H18.
3) Electrophoresis power supply.
4) Fraction Collector-SF-160 (Advantec, Japan).
5) Homogenizer - Model AM - 5
6) Incubator - Gallenkamp, England
7) Micropipette
8) pH - Meter
9) Shimadzu Double beam Spectrophotometer
10) Stirrer / Hotplate MR - 2000.
11) Super mixer, Labline instrument.
12) Slab gel electrophoresis apparatus
13) Volac pipette controller - Model 958241, U.S.A.
14) Water Bath.
15) Cold Chamber.
16) Blank filter paper discs (sterilized)
17) Standard kanamycin disc (K-30)
18) Petridishes
19) Inoculating loop
20) Test tubes
21) Sterile forceps
22) Micro-pipette
23) Bunsen burner
24) Laminar air flow unit (BIOCRAFT & SCIENTIFIC INDUSTRIES, INDIA)
25) Methanol
26) Rectified spirit.
27) Nutrient agar (DIFCO)
28) Incubator (OSK, 9639A, JAPAN)
29) Refrigerator (ARISTON, ITALY)
30) Autoclave (ALP Co. Ltd. KT-30L, TOKYO)

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