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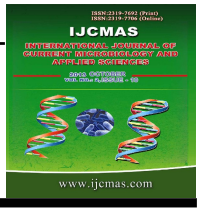

Original Research Article

Production of α-amylase from banana peels with *Bacillus subtilis* using solid state fermentation

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A B S T R A C T

Banana is the common name for an edible fruit produced by several kinds of large herbaceous flowering plants of the genus Musa. The fruit is variable in size, color and firmness, but is usually elongated and curved, with soft flesh rich in starch covered with a rind which may be yellow, purple or red when ripe. The fruits grow in clusters hanging from the top of the plant. Almost all modern edible parthenocarpic (seedless) bananas come from two wild species – *Musa acuminata* and *Musa balbisiana*. They are grown in at least 107 countries, primarily for their fruit, and to a lesser extent to make fiber, banana wine and as ornamental plants. Amylases are used in industry due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization. The increased demand for these enzymes in various industries has led to an enormous interest in developing enzymes with better properties such as raw starch degrading amylases. It is suggested that banana peel could employ as a promising substrate for the production of α amylase by *Bacillus subtilis*. Further, solid state fermentation is a better choice for amylase production. The addition of external growth medium is also found beneficial for increasing enzyme production. The present study was undertaken to isolate, identify and characterize the *Bacillus subtilis* in the culture medium followed by alpha amylase extraction from the fermented carrier, Banana peel and *Bacillus subtilis.* Optimization of fermentation parameters for α-amylase production, the effect on pH, Incubation temperature and Substrate concentration was also assessed. Finally amount of minerals in Banana peel and *Bacillus subtilis* was carried out using FT-IR Spectrometer.

K e y w o r d s

Banana; Amylase; *Bacillus subtilis*; FT – IR; Enzyme Production.

Introduction

Worldwide, there is no sharp distinction between "bananas" and "plantains". Especially in the Americas and Europe, "banana" usually refers to soft, sweet, dessert bananas, particularly those of the Cavendish group, which are the main exports from banana-growing countries. By contrast, Musa cultivars with firmer, starchier fruit are called "plantains". In other regions, such as Southeast Asia, many more kinds of banana are grown and eaten, so that the simple two-fold

distinction is not useful and is not made in local languages.

The term "banana" is also used as the common name for the plants which produce the fruit. This can extend to other members of the genus Musa like the scarlet banana (*Musa coccinea*), pink banana (*Musa velutina*). It can also refer to members of the genus Ensete, like the snow banana (*Ensete glaucum*) and the economically important false banana (*Ensete ventricosum*). Both genera are classified under the banana family, Musaceae. Bananas are naturally slightly radioactive, more so than most other fruits, because of their potassium content and the small amounts of the isotope potassium-40 found in naturally occurring potassium. Proponents of nuclear power sometimes refer to the banana equivalent dose of radiation to support their arguments (Krishna C and Chandrasakaran M, 1996). *The genus Musa is in the family Musaceae. The* APG III system assigns Musaceae to the order Zingiberales, part of the commelinid clade of the monocotyledonous flowering plants. Some sources assert that Musa is named for Antonius Musa, physician to the Emperor Augustus. Others say that Linnaeus, who named the genus in 1750, simply adapted an Arabic word for banana, mauz. The word banana is generally said to be derived from the Wolof word banana. Some 70 species of Musa were recognized by the World Checklist of Selected Plant Families as of January 2013 several produce edible fruit, while others are cultivated as ornamentals. Banana classification has long been a problematic issue for taxonomists. Linnaeus originally classified bananas into two species based only on their uses as food: *Musa sapientum* for dessert bananas and *Musa paradisiaca* for plantains. Subsequently further species names were added. However, this approach proved inadequate to address the sheer number of cultivars existing in the primary center of diversity of the genus, Southeast Asia.

All widely cultivated bananas today descend from the two wild bananas *Musa acuminata* and *Musa balbisiana*. While the original wild bananas contained large seeds, diploid or polyploid cultivars (some being hybrids) with tiny seeds are preferred for human raw fruit consumption. These are propagated asexually from offshoots. The plant is allowed to produce two shoots at a time; a larger one for immediate fruiting and a smaller "sucker" or "follower" to produce fruit in 6–8 months. The life of a banana plantation is 25 years or longer, during which time the individual stools or planting sites may move slightly from their original positions as lateral rhizome formation dictates.

Export bananas are picked green, and ripen in special rooms upon arrival in the destination country. These rooms are air-tight and filled with ethylene gas to induce ripening. The vivid yellow color normally associated with supermarket bananas is in fact a side effect of the artificial ripening process. Flavor and texture are also affected by ripening temperature. Bananas are refrigerated to between 13.5 and 15 °C (56 and 59 °F) during transport. At lower temperatures, ripening permanently stalls, and turns the bananas gray as cell walls break down. The skin of ripe bananas quickly blackens in the 4 °C (39 °F) environment of a domestic refrigerator, although the fruit inside remains unaffected.

Bananas are a staple starch for many tropical populations. Depending upon

cultivar and ripeness, the flesh can vary in taste from starchy to sweet, and texture from firm to mushy. Both the skin and inner part can be eaten raw or cooked. The banana's flavor is due, amongst other chemicals, to isoamyl acetate which is one of the main constituents of banana oil. During the ripening process, bananas produce a plant hormone called ethylene, which indirectly affects the flavor. Among other things, ethylene stimulates the formation of amylase, an enzyme that breaks down starch into sugar, influencing the taste of bananas. The greener, less ripe bananas contain higher levels of starch and, consequently, have a "starchier" taste. On the other hand, yellow bananas taste sweeter due to higher sugar concentrations. Furthermore, ethylene signals the production of pectinase, an enzyme which breaks down the pectin between the cells of the banana, causing the banana to soften as it ripens.

Amylases are one of the main enzymes used in industry. Amylases have been reported to occur in micro-organisms, although they are also found in plants and animals. Alpha Amylase's official name is 1,4-a-D-Glucan glucanohydrolase; EC 3.2.1.1. The official names of enzymes are maintained by a commission on enzyme nomenclature (Kokab S *et. al.*, 2003).

Two major classes of amylases have been identified in micro-organisms, namely α-amylase and glucoamylase. α-Amylases (endo-1, 4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the 1, 4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain (Monteiro de Souza *et.al.*, 2010). Glucoamylase (exo-1, 4-α-D-glucan glucanohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a step-wise manner.

Bacillus strains such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known as good producers of alpha-amylase for various applications (Vijayabaskar et al., 2012). *Bacillus* are widely used for production of alpha-amylases and these bacteria need rich source of nutritional medium to grow, different fruit and vegetable peels usually considered a waste provide rich source of starch and nutrients for bacteria. Production of amylases from bacteria is beneficial for human population as their starch degrading ability can be exploited for preparation of special food items, easily digestible for infants, patients and elderly people Rick W and Stegbauer HP 1974).

Materials and Methods

Isolation, identification and growth of the *bacillus subtilis*

One gram of each soil sample was added in 99 ml of sterile distilled water and stirred for 20 mins. It was heated at 60°C for 60 min in water bath to make a soil suspension. Ten-fold serial dilutions of soil samples in sterile distilled water were prepared in duplicates up to 10-8 dilution and plated on 1.5% nutrient agar. Pour-plate method was used to isolate bacterial species from the soil samples. Plates were incubated at 37^{o}C for 24 to 48 h.

Preparation of substrate

Banana peel used as substrate was obtained from fruit market and chopped into small pieces of uniform size and stored in polythene bags at room temperature.

Production of *bacillus subtilis* spores in sporulation media

A loop full of *bacillus subtilis* strain was transferred aseptically to a 500 ml conical flask containing 100ml of sporulation media in laminar air. The flask was kept on shaker at 120 rpm at 37^{o}C for 72 hours.

Inoculum preparation

The spores of *B. subtilis* were transferred aseptically to a 500 ml conical flask containing 100 ml of pre-sterilized inoculum medium in laminar air flow. The flask was then kept on shaker (120 rpm) at 37^{o}C for 24 h. The homogenous spore suspension (106-107 spores/mL) was used as inoculum.

Solid state fermentation

The pH pf the fresh chopped banana peel (80% moisture) was adjusted to pH 7 and sterilized in autoclave for 15 min at 121^{o}C. After cooling, inoculum (1mL) was added to each flask in the laminar air flow with the help of sterilized pipette. The flasks were then incubated at 35^{o}C for 24 hr without shaking in incubator. The SSF media flasks were gently shaken after every 12 h for uniform mixing of the substrate and microorganism.

Crude enzyme extraction

Fermented carrier was taken, after 24 to 48 h of incubation, eluted with 20 ml 0.02 M phosphate buffer, pH 7.0, It was shaken properly at 175 rpm for 60 min and filtered with muslin cloth. The filtrate was centrifuged at 9000 rpm for 15 min at room temperature. The culture filtrate was used as a crude enzyme extract.

Enzyme assay

Estimation of maltose

200mg of maltose is dissolved in 100ml of distilled water (concentration = 2 mg/ml). From this stock solution, 10 appropriate dilutions with concentrations of 0.4 to 2.0 mg/ml were prepared.

To 1 ml of each dilution, 1 ml of 3, 5 dinitrosalicylic acid reagent was added. Blank was prepared by adding 1 ml of DNS in 1 ml of distilled water. These test tubes were placed in boiling water bath for 5 min, cooled at room temperature. After cooling the contents of the tube were diluted up to 20 ml. Absorbance was measured at 540 nm. Standard curve was prepared by plotting absorbance on y-axis and maltose concentration on x-axis. Crude enzyme extract (1 ml) was added to the test tube containing 1 ml of 1 % starch solution prepared in 0.02 M phosphate buffer pH 7.0.This mixture was incubated at 25°C for 10 min. Two ml of DNSA was added to the test tube and placed in water bath for 5 min. The absorbance of the reaction mixture was determined at 540 nm against maltose as standard. The amylase activity was determined in IU/ml/min by applying the standard formula.

$$\text{Amylase Activity((IU/ml/min)} = \frac{\text{Amount of sugar released x 1000}}{\text{Molecular weight of maltose x Time of incubation}}$$

Optimization of process parameters

The growth medium of banana peel was fermented with *B. subtilis* for optimization of different parameters for α-amylase production.

The experiments were carried out systematically in such a way that the parameter optimized in one experiment was maintained at its optimum level in the subsequent experiments.

Study of different parameters

Effect of pH of the medium on α-amylase production

pH of 10g (optimum) chopped banana peel was adjusted at different levels viz., 5.7,6.7,7.5,8.0 before inoculation and incubation for 24 hr. Alpha-amylase exhibits maximum activity at its definite pH. The pH at which the enzyme exhibits maximum activity is called its optimum pH.

Effect of incubation temperature on α-amylase production

SSF media of banana peel (10gm) were inoculated (1 mL) and incubated at pH 7 under different conditions of temperature as 7^oC, 30^oC, 37^oC, 65^oC for 24 hours. Alpha-amylase exhibits maximum activity at its optimum temperature. The temperature at which the enzyme exhibits maximum activity is called its optimum temperature.

Effect of substrate concentration on α-amylase production

Conical flasks containing different substrate levels (5-20 g) were inoculated (1 mL) and incubated for 24 hours at pH 7 and 35^Oc. α-amylase catalyses the hydrolysis of α 1-4 glycosidic linkages and producing reducing sugars. Reducing sugars like maltose is then coupled with DNSA in alkaline medium .It produces an orange coloured complex.The intensity of the colour produced can be measured at 540nm which is directly proportional to the activity of the enzyme.

Fourier transforms infrared spectrometer

FT-IR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitate some components of an unknown mixture. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FT-IR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. Today's FT-IR instruments are computerised which makes them faster and more sensitive than the older dispersive instruments.

Light covering the whole frequency range, typically 5000-400cm^{-1}, is split into two beams. Either one beam is passed through the sample or both are passed, but one beam is made to transverse a longer path than the other. Recombination of the two beams produces an interference pattern that is the sum of all the interference patterns created by each wavelength in the beam. By systematically changing the difference in the two paths, the interference patterns change to produce a detected signal varying with optical path difference. This pattern is known as the 'Interferogram',

Wavelength of Different Light Regions

The infrared spectra usually have sharp features that are characteristic of specific types of molecular vibrations, making the spectra useful for sample identification. Infrared band characteristics are shown table.1

Table.1 Characteristic IR Bands

X-H vibrations	Bond	Wave numbers (cm^{-1})
Hydroxyl	O-H	3610-3640
Aromatic rings	C-H	3000-3100
Amines	N-H	3300-3500
Alkenes	C-H	3020-3080
Alkanes	C-H	2850-2960
Triple bonds	C=C	2500-1900
Double bonds	C=C	1900-1500
Deformation/heavy atoms		1500-

Perkin-elmer spectrum one ft-ir spectrometer

The Perkin-Elmer Spectrum One FT-IR Spectrometer is capable of data collection over a wavenumber range of 370-7800 cm^{-1}. It can be configured to run in single-beam, ratio, or interferogram modes. The best resolution is 0.5 cm^{-1}. The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when Fourier transformed, gives rise to the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated online computer. The Perkin-Elmer Spectrum One FT-IR spectrometer instrument consists of globar and mercury vapour lamp as sources, an interferometer chamber comprising of KBr and mylar beam splitters followed by a sample chamber and detector. Entire region of 450-4000 cm^{-1} is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr or polyethylene pellets depending on the region of interest. This instrument has a typical resolution of 1.0 cm^{-1}. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible (Figures. 8, 9).

Results and Discussion

Selection of a suitable solid substrate and its level are important factors for solid state fermentation. Banana peels added to mineral salt medium for alpha-amylase production have an important role in providing nutrients for microbial growth. The ultimate benefit of utilizing agro-industrial waste is to reduce pollution problems for human beings, which otherwise need to be disposed off thus adding to environmental pollution. *Bacillus* strains were identified and characterized as *B. subtilis* indicating that are prelavent. Bacillus strain shows there behavior by colony characterization with a light whitish cream colour which indicates the presence of *Bacillus subtilis*. The petri plates were covered with the paraffin and kept in the refrigerator

Solid state fermentation

In 250ml conical flask banana peel is chopped into pieces (10gm), 1 ml of the inoculum is added and is incubated at 37^{oC} for 24 hours. The cultivation of micro-organisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. It holds tremendous potential for the production of alpha amylase. The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which micro-organisms are adapted. The growth of the bacillus subtilis on the banana peel is observed.

Extraction of fermented carrier (banana with bacillus), banana peel and *bacillus subtilis*

Phosphate buffer helps to extract the alpha amylase from the source and after centrifugation the supernatant is used for

the following parameters. The absorbance values of crude enzyme extracts obtained from 24 and 48 hr bacterial cultures in the presence of three substrates at 540 nm, and the respective enzyme units is given below

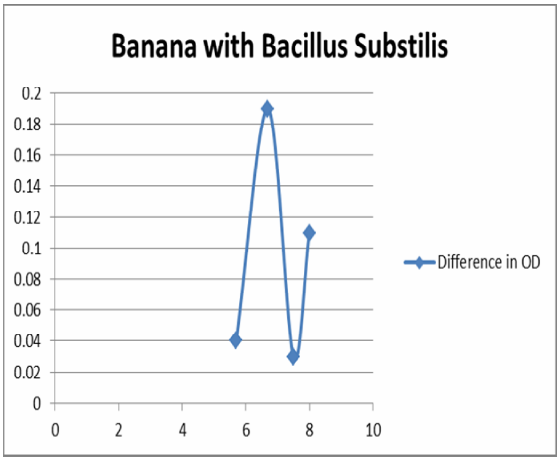
substrate level	amylase activity range
Banana with *bacillus subtilis*	1805.55 IU/mL/min
Banana peel	1388.88 IU/mL/min
Bacillus subtilis	972.22 IU/mL/min.

Highest alpha-amylase activity obtained with 10 g of banana peels for the *Bacillus* strains range from 1805.55 IU/ml/min in 24 h of SSF at pH 7 and 37°C Banana peel producing 1388.88 IU/ml/min *and B.* subtilis producing 972.22IU/ml/min are the next to fermented carrier

Optimization of fermentation parameters for α-amylase production

Different fermentation parameters were optimized for α-amylase production by conducting a series of experiments and the results are presented as under:

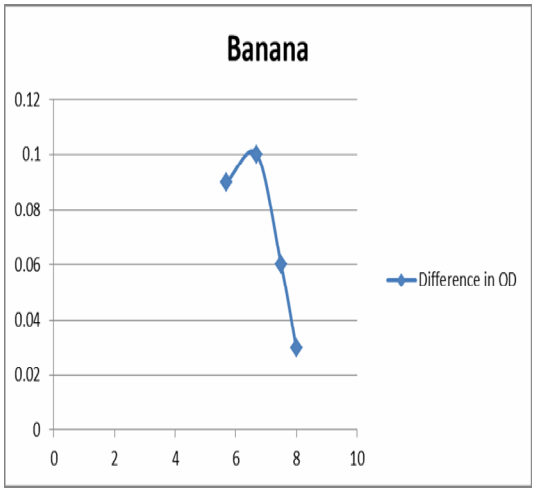
Figure.1. Effect of pH of the medium on α-amylase production



Banana with *Bacillus subtilis*

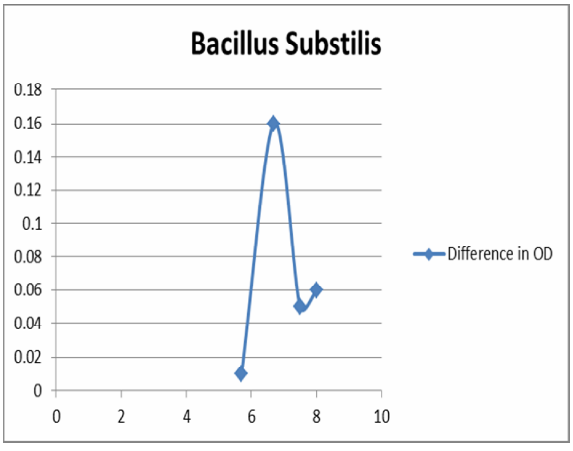
The α-amylase activity at pH 6.7 showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in pH caused a decrease in enzyme production.

Figure.2 Effect of Temperature on α-amylase production



The α-amylase activity at pH 6.7 showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in pH caused a decrease in enzyme production (Figure.1).

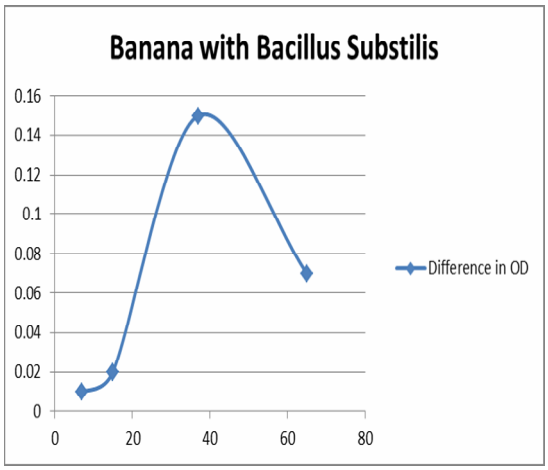
Figure.3 Effect of substrate on α-amylase production



The α-amylase activity at pH 6.7 showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in pH caused a decrease in enzyme production. It is obvious from the table , the maximum activity of α-amylase is observed in all the three substrate was in pH 6.7, but the maximum production of α-amylase from the substrate was in fermented carrier

The results showed that the effect of pH on α-amylase production by *Bacillus subtilis* in SSF of banana peel were pH 5.7, 6.7, 7.5and 8.0 respectively. The maximum activity of α-amylase was observed in pH 6.7 in the fermentation medium adjusted at pH 7. At pH 5.7,7.5and8.0, α-amylase activity was low due to more acidity. . Banana peel and *Bacillus subtilis* are next to fermented carrier.

Figure.4 Effect of Incubation Temperature on α-amylase production



The α-amylase activity at 37 ^{o}C showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in incubation temperature caused a decrease in enzyme production. The α-amylase activity at 37^{o}C showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in incubation temperature caused a decrease in enzyme production (Figures.2, 3, 4).

Figure.5 Banana as a substrate

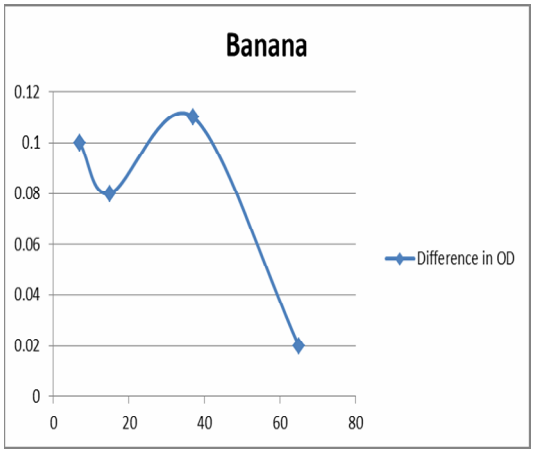
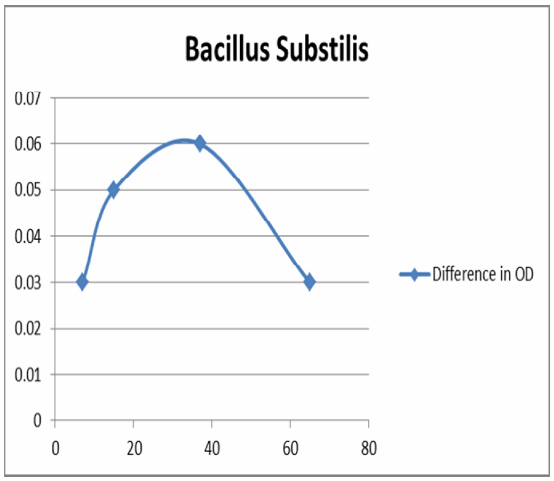


Figure.6 Production by *B. subtilis*



The α-amylase activity at 37 ^{o}C showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in incubation temperature caused a decrease in enzyme production .It is obvious from the table , the maximum activity of α-amylase is observed in all the three substrate was in 37 ^{o}C, but the maximum production of α-amylase from the substrate was in fermented carrier.

Table.2 Comparative analysis Banana peel with *bacillus subtilis*

Different parameters	**Maximum α amylase activity**
Specific α amylase activity	1805.55IU/mL/min
pH at 6.7	0.19
Incubation temperature at 37°C	0.15
Substrate concentration 20 g	0.05

Table.3 Banana peel

Different parameters	**Maximum α amylase activity**
Specific α amylase activity	1388.88IU/mL/min
pH at 6.7	0.1
Incubation temperature at 37°C	0.11
Substrate concentration 20 g	0.06

Table.4 *Bacillus subtilis*

Different parameters	**Maximum α amylase activity**
Specific α amylase activity	972.22IU/mL/min
pH at 6.7	0.16
Incubation temperature at 37°C	0.06
Substrate concentration 20 g	0.05

The study was carried out to check the effect of temperature on α-amylase activity. The α-amylase activity at7, 15, 37, and 65°C, Banana with Bacillus subtilis showed maximum production of α-amylase, which is incubated at 37°C for 24 hours at pH 7. A decrease or increase in incubation temperature caused a decrease in enzyme production. Banana peel and *Bacillus subtilis* are next to fermented carrier (Figure.7)..

Effect of substrate concentration on α-amylase production

Fermentation media containing 5, 10, 15, and 20 g banana peel were sterilized, inoculated and incubated for 24 h at pH 7 and 35°C. The α-amylase activity in different substrates.

Figure.7 Banana with *Bacillus subtilis*

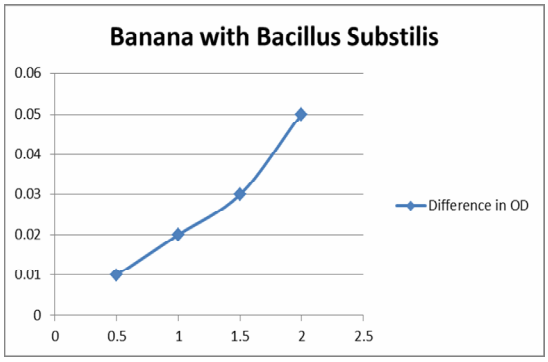
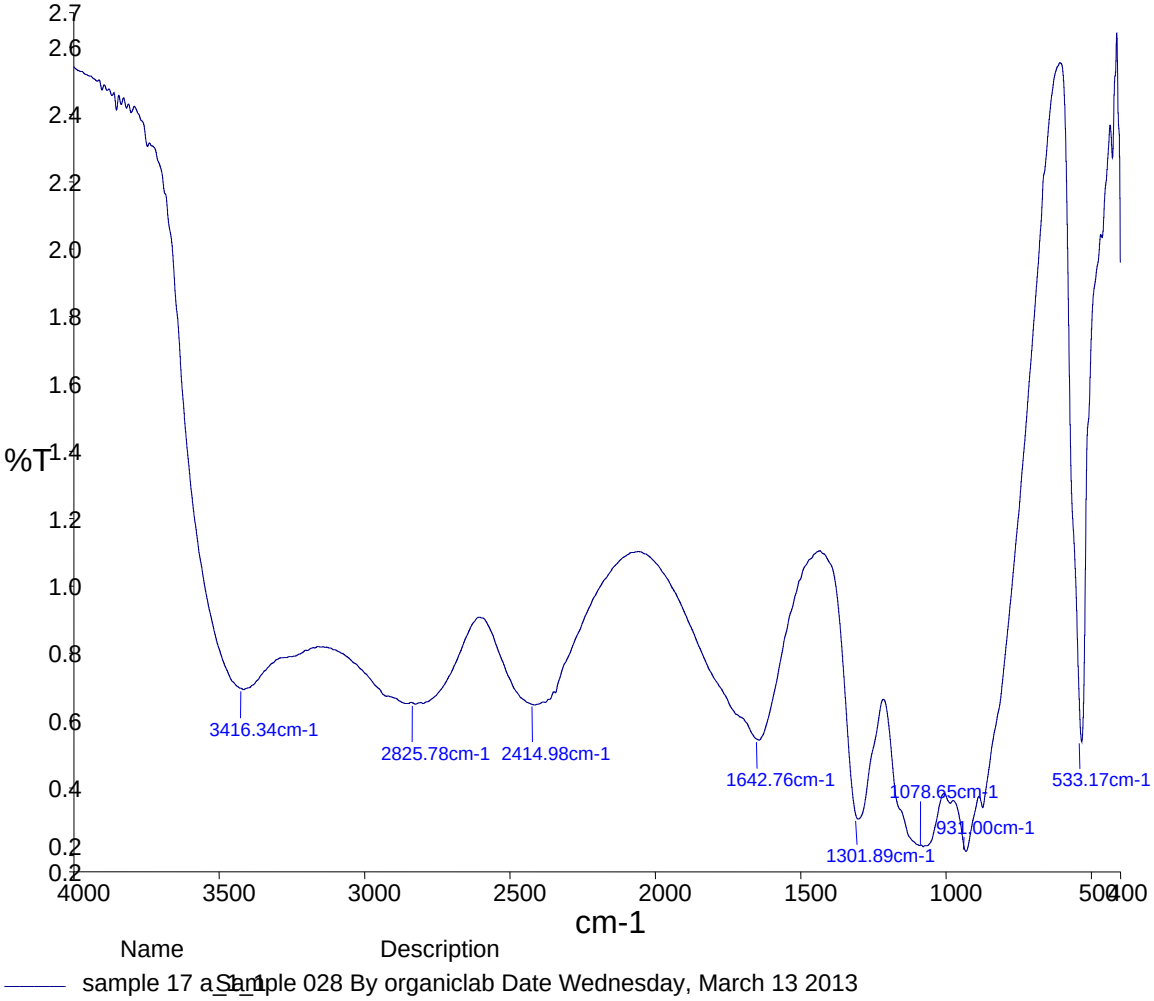


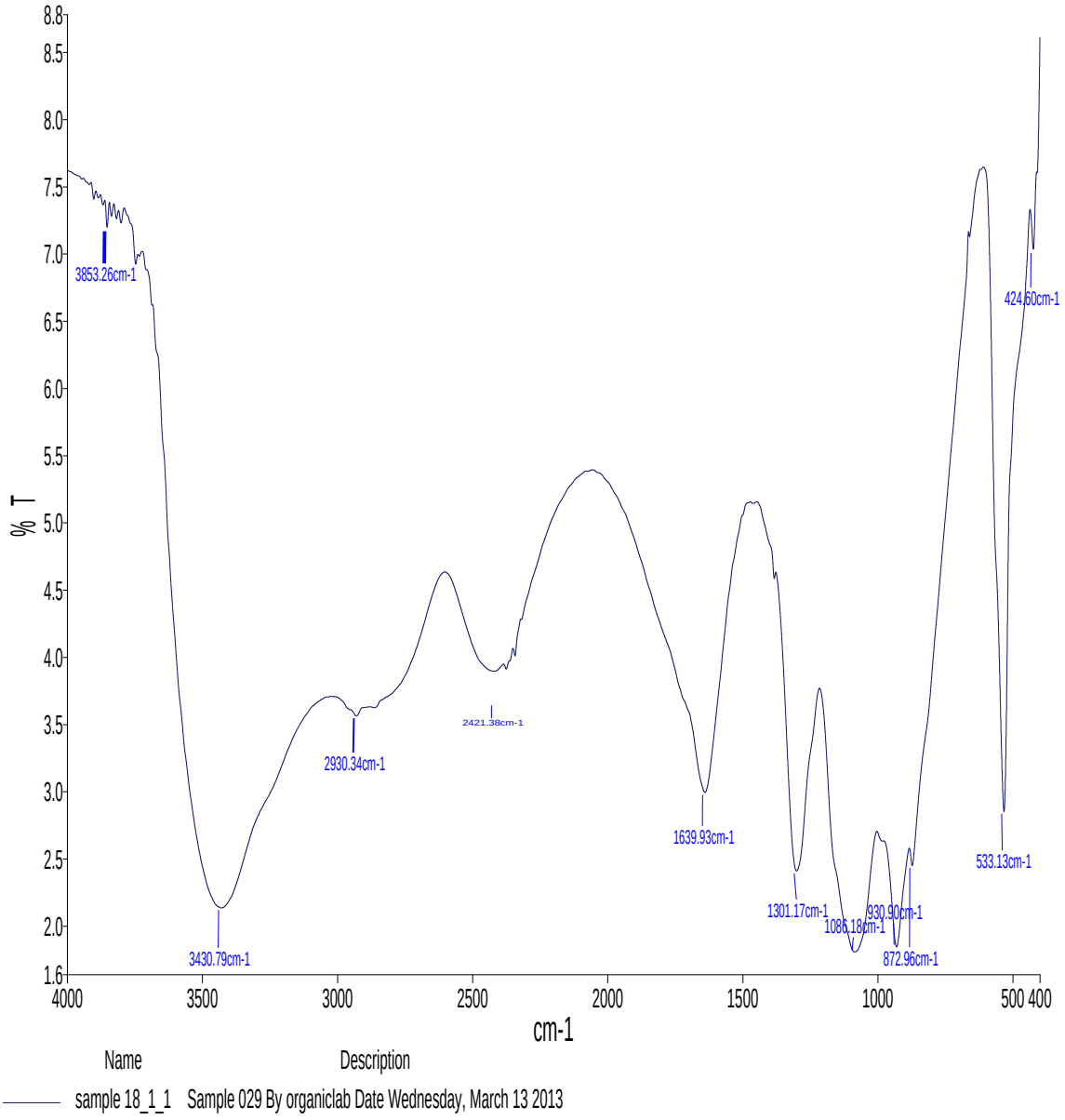
Figure.8 IR spectroscopy of Banana peel Extract



Interpretation

The IR spectroscopy of Banana Extract shows the range of phenols compound 3416nm (3200-3550nm), aldehydes 2825nm (2690-2840nm), and alkenes 1642nm (1630-1680nm).

Figure.9 IR spectroscopy of Banana peel with *Bacillus subtilis*



Interpretation

The IR spectroscopy of Banana with Bacillus shows the range of phenols compound 3430nm (3200-3550nm), alkanes 2930nm (2850-3000nm), alkenes 1639nm (1630-1680nm), carboxylic acid 1301nm (1210-1320nm), and amines 1086nm (1000-1250nm).

concentrations was found to be maximum in fermented carrier is 1.111 x 10^3 µ mol/litre respectively. It was observed that 20 g Banana with *Bacillus subtilis* in the fermentation medium yielded maximum α-amylase activity after 24 h. A further increase in substrate did not increase the enzyme yield significantly because 1mL inoculum was added to each flask and increase in substrate concentration could not affect the growth of organism.

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