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***RESEARCH ARTICLE***

# Isolation and Characterization of Glucanase producing bacteria from Forest Tree Litter

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## ABSTRACT:

The main objective of the project is to isolate bacteria from Forest soil and characterization of bacteria producing Glucanase. Three different bacteria are isolated from the forest soil. The Bacteria are identified using Biochemical Test. The bacterial strain identified are Bacillus, E. coli and Klebsiella. Two screening for Glucanase producing bacteria are made. The primary screening involves the modified agar diffusion with Congo red assay. The clear white zones are observed in the primary screening. The sample 3 has large white zone when compared to sample 2 and sample 1. The secondary screening involves the Glucanse assay stated by Mendal and Webber. The bacterial sample 3 produced higher concentration of Glucanase than other two bacteria. The sample 3 has produced 0.473mg of Glucose after the conversion of cellulose by the Glucanase enzyme.

**KEYWORDS:** Glucanase, Congo red assay, Biochemical test, DNS method by Mandel and Webber.

## INTRODUCTION:

Enzymes produced from microbes are responsible for many reactions that happen in our surroundings. Enzymes are biocatalysts that regulate multiple chemical reaction that occur in living cell such as energy conversion, food digestion, etc. Every year 3.45-ton litter has been produced in the forest. The term litter has defined has dead plant parts such as leaves, fruits, barks, twigs. This litter were decomposed with the help of bacteria and fungi. The litter composed of 78% leaves, 20% flowers, seeds, 2% twigs and barks[1,2].

Among the soil micro flora, the fungi are the leading decomposers of litter and accounts 70% of total biomass decomposed by fungi and bacteria with 20-30% of total biomass decomposed. Various soil factor such as pH, temperature, moisture, climatic conditions such as rainfall, seasonal variations, and litter quality affect the rate of the decomposition.

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The quality of the litter is measured by chemical composition of nitrogen, phosphorus, potassium and cell wall components, such as lignin, cellulose and hemicelluloses that influence the litter decomposition and nutrient release. Slow decomposition of litter builds up the organic matter in the soil. Main factor affect decomposition rate is temperature. Cellulose, hemicellulose and lignin are major macromolecular found on the litter. Cellulose and hemicellulose accounts for 40-50% of the litter composition whereas 40% of the composition is lignin[3]. The microbes such as fungi and bacteria cannot absorb the cellulose in the litter as cellulose is insoluble and large to absorb, so these organisms produce extracellular enzymes such as β-Glucanase, xylanase to degrade the cellulose and hemicellulose present in the litter. The extracellular enzymes are released onto litter or the bound to cell wall by the microbes[4].

Although the fungi are considered to major decomposers of forest litter, studies show that at $1m^2$ of soil there are 1,000-5,000 bacteria and 100 dominant species of fungi are found[5]. Bacteria degrade cellulose in different manner when compared to fungi[6]. The enzyme β-Glucanase hydrolyse the long chains of cellulose

resulting in the release of cellobiose, two glucose units and $CO_2$[7]. The endo and exoglucanase can breakdown the crystalline and amorphous cellulose with their synergic actions. The cellulolytic enzymes in the bacteria are arranged in groups and perform in collective manner [8]. The β- Glucanase can be used in different applications such as paper industry, bioethanol production and agricultural wastes can be bio conversed to replace pumice in the textile industry[9,10].

## MATERIALS AND METHODS:

### Sample Collection:

The forest tree litter samples were collected from the Vandalur forest reserve area. Three different soil samples were collected from under three different plants. The plants were Bamboo, Sweetenia and Khaya.

### Serial Dilution:

Serial dilution is the method of sequential diluting the concentration of microbe presents in the sample. This makes the concentrations of sample more usable. 1g of three soil samples were taken in three different test tubes. 10ml of distilled water was added and stock solution was prepared. The stock solution was diluted to 7 concentrations. 1ml of stock solution was taken and added to 9ml of distilled water in the test tube. Further, the dilution was carried out for 6 test tubes containing 9 ml of distilled water.

### Culturing:

100ml of nutrient agar was prepared and 3 plates were taken. The nutrient agar and the plates were autoclaved at 121°C for 20 min. The plates were kept in laminar air flow and marked with the initials and the Bunsen burner was set up. The nutrient agar was poured into poured into the plates inside the laminar air flow under sterile conditions and kept untouched until the media solidifies. After the media got solidified, 100µl the seventh concentration of serial diluted sample is taken and inoculated in the media by spread plate method. The plates were incubated for 24 hours at the incubator.

### Sub culturing:

Sterile nutrient agar plate was prepared and marked the bottom of the plate with the initials and setting up the required materials in a sterile environment such as a laminar flow hood and turn on the Bunsen burner. Aseptically transferred a very small amount of the given bacterial sample to the one of the quadrant edges of the agar plate. The plate was streaked by quadrant streak method. The plates were incubated for 24 hours at incubator.

### Preparation of Pure culture:

Nutrient broth was prepared and autoclaved. The nutrient broth was kept in the laminar air flow chamber and the Bunsen burner was set up. The quadrant streak plate was taken and observed. A loop of bacteria was scraped form the individual colony in the quadrant streak plate. The loop of bacteria was inoculated in the nutrient broth. The nutrient broth was incubated for 24 hours.

### Biochemical Test:

The Biochemical identification is done to identify the bacteria that were isolated. The various tests done to identify the bacteria are explained below.

### Gram Staining:

A sterile glass slide was taken. A drop or loop of pure culture was taken and a smear was made in the Glass slide. The smear is heat fixed with the Bunsen burner. The smear is flooded with crystal violet (Primary strain) for 1 minute. Then rinsed with distilled water. The drops of Gram's Iodine (Mordant dye) were added to smear and kept for 1minute. Then decolourized using 95% ethyl alcohol or acetone (Decolourizing agent). After 10 sec the smear was rinsed with distilled water. One drop of Safranin (Counter strain) was added to the smear. Then rinsed with distilled water. The slide was observed under microscope. The Gram-Positive bacteria has thick peptidoglycan layer and the bacteria will be stained violet colour. The Gram-negative bacteria has thin peptidoglycan layer and the bacteria will be stained pink colour. Since the Gram-negative bacteria's peptidoglycan layer dissolved by the ethanol and the bacteria absorbed the Safranin.

### Indole Test:

The Indole Test is a biochemical test to identify bacteria that can convert tryptophan into indole. The indole test is performed after the bacteria incubated for 24 hours prior to test in the peptone broth (0.35g/25ml). After incubation for 24 hours, five drops of Kovac's reagent was added to the culture broth. The change of colour to red indicate the positive indole bacteria. When there is no change in colour, then it is negative indole bacteria.

### Methyl red test:

The methyl red test is a quantitative test that measures the amount of acid produced by different bacterial species. The Methyl red performed after the bacteria incubated for 24 hours prior to test in the MR-VR broth (0.75g/50ml). After incubation for 24 hours, few drops of Methyl red reagent to the culture broth. The Colour change to methyl red colour indicates the acid nature of bacteria and test is Positive. When there is no colour change the test is negative.

### Voges–Proskauer test:

The test identifies the bacteria that can convert glucose into acetylmethylcarbinol. The Voges–Proskauer test performed after the bacteria incubated for 24 hours prior

to test in the MR-VR broth (0.75g/50ml). After incubation for 24 hours, few drops of alpha-naphthol and potassium hydroxide to the culture broth. The observation of pink- red colour indicates the positive Voges–Proskauer. No Colour change indicate negative Voges–Proskauer.

**Citrate Test:**
The citrate is a test that is performed to determine the ability of bacteria to utilize the citrate as carbon source. The Simmons Citrate agar was prepared (0.61g/25ml). The Simmons citrate agar poured in a test tube and the test tube is kept in slant position. After the agar solidifies, inoculate the bacteria and kept for 24 hours incubation. The Colour change for green to blue indicates the positive citrate bacteria. When there is no colour change is observed, the test is negative.

**Primary Screening of Glucanase producing bacteria:**
The bacteria subjected to screening for the Glucanase property are by using modified agar diffusion method [11]. The Carboxyl Methyl Cellulose medium as main carbon source is used for modified agar diffusion method for Glucanase property. The CMC medium contained the following 0.05g $MgSO_4$. $7H_2O$, 0.005g $CaCl_2$, 0.005g $NaNO_3$,0.009g $FeSO_4.7H_2O$,0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g $KH_2PO_4$, 2g peptone, 19g Agar. The CMC medium is used so that bacteria which has ability to produce the Glucananse enzyme and break down the Cellulose present in the medium into glucose and use the glucose for the carbon source of the bacteria. The 3 sterile 1% CMC agar medium plates were prepared. A loop of Bacteria was inoculated in each of the CMC medium. The CMC medium plates were kept for incubation for 2 days in the incubator. After 48 hours, the plates were flooded with 0.4% Congo red indicator for 30 min and then destained with 0.1M NaCl.

The clear wide zone indicates the breakdown of cellulose into glucose by the bacteria. Isolates with higher Glucanase where used for further work.

**Secondary Screening of Glucanase producing Bacteria:**
The bacterial isolates where subjected to secondary screening by using the CMC medium as main carbon source. The CMC medium contained the following 0.05 g $MgSO_4$. $7H_2O$, 0.005 g $CaCl_2$, 0.005g $NaNO_3$,0.009g $FeSO_4.7H_2O$,0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g $KH_2PO_4$, 7g peptone. 1% CMC medium for 50 ml was prepared. The bacterial isolates were inoculated and incubated for 2 days at 28± 2°C. After incubation, the medium was centrifuged at 12,000rpm for 15 minutes in a cold centrifuge and supernatant was recovered. The Glucanase activity was calculated from the amount of reducing sugar present in the medium estimated using the method [12].

**Glucanase assay:**
The method of Mandels and Webber are used to determined β – Glucanase activity. 0.1M Citrate buffer pH 5.6 and DNS reagent was prepared. 1% CMC medium in 0.1M citrate buffer was prepared. To 0.5ml of 1% CMC medium in 0.1M Citrate buffer, 1ml of culture filtrate was added and taken in test tube. The test tubes were incubated at 40°C in a water bath for 30min. The reaction was terminated with 2ml of DNS reagent added to the test tube and boiled for 5 minutes. The absorbance of the reaction mixture is measured at 540nm using a spectrometer. One unit of glucanase was defined as the amount of enzyme that released 1µg/ml reducing sugar as glucose equivalent per minute in the reaction mixture under the specified assay conditions. The absorbance value is plotted against the graph.

## RESULTS AND DISCUSSION:

After the bacteria are isolated and pure culture were made. The biochemical identification has been done to identify the bacteria.

**Gram Staining:**
Gram staining was performed on 3 bacterial isolates. The bacterial isolates were found to be Gram positive bacteria. When observed in the microscope the bacteria sample 2 stained as violet colour and it is confirmed as Gram positive bacteria. The sample 1 and sample 3 are stained red colour and confirmed as Gram negative bacteria. Felix, C. R., and Ljungdahl has discussed about the production of Glucanase by Gram negative bacteria in their research work[13].

**Table 1: Gram staining**

| Sample | Gram positive bacteria | Gram Negative Bacteria |
|---|---|---|
| 1 | | - |
| 2 | + | |
| 3 | | - |

**Biochemical Test:**
The results of biochemical tests are listed in the below table. With respect to the results obtained performing the test the bacteria are identified as *Bacillus, E. coli, Klebsiella*. The sample 1 has got positive result in the Indole test, Methyl red test, citrate test and negative result in VP test. The sample 2 has got positive result in the Indole test, Methyl red test, and negative result in citrate test, VP test. The sample 3 has got positive result in the Methyl red test, and negative result in Indole test, citrate test, and VP test. In this work they identified stains of *Bacillus, Pseudomonas, Klebsiella* are isolated and confirm by Biochemical test which are similar to the similar work[14].

**Table 2: Biochemical Identification**

| Sample | Indole test | Methyl red Test | VP test | Citrate test |
|---|---|---|---|---|
| Sample 1 | + | + | - | + |
| Sample 2 | + | + | - | - |
| Sample 3 | - | + | - | - |

**Primary Screening of Glucanase producing Bacteria:**

The primary screening of glucanase producing bacteria was done with CMC medium by the modified agar diffusion method. The three different bacterial isolates were screened. All the three bacterial isolates showed glucanase activity with clear white zones. The sample 3 has large clear white zone when compared to sample 2 and sample 1. The sample 1 has moderate white zone. A researcher in his research work screened the wood decaying fungi with CMC medium and performed Congo red assay and observed the production of glucanase enzyme with white clear zones [15]. They stated that the microbes such as bacteria, fungi take Cellulose has the carbon sources which leads to the white zones. These white zones are examined with Congo red assay.

**Secondary Screening of Glucanase producing Bacteria:**

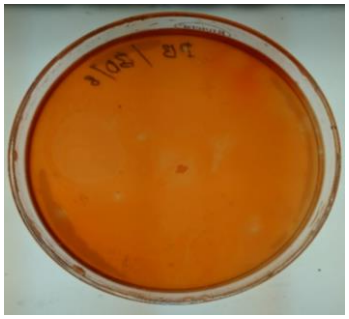
The Glucanase activity were measured at 540 nm in spectrometer [13]. The glucanase activity of the bacteria was determined by the DNS method. The glucanase activity of bacteria was compared to the standard glucose solution. The standard glucose was observed in different concentrations and compared to the Glucanase activity in 1% CMC medium in 0.1M citrate buffer for 30 mins in the boiling water. Then the reaction was stopped with DNS reagent for 5 mins. The sample 3 showed greater concentration of glucose produced by converting cellulose into cellobiose such as monomeric sugars e.g. Glucose, fructose. The sample 3 has 0.473 mg in the citrate buffer is greater than the sample 2 has 0.0146 mg. The sample 1 0.0351 has in the citrate buffer is lesser than the sample 3 has 0.473 mg. A researcher studied the amount of glucose produced due to Glucanase enzyme produced by the fungi and observed that strains of *A. niger* has produced higher concentration of 1.2 mg/ml [15].

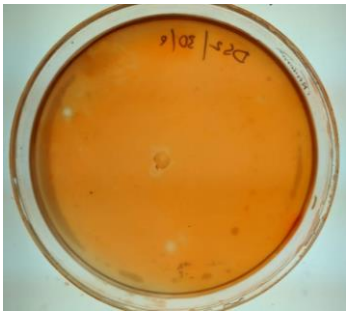
**Table 3: Concentration of Glucose**

| Concentration (mg/ml) | Abs (540nm) |
|---|---|
| 0.2 | 0.08966 |
| 0.4 | 0.16492 |
| 0.6 | 0.24018 |
| 0.8 | 0.31544 |
| 1 | 0.3507 |

**Table 4: Concentration of Glucose converted by bacteria by producing Glucanase**

| Concentration (mg/ml) | Abs | Concentration (mg/ml) | Concentration (µg/ml) |
|---|---|---|---|
| Sample 1 | 0.0351 | 0.013975617 | 13.97561701 |
| Sample 2 | 0.0146 | -0.046981861 | -46.98186143 |
| Sample 3 | 0.1897 | 0.473684211 | 473.6842105 |





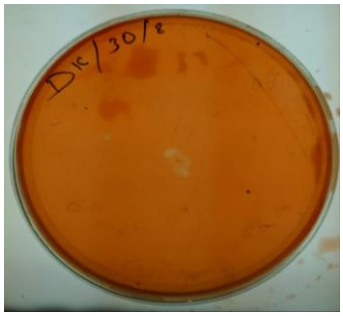
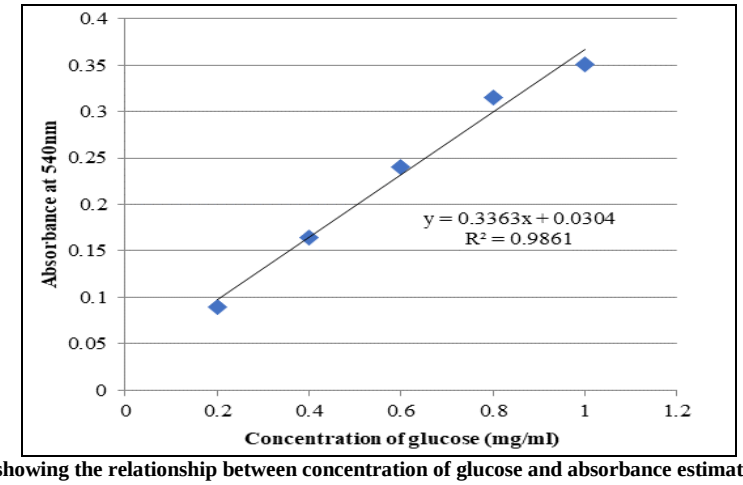


**Figure 1: Primary screening of sample 1 Figure 2: Primary Screening of sample 2 Figure 3: Primary Screening of sample 3**




**Graph 1: Standard graph showing the relationship between concentration of glucose and absorbance estimated by DNS method**

## CONCLUSION:

Three different type of bacteria has been isolated from the soil collected from the Vandalur Forest. The 3 bacteria are sub- cultured and pure culture has been obtained. The biochemical identification is done to 3 bacteria. Out of three bacteria isolated one of the bacteria is Gram positive bacteria and 2 bacteria are gram negative. The bacteria analysed by Biochemical identification. The biochemical test has identified that are bacteria of species *Bacillus, E. coli, Klebsiella*. The bacteria analysed for the production of Glucanase using the modified agar diffusion method using CMC medium and Congo red assay. The Glucanase assay has been done the sample 3 bacteria has produced higher concentration of Glucanase.

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