Screening and Characterization of Lignocellulolytic Bacteria from Agricultural Waste and Contaminated Soil

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(21581)

This project is submitted in partial fulfilment of the requirement of the degree of Bachelor of Science with Honours (Resource Biotechnology)

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Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

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<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose Gel Electrophoresis</td>
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<tr>
<td>min</td>
<td>Minutes</td>
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<td>s</td>
<td>Seconds</td>
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<td>nm</td>
<td>nanometer</td>
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<td>degree Celcius</td>
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<td>µL</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>h</td>
<td>hours</td>
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<td>cm</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>MR-VP</td>
<td>Methyl red- Voges-Proskauer</td>
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Screening and Characterization of Lignocellulolytic Bacteria from Agricultural Waste and Contaminated Soil

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Abstract

Lignocellulolytic bacteria are the types of bacteria that are essential in degrading lignocellulosic material such as agricultural waste and plant material. The intention on conducting this study is to gain knowledge in the degradation of lignocellulose by the microorganism especially bacteria. There is not much of knowledge about the degradation of lignocellulose by bacteria such as how the bacteria able to degrade lignocellulose materials, which strains of bacteria is capable of degrading lignocelluloses materials and what mechanism are used by the bacteria to degrade the lignocelluloses. The main aim of the study is to screen, isolate and characterize the lignocellulolytic bacteria. Through this study, bacteria that capable of degrading lignocelluloses materials were screened, isolated and then characterized. The best lignocellulose bacteria were identified by the morphological, molecular and biochemical characteristics. The bacteria were screened for ligninolytic properties using carboxymethylcellulose (CMC) and Remazol Brilliant Blue reagent (RBBR) substrate specific plate assay. Besides that, DNS enzyme assay was also conducted in order to gain the information on the ligninolytic enzyme activity produced. After that, the bacterial cells were characterized based on morphological characteristic using staining methods, biochemical characteristics and molecular characteristics using colony PCR and AGE. The PCR product was sequenced and then BLAST search conducted in order to identified the species of the bacteria. The result of BLAST identified the S3 sample as Burkholderia sp. while the C4 as Pantoea sp.

Keywords: lignocellulolytic, bacteria, morphological characteristics, biochemical characteristic, molecular characteristics

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Abstract


Kata kunci: lignocellulolitik, bakteria, ciri-ciri morfologi, ciri-ciri biokimia, ciri-ciri molekul
1.0 Introduction

Interest in the exploitation of plant biomass as a renewable resource has increased throughout the years since the demand for biofuels increases. This has significant effect to the research on microbial degradation of lignocellulose since most of the organic carbon that is produced as plant biomass is accumulated in lignocellulose which is the main structural component of plant cell walls. The lignocellulose is not only a resource that can provide fuels but also a resource for structural materials such as paper and fiber and a potential source for human food, animal feed and chemical feedstock.

These researches are mostly concentrated on the roles of fungi as lignocelluloses degrader however, recently, lignocelluloses degrading prokaryotes especially bacteria are beginning to receive more attention since manipulation of the bacteria easily achieve compare to fungi. The research on prokaryote has been concerned on obtaining good evidence for the existence of ligninolytic activity and has been achieved for several actinomycete species.

In the nature, lignocellulose is digested in the rumen of ruminants and in the digestive system of insect such as termites and other animals that feed on lignocellulose source which is the plants. Lignocellulose materials are made up of 3 major components which are the cellulose, hemicelluloses and lignin. The most recalcitrant is the lignin which protects the cellulose and the hemicelluloses from enzymatic attack by some microorganisms (Bonnarme and Jefferies, 1998). One-third to one-half of the approximately 150 billion tones of organic matter synthesized annually is constituted by cellulose (Bayer and Moray, 1994). The hemicellulose is a major plant constituent that is second in quantity to cellulose and an ill-defined group of carbohydrate. According to Erikson et al. (1990), the general recalcitrance of
cellulose, hemicelluloses and lignin and the importance of their biodegradation in the environment have received much attention for several years.

The industries such as forestry and agroindustries generated large amounts of lignocellulosic “waste” which is often disposed of by global biomass burning which has been considered as a global phenomenon (Levine, 1996). However this “waste” has multiple functions and can be converted into various value added product such biofuels, chemicals and cheap energy sources for fermentation. The important component in the conversion process is the decomposers which are the microorganism such as bacteria. The problem statements of this study are which bacteria that capable of degrading the lignocelluloses materials and what make the bacteria capable of degrading lignocelluloses materials.

The main aims of this research study is to screen, isolate and identify the bacteria that capable in degrading lignocelluloses materials and then to characterize the bacteria. Through the morphological and the biochemical characteristics, the best lignocelluloses degrading bacteria were chosen.

Therefore in order to achive the aim, the objectives are as follows:

1) to screen and isolate ligninocellulolytic bacteria from agricultural waste and contaminated soil.

2) to identify and characterize the bacterial strains isolated.
2.0 Literature Review

2.1 Bacterial Characterization

Characterization is an important step in order to differentiate the different species of bacteria. Characterization of the bacteria can be done in three ways which are morphological characteristic, biochemical characteristic and molecular characteristic.

For morphological characteristic, the bacteria will be distinguished through physical appearances such as the colony shape and size, shape and arrangement of the bacterial cell and the cell wall of the bacteria. According to Singleton (1992), bacterial cells vary widely in shape, according to species such as rounded or spherical cell of any species are called cocci and elongated, rod-shaped cells of any species are called bacilli. As for the cell walls, there are two types of bacterial cell walls which are Gram-positive cell walls and Gram-negative cell walls. The Gram-positive cell wall is the cell wall that retain the dye colour when stained with Gram staining and this wall is relatively thick which about 30-100 nm and generally has a simple, uniform appearance under the electron microscope. Example of Gram-positive cell wall bacteria are Staphylococcus and mycobacterium. The Gram-negative cell wall is the wall that can be decolourised and this wall is 20-30 nm thick and has a distinctly layered appearance under the electron microscope. Example of Gram negative cell wall bacteria is E. coli. The motility of the bacteria can be observed under microscope by using a ‘hanging drop’ preparation.

As for biochemical characteristic, the tests required are the Voges-Proskauer test, the methyl red test, the citrate utilization test, and the hydrogen sulphide production test. Voges-Proskauer test is a detection test for the butanediol pathway which carried out by Enterobacter aerogenes but not by Escherichia coli. This test detects the presence of intermediary
metabolite of the pathway rather that the end product since the end product is a neutral product. The methyl red test is a test to detect whether the bacteria carry the mixed acid fermentation or not. The basis of this test is the colour changes that occur to the methyl red solution in different pH value. At pH below 5.0, the methyl red is red in colour and this is the positive result for the test. This is because the mixed acid fermentation pathway produce various end products such as ethanol, acetate, formate and carbon dioxide which will contribute to acidic condition that makes the methyl red solution red. The citrate utilization test is a test for determination of the ability of the bacteria to utilize citrate as its sole carbon source. In this test, Simmon’s citrate agar is used. This medium contains sodium citrate which act as the carbon source, ammonium salts as nitrogen source and bromthymol blue as pH indicator. Growth of bacteria on the medium will result in the rise of pH and this will cause the pH indicator to turn from green to blue. For the hydrogen sulfide production test, the SIM medium is used. The SIM medium contains sodium thiosulfate and ferrous ammonium sulfate which act as indicators of hydrogen sulfide production. Black precipitate will be produced if H₂S gas reacts with the indicators which produce ferrous sulfide

As for molecular characteristic, colony PCR is used. Colony PCR is a type of PCR that conducted without conducting the DNA extraction first. After the colony PCR is conducted, the PCR products ran on agarose gel electrophoresis to view the band formation and detect the PCR product.
2.2 Lignocelluloses

2.2.1 Composition of lignocellulosic materials

Lignin, the second most abundant renewable resource on the earth is difficult to degrade (Huang et al., 2006;) and also slows down the biodegradation of cellulose and hemicelluloses in lignocellulosic plant materials because it acts as a physical barrier protecting the carbohydrates. According to Mitchell et al. (1992), lignocellulosic materials are composed of three major components which are lignin, polysaccharides and extraneous substances. The lignin is a three dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds. The polysaccharide component is comprised of cellulose and hemicellulose which made up 60-80% of the total wood where cellulose as the major wood component whiles the hemicellulose is in close association with cellulose in the cell wall. Besides that, the polysaccharide component also comprised of starch and pectin substances and also water-soluble polysaccharides such as the arabinogalactants. The extraneous components which contribute only a few percent of the wood are comprised of inorganic substances which are analyzed as ash and organic substances such as terpenes, resins and phenols (Sanchez, 2009).

2.2.2 Mechanism of lignocelluloses degradation

Mechanism of degradation of lignocelluloses can be divided into two which are degradation of cellulose and degradation of lignin. In the degradation of cellulose or cellulose hydrolysis, several cellulases enzymes are involve and it is a complex process. Cellulose is composed of \( \beta\)-D-glucopyranose units which are linked by \( \beta\)-(1-4)-glycosidic bonds. The cellulases
enzymes can be divided into three major types which are the endoglucanase, the
cellbiohydrolase and the cellobiase. The endoglucanase or 1,4-β-D-glucan glucanohydrolase
is an enzyme that randomly attacks the internal linkages in the cellulose chain thus releasing
oligosaccharides. The cellbiohydrolase or 1,4-β-D-glucan cellbiohydrolase is an enzyme
that cleaves cellobiose from non-reducing end of cellulose chains. As for the cellobiase or 1,4-
β-D-glucosidase, it is function to hydrolyse the cellobiose and other oligosaccharides that had
been produced to glucose. This step is crucial since cellobiose inhibits the endoglucanase and
the cellbiohydrolase. In the degradation or hydrolysis of lignin, the lignin will be converted
into ethanol since lignin contains no sugar which makes it impossible to convert lignin into
sugar. The major enzyme that involved in degradation of lignin is lignin peroxidase.. The
enzyme catalyses the oxidative cleavage of bonds between phenylpropane units of lignin.
Another enzymes that usually involve in the degradation of lignin are glyoxal oxidase and
Mn(II) dependent peroxidases, although their catalytic mechanisms are different (Bonnarme
and Jeffries, 1998).

There have been studies on lignin-induced peroxidases which are extracellular and cell
associated were identified and characterized in Streptomyces spp.. There is an equivocal
evidence for the involvement of the other enzyme activities such as phenol oxidase and
cellulose, however, the nature of lignocelluloses structure suggesting that a number of enzyme
activities that contribute to degradation of the lignin component.
2.3 Lignocellulytic Bacteria

According to Singleton (1992), bacteria are minute organisms which occur almost everywhere and their presence are always unaware because their activity are less obvious and they are so small. However, the bacteria plays many essential roles in the natural cycles such as the nitrogen cycle and bacteria also contribute a lot in so many industrial sectors such as in the production of antibiotics. Each species of bacteria produce different metabolite products dependent on the habitat, environment and ways of getting nutrients. Some of the bacteria can produce specific enzymes that can act on specific substances. There are bacteria that can degrade lignocellulosic materials which contain lignin and cellulose. These bacteria are known as lignocellulolytic bacteria and these bacteria play a major role in the bioconversion of plant biomass and degradation of plant materials in rumens and insects. An example of lignocellulolytic bacteria are the *Bacillus shackletonni*, *Streptomyces thermovulgaris* and *Ureibacillus thermosphaericus*. Another example are the actinomycetes. The hyphal growth of the actinomycetes is well suited for the colonization of plant biomass and also they secrete a range of enzymes that active against lignocellulose materials (McCarthy and Ball, 1991). The research has been focused on the screening and detection of good lignocelluloses degraders among the actinomycetes.
3.0 Materials and Methods

3.1 Sampling

Samples used were oil palm empty fruit bunch (OPEFB) compost and soil that was contaminated with hydrocarbon. OPEFB that used are in the final stage of decomposition and obtained from the stock at the lab and the oil contaminated soil was also obtained from the lab stock. OPEFB was used because of the composition of lignin and cellulose in the OPEFB whereas soil contaminated with hydrocarbon was used because some of hydrocarbon degrading bacteria are capable of degrading lignocellulolytic materials.

3.2 Media preparation

Nutrient broth was prepared was and used in order to enriched the bacterial growth. Minimal salt medium (MSM) broth was prepared in the lab by adding the following ingredients 7.0g/L $K_2HPO_4$, 3.0g/L $KH_2PO_4$, 1.0g/L $(NH_4)2SO_4$ and 2.05g/L $MgSO_4.7H_2O$. Nutrient agar (NA) was prepared and used as growth media to observe the colony characteristics of the bacteria.

As for the secondary screening, the media used were MSM media which consist in of 7.0g/L $K_2HPO_4$, 3.0g/L $KH_2PO_4$, 1.0g/L $(NH_4)2SO_4$ and 2.05g/L $MgSO_4.7H_2O$. 100ml of the MSM media was prepared. For the RBBR decolourization, 0.01% RBBR was added into the media while for the cellulase enzyme activity, 1% of CMC was added.
3.3 Enrichment and screening

The method used was enrichment method where each of the samples was enriched in the nutrient broth first. The method used is based on the method by Chan et al (2007). The nutrient broth (NB) was prepared using BD nutrient broth powder where 8 g was needed to make 1 L of nutrient broth. Approximately, 200 ml of NB was prepared where each of the samples was enriched in conical flask containing 100 ml of NB. Two gram of each samples were weighted on digital balance and transferred into the broth. The conical flasks were then placed on the shaker for 24 to 48 hours at room temperature. After 24 to 48 hours, 1 ml of the enriched sample were transferred into prepared MSM media. The MSM media acted as primary selective media. The samples were again placed on the shaker for 48 hours. After 1 week (168 hours), an aliquot were sampled and diluted using serial dilution method until $10^7$. Sterile distilled water was used to dilute the samples. In the serial dilution experiment, 7 test tubes were filled with 9 ml of sterile distilled water and then 1 ml of sample was added into the first tube and from the first tube ($10^1$), 1 ml of the sample was transferred to the second test tube ($10^2$) and so on until the last test tube ($10^7$). From each of the samples, the samples diluted to $10^5$, $10^6$ and $10^7$ were selected and spread onto NA plate and incubated for 24 to 48 hours at 37°C. After incubation, the growth of the bacterial colonies was observed. The bacterial colonies were then restreaked onto new NA plate based on the size, colour, and different visible morphological characteristics between the colonies. The bacterial isolates were then cultured into LB broth as stock culture and working culture were cultured on the NA plate.
3.4 Secondary screening

Secondary screenings were done by using RBBR decolourization in order to detect lignin degrading ability (Machado et al., 2005) and conducting enzyme assay to detect the activity of lignocellulosic enzyme produced by the bacteria.

As for RBBR decolourization, 2 ways were done which were the plate method and the liquid method. In the plate method, MSM agar containing 0.01% RBBR was used to culture the bacteria. All the different colonies were stab on MSM plates containing 0.01% RBBR and incubated for 1 week at 37°C. The production of halo around the colonies was observed everyday starting from the first 24 hours until 1 week. As for the liquid method, liquid media which was also MSM containing 0.01% RBBR, was used. The media was placed in bijou bottle and the bacteria were inoculated into the media. The inoculated media then incubated by shaking at 120 rpm at the temperature of 37°C for the period of 1 week. After 1 week, the optical density (OD) of the each of the cultures was observed using UV spectrophotometer at 595 nm. Approximately, 1.5ml culture from each Bijou bottles were transferred into fresh Eppendorf tubes and centrifuged for 5 minutes at 13000 rpm. The supernatant then transferred into cuvettes for the OD reading. For each of the cultures, the readings were done twice and the average of the reading calculated. From the average readings calculated, the percentage of RBBR decolourization calculated using following formula:

\[
\text{Percentage of decolourization} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}}
\]
For the detection of cellulose degrader, DNS method was conducted. DNS assay was used to determine the amount of reducing sugars in crude enzyme (Bharathidhasan et al. 2009). Before conducting the assay, the DNS reagent was prepared at room temperature by mixing 2 g NaOH, 2 g DNS, 0.4 g phenol and 0.1 g sodium sulphite in 200 ml of distilled water. Before adding the sodium sulphite, the NaOH, DNS and Phenol were mixed well. For the DNS assay, the bacterial culture was harvested once in every 6 hours for the first 24 hours and then every 12 hours for the next 24 hours. The harvested cultures were transferred into a fresh Eppendorf tube and centrifuged for 5 minute at 4°C and 3000 rpm. Then, the 1ml of supernatant which is the crude enzyme were transferred into a fresh test tube containing 400 µL of 1% CMC solvated in 0.05 M, pH 7.2 tris HCl buffer. The test tubes were vortexed to mix them well and then incubated for 10 minutes at 55°C. After 10 minutes, 800 µL of DNS was added into each tubes, vortexed to mix them well and then boil at 100°C for 20 minutes. After 20 minutes, 400 µL Rochelle salt was added to each tubes and let the mixture cooled down. The tubes were boiled to develop red-brown colour while the Rochelle salt was added to stabilize the colour. After the solution has cooled down, the optical density (OD) was read at 575 nm.
3.5 Characterization of the isolates

3.5.1 Morphological Characteristic

The morphological characteristics are based on the Bergey’s Manual of Determinative Bacteriology. As for the colony morphology, the shape of the colony, the size of the colony, and the colour of the colony were observed. There are several difference characteristics that can be distinguished using the naked eyes.

For the Gram staining, the sample smears were prepared by picking up the colony using a loop and then smeared on the glass slides that have been dropped with 1 drop of distilled water. The smears were heat fixed before the staining process started. Firstly, the primary staining which is the crystal violet were dropped onto the smears and cover up the smear for 1 minute. After 1 minute, the excess crystal violet were washed using distilled water. The slides were blotting using tissue paper to remove the excess water. Then, the smears were flooded with Gram iodine which functions as the mordant for 1 minute. After 1 minute, the excess iodine was washed using distilled water. The excess water was dried using tissue paper. After that, 70% ethanol was poured through the slide for about 30 s. Excessive ethanol was washed using distilled water. The slides were dried. Then, the counter stain which is the safranin was flooded onto the slides for 30s. Excessive safranin was washed using distilled water. The slides were blotted dry before the slides viewed under the microscope.

3.5.2 Biochemical Characteristics

As for the biochemical test, there were few basic biochemical tests conducted such as the Voges-Proskauer test, methyl red test, citrate utilization test, and hydrogen sulfide production test.
For the Voges-Proskauer test, the materials needed are the MR-VP medium, Barritt’s reagent A and B. The samples were inoculated into 5 ml of MR-VP medium and incubated in 37°C for 24-48 hours. After incubation, half or 2.5 ml of the broth were transferred into clean test tubes. Few drops of Barritt’s reagent A and B were added into the broth. The colour changes in the medium were observed.

For the methyl red test, the materials are MR-VP medium and methyl red solution. The remaining cultured MR-VP medium from the Voges-Proskauer test were used where few drops of methyl red were added into the solution. The immediate colour changes in the media was observed.

For the citrate utilization test, the materials are Simmon’s citrate agar which prepared using Oxoid Simmon’s citrate agar powder. The agar were poured into plates after autolave and waited for the agar to solidify. After the agar had solidified, the samples were streaked onto the Simmon’s citrate agar and incubated for 24-48 hours at 37°C. The colour changes of the agar were observed.

For the hydrogen sulfide production test, SIM media was used. 10 ml of SIM media were prepared in 5 sterile test tubes and each of the tubes were stabbed with the samples with 1 tube act as control tube without inoculate. The tubes were incubated for 24-48 hours at 37°C.

3.5.3 Molecular Characterictics

For the molecular characteristics, colony PCR was conducted in order to get the DNA of the bacterial sample (Hiraishi, 1992). This colony PCR was done using PA as forward primer and PH as reverse primer. The master mix of the PCR are prepared as follow for the total of 50 μL where 10 μL 10X PCR buffer was added into PCR tube first, then 6 μL 2mM MgCl2 was
added into the tube. After that, 2 μL 10 mM dNTPs was added and 31 μL double distilled water was also added into the tube. Finally, taq added into the tube and the mixture was centrifuged for a couple of second to mix them well.

For each of the PCR tubes, 12.5 μL of PCR master mix were added. After that, the forward primer, PA was added with the amount of 1.25 μL and the reverse primer, PH was also added into the tube with the equal amount as PA. Then, double distilled water was added into the tube to make the total volume of the reaction equal to 25 μL. the sample or the colony was picked using the pipette tip and then mixed slowly into the PCR tube.

The colony PCR was conducted using the ESCO Swift.MiniPro PCR machine with the condition where for the initial denaturation, the temperature needed is 96°C for 5 min., denaturation is done at 96°C for 30 sec for each cycle, annealing is done at 55°C for 1 min. for each cycle, extension stage is done at 72°C for each cycle, the final extension stage is done at 72°C for 7 min. and the end stage is at 4°C. For the denaturation, annealing and the extension stage, a complete 30 cycles are done for each of the stages.

After the colony PCR, the PCR products were ran on the agarose gel electrophoresis for 45 minute using 110 V. The PCR products were run on 1% agarose gel. The gel then viewed under UV. The PCR product obtained were then purified before sending to 1st Base for DNA sequencing using direct PCR product purification kit. After the purification, the PCR products were sent to 1st Base for DNA sequencing. The results from the sequencing were used to conduct nucleotide BLAST in order to identify the bacteria species.
4. 0 Result and discussion

4.1 Spread plates

From the serial dilution, the last three of the dilution factor \((10^5, 10^6, \text{ and } 10^7)\) from each of the samples was picked and spread into nutrient agar (NA) plate. This was because the last three of the dilution factors were assumed to have the less bacterial colony and there is also possibility that no bacteria will grow from the dilution. The plates were incubated for 24-48 h at room temperature for the bacteria to grow. The bacterial growth on the plate can be view at appendix. The plates that spread with the samples at the dilution factor \(10^5\) showed more colony growth compare to the \(10^6\) and the \(10^7\) (refer to Appendix 1). This is because at each of the dilution factor which starting from 1-7, the concentration of bacteria colony has been reduced.

4.2 RBBR decolourization

The RBBR decolourization was conducted for a period of 1 week where isolates were inoculated into bijou bottles containing MSM media and 0.01% RBBR. The bijou bottles were incubated by shaking at 120 rpm at the temperature of 37°C. After 1 week, the optical densities (OD) of the media were read using UV spectrophotometer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average OD</th>
<th>% of decolourization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.598</td>
<td>0</td>
</tr>
<tr>
<td>S1</td>
<td>0.277</td>
<td>53.68</td>
</tr>
<tr>
<td>S2</td>
<td>0.385</td>
<td>35.62</td>
</tr>
<tr>
<td>S3</td>
<td>0.257</td>
<td>57.02</td>
</tr>
<tr>
<td>S4</td>
<td>0.548</td>
<td>8.36</td>
</tr>
</tbody>
</table>

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Table 4.1 shows the percentage of decolourization of the RBBR after 1 week of incubation. The percentage of the decolourization was compared and the samples with the highest percentage were chosen to be further analysis. The samples that were chosen were S3 and C4 (highlighted). The entire test such as the enzyme assay, biochemical test, and the colony PCR were conducted only on these two samples instead of all the 14 samples.

Reduction in the OD reading shows that the bacterial cultures were consuming the RBBR dye and making the RBBR as their carbon source. In the MSM media provided to the bacterial cultures, there was no carbon source instead of RBBR. In order to obtain energy, the bacteria consume RBBR and convert the RBBR to energy which result in the decolourization of the medium.
4.3 DNS Assay

This assay is conducted to determine the enzymatic activity of cellulase by calculating the reducing sugar in the crude enzyme. The OD reading of the samples were harvest at the interval of 6 hours for the first 24 hours and then 12 hours for the next 24 hours. Bharathidhasan, et al, (2009) stated that one IU of cellulose activity is defined as the amount of enzyme that liberates 1 mM of reducing sugars per minute under the assay condition. The enzyme activity was calculated based on the glucose standard on Appendix 2.

![Enzymatic activity graph](image)

**Figure 4.1:** The cellulase enzyme activity graph for control, S3 and C4 for 48 hours.

<table>
<thead>
<tr>
<th>Hours</th>
<th>Enzyme activity (U/ml)</th>
<th>Control</th>
<th>S3</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6th</td>
<td></td>
<td>0.151</td>
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**Table 4.2:** Enzyme activity for Control, S3 and C4 for 48 hours.
Figure 4.1 shows the cellulose enzyme activity graph for the control, S3 and C4 sample for the period of 48 hours. In the control sample there was no inoculate so the reading of the enzymatic activity should be constant throughout the experiment. According to the graph, the enzyme activity increase at the first 6 h and then decrease during the second harvest. However, after second harvest, the enzyme activity increase and reached the maximum point at the fifth harvest. This is showing that for sample S3, the highest activity of enzyme cellulose produce by S3 is after 36 h of incubation. After reaching the maximum point, the enzyme activity decreased again. The graph of enzyme activity for C4 is showing the same pattern as S3 sample where during the first 6 h higher compare to the reading on the second harvest is. During the third harvest, the reading increase again and then reaching maximum point during the fourth harvest then the reading decrease until the final harvest. The OD reading for the assay can be referred at Appendix 3.

There are many factors that can affect enzyme activity such as temperature, pH, relative humidity and the preparation of the crude enzyme (Bharathidhasan, et al, 2009).