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Isolation, screening, and cellulase assay of cellulolytic bacteria from rice field soils in Changwat Kalasin

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รวิชญ์ อิ่มรักษา : การคัดแยก คัดเลือก และวัดกิจกรรมเอนไซม์เซลลูเลสของแบคทีเรียที่ย่อยสลายเซลลูโลสจากดินนาข้าวในจังหวัดกาฬสินธุ์

อาจารย์ที่ปรึกษา : อ.ดร. ศุภวิน วัชรมูล

การศึกษานี้มีวัตถุประสงค์เพื่อคัดแยกและคัดเลือกแบคทีเรียที่มีความสามารถย่อยสลายเซลลูโลส และประเมินศักยภาพของการแบ่งส่วนทางชีวภาพโดยใช้แบคทีเรียเซลลูโลสที่แยกได้ โดยคัดแยกแบคทีเรียที่นำมาศึกษาถูกคัดแยกจากดินนาข้าวในจังหวัดกาฬสินธุ์ด้วยวิธีเพาะเลี้ยงเชื้อบนอาหารเลี้ยงเชื้อ จากการศึกษาพบว่าจากแบคทีเรียจำนวน 60 สายพันธุ์ มีจำนวน 40 สายพันธุ์ที่มีความสามารถในการย่อยสลายเซลลูโลส ทดสอบโดยการย้อมสีโดย Gram's iodine โดยการคำนวณ Hydrolysis capacity (HC) และในการวิเคราะห์ทางสถิติ แบคทีเรียที่มีความสามารถในการย่อยสลายเซลลูโลสสูงมี 10 สายพันธุ์ หรือที่มีค่า HC value สูงที่สุด ได้แก่สายพันธุ์ SB 13, SB 19, SB 22, SB 24 SB 29, SB 33, SB 39, SB 50, SB 52, SB 59 จากนั้นทำการทดสอบประสิทธิภาพการย่อยสลาย และศึกษาลักษณะรูปร่างโคโลนี พบว่าจุลินทรีย์สายพันธุ์ SB 24 มีค่า HC สูงสุด จึงสรุปได้ว่าแบคทีเรียที่คัดแยกจากนาข้าวนี้มีความสามารถในการย่อยสลายเซลลูโลส และเนื่องจากสถานการณ์ Covid-19 ส่งผลให้ห้องปฏิบัติการไม่สามารถใช้งานได้ จึงไม่ได้ทำการทดลอง cellulose assay และทดสอบประสิทธิภาพในการย่อยสลาย

คำสำคัญ : แบคทีเรียย่อยสลายเซลลูโลส, เซลลูเลส, การวัดกิจกรรมเอนไซม์เซลลูเลส
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Rawit Aimraksa : Isolation, screening, and cellulase assay of cellulolytic bacteria from rice field soils in Changwat Kalasin

Project Advisor: Dr. Supawin Watcharamul

This study is focused on the isolation and screening the cellulolytic bacteria and assess the potential of bioaugmentation using isolated cellulolytic bacteria. The rice field soil from Kalasin province collected and screened for the active cellulolytic bacteria by Culturing technique. From the experiment, 40 of 60 isolates were defined as a cellulolytic bacterium by the Gram's iodine staining test. The Hydrolysis Capacity (HC) estimation and statistical analysis showed that 10 isolates had a highest HC value and selected for cellulase assay and biodegradation efficiency test. They were named SB 13, SB 19, SB 22, SB 24 SB 29, SB 33, SB 39, SB 50, SB 52 and SB 59, respectively. Then the degradation efficiency test and morphological study confirmed that the SB 24 isolate is bacteria and showed the highest HC value. This can be concluded that active cellulolytic bacteria can be isolated from the rice field. And due to the Covid-19 situation, the laboratory was closed, cellulose assays were not conducted and the efficacy of degradation was tested.

Keyword: cellulolytic bacteria, cellulase, cellulase assay, bioaugmentation

rice straw degradation

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ABBREVIATION

$CaCl_2$	Calcium chloride
CH_4	Methane
CO	Carbon monoxide
cm	Centimeter
CMC	Carboxymethylcellulose
DNS	3,5 Dinitrosalicylic acid
FPase	Cellulase assay by Filter paper
HC	Hydrolysis Capacity
g	Gram
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KCl	Potassium chloride
KI	Potassium iodide
l	liter
M	Molar
$MgSO_4$	Magnesium sulfate
mg	Milligram
ml	Milliliter
$MgSO_4 \bullet 7H_2O$	Magnesium sulfate heptahydrate
Na	metabisulfite Sodium metabisulfite
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
NO_x	Nitrogen oxide

rpm	Revolutions per Minute
mg/l	Milligram/Liter
TSB	Tryptic Soy Broth

CHAPTER 1

INTRODUCTION

Rice is a major agricultural product in Thailand. In the cultivation process, rice straw waste is available in the field. In practical, over 90% of the rice field during the harvesting season (November-December) is burned (Tipayarom and Oanh, 2007) to eradicate the rice straw for preparing the next cultivation. The main problem of rice field open burning is the pollutant emission from incomplete combustion such as CO, CH_4, NO_x, and hydrocarbons to the troposphere (Kumar *et al.,* 2018). To solve this problem, there are many options to use rice straw as a raw material such as making roof, animal feed, fermentation of biofuel production, improving the soil quality as fertilizer, and plastic mulch replacement to improve agricultural products (Jin *et al.,* 2019; Kim *et al.,* 2017; Abrantes *et al.,* 2018). Plowing is another method that can solve the problem of rice straw burning by adding oxygen to soil causing the decomposition rate of bacteria (Ubon *et al.,* 2015) and increase the organic carbon in the soil.

Plowing the rice field soil is a preparation step for the next cultivation in which rice straw will be amended to the field. The rice straw main components are cellulose, hemicellulose, and lignin approximately consist of 35%, 18%, 15% respectively (Jiang *et al.,* 2011). These components can be degraded by microbes. Cellulose is a major component of plant biomass consisted of a linear chain of β-(1,4) linked D-glucose units. In the biodegradation process, cellulose can be biodegraded by cellulases into glucose by cellulolytic microbes. Cellulases are a group of enzymes that hydrolyze the β -glycosidic bond in cellulose. Endoglucanase randomly hydrolyzes internal amorphous regions of cellulose to produce oligosaccharides, exoglucanase hydrolyzes the ends of cellulose chains to generate either glucose or cellobiose, and β -glucosidase hydrolyzes soluble cellodextrins and cellobiose into glucose (Hasunuma *et al.,* 2013).

In bioremediation, the application can be divided into three approaches. Firstly, adding nutrients and an electron acceptor called biostimulation. Secondly, called bioaugmentation (adding microbial strain). Lastly, is the remediation by not to disturb the contaminated site or called natural attenuation. Bioaugmentation is an addition of microorganisms into the environment to increase the microbial population and enhance the performance of biodegradation. The alternative way to improve the biodegradation performance is to increase the cellulolytic microorganisms by isolation, screening and enrichment technique and add back into the remediation site or called bioaugmentation. There are several types of research that use bioaugmentation for the bioremediation process (Plangklang, 2009; Ecem Öner *et al.,* 2018; Nwankwegu and Onwosi, 2017). Bioaugmentation process can be used in either in situ or ex situ bioremediation. In various researches, both bacteria and fungi were studied for biodegradation process. Due to the reduced incubation time, reduced need for pH control and bacteria availability of the rice field community, these are the advantages of bacteria over fungi for cellulase production (Pandey *et al.,* 2019). Cellulolytic bacteria can be isolated from various environments such as rice straw and hot spring (Pore *et al.,* 2019; Singh *et al.,* 2018). The cellulolytic microbes have an ability to produce cellulase to enhance the biodegradation process, increase the humus quality (Barker *et al.,* 2006) and improve the soil abundant. The advantages of cellulolytic bacteria isolation from rice field soil are to obtain a cellulolytic bacterium that is available in the soil, increase the population to improve the biodegradation rate, decreasing the combustion of rice straw, and can be easily found in the agricultural area.

In Thailand, researches in cellulolytic bacteria are available which isolated from various environment such as mangrove swamps, ruminant feces, waste disposal site, and soil (Chantarasiri, 2015; Chantarasiri, 2014; Sawangjit, 2017; Akaracharanya *et al.,* 2009). There are also some studies in different types of microorganisms such as aerobes, anaerobes, mesophiles, thermophiles, and halotolerant microbes (Kim, 2018; Doi, 2008; Suchardová *et al.,* 1986; Chantarasiri, 2014; Rachamontree, 2017). In this study, the microorganism study in rice field soil at Kalasin province which is a rice agriculture area have not been studied and most of the rice straw in the field are burnt. In the study site have not been burned for preparing the next cultivation but prepared by plowing the soil and the rice straw in the field. The rice straw was decomposed by microorganism and increase the soil organic content.

In this study, the cellulolytic bacteria are isolated from rice field soil for cellulase measurement and bioaugmentation potential. The biodegradation rate can be determined by measuring the enzyme activity of microorganisms. Hydrolysis capacity (HC) was used for screening the high cellulose degrading potential bacteria and choose for bioremediation test. To measure the activity of these enzymes for biodegradation assessment, the widespread technique is determining the cellulase activity by Filter paper (FPase) activity using the 3,5 dinitrosalicylic acid (DNS) method (Ghose, 1987) in terms of Filter paper unit (FPU/ml). The biodegradation efficiency can be determined by calculating the weight loss of rice straw that inoculated by the isolated microbes.

1.1 Objectives

1. To isolate and screen cellulolytic bacteria from rice field containing rice straw.
2. To study the growth and determine the cellulase enzyme activity of cellulolytic bacteria.
3. To assess the potential of bioaugmentation using isolated cellulolytic bacteria.

1.2 Scope of the study

1. Sample collection: Samples for isolation and prepare for bioaugmentation were collected from unburnt rice field containing rice straw.
2. Parameter: Measure the Hydrolysis capacity (HC), Cellulase activity (FPU/ml)
3. Duration: August 2020 – April 2021 (9 months)

1.3 Expected benefits

Effective cellulolytic bacteria could be obtained and applied to increase the biodegradability of rice straw in the rice straw field

CHAPTER 2

LITERATURE REVIEW

2.1 Cellulose

Cellulose is the most abundant natural raw material. It is the main component of all-natural fiber (Thakur and Voicu, 2016). The fibrous, tough, and water insoluble of this biodegradable polymer helps the cell wall structure of plants to be more sustain which consist up to 47% in wood and 38.3% in rice straw (Pinkert *et al.,* 2009; Fan *et al.,* 2013). Cellulose also can be extracted from other source such as algae, bacteria, and annual crops.

Cellulose is a linear polymer of two glucose sugar unit that linked by glycosidic linkage (C-O-C) at the C1 and C4 position (or cellobiose) as shown in **Figure 2.1.** One end of the chain has a reducing acetal group at C1 position, and the other end has and hydroxy group at C4 position. In natural cellulose is a polymer that consist of microfibril or crystalline region and amorphous region. In **Figure 2.2** shows the β-(1→4) link of intramolecular and intermolecular hydrogen bonds between the C3 hydroxy group and nearby in-ring oxygen to form crystalline structure.

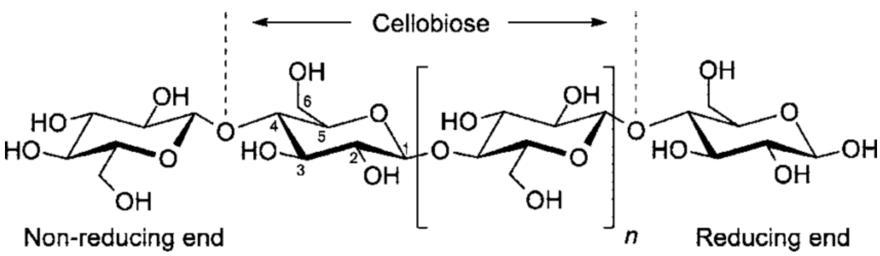


Figure 2.1 Cellulose structure (Pinkert *et al.,* 2009)

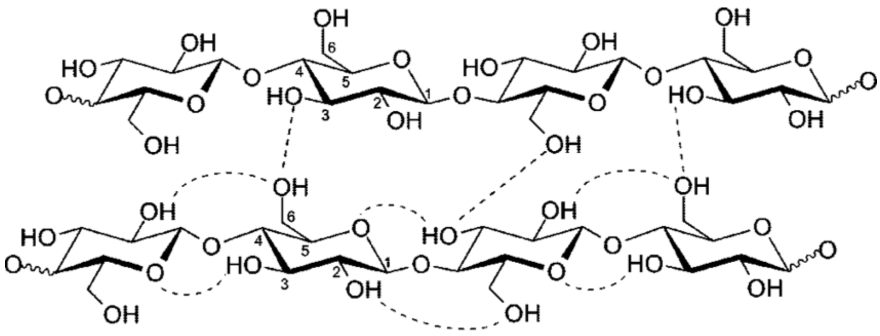


Figure 2.2 Intramolecular and intermolecular hydrogen bonds in cellulose

(Pinkert *et al.,* 2009)

2.2 Cellulose degradation pathway by Cellulases

Cellulose can be degraded by a group of enzymes called cellulases. Cellulases are inducible enzyme for lignocellulose decomposition from agricultural, municipal, forestry and industry (Amore *et al.,* 2012). Cellulases can be divided into three type by degradation function: (1) endoglucase (E.C.3.2.1.4), which randomly hydrolyze internal bonds in cellulose chain to produce oligosaccharides; (2) xoglucanase (E.C.3.2.1.9), which drolyze 2 – 4 units from the end of cellulose chain to produce either glucose or cellobiose; (3) β-glucosidase (E.C.3.2.1.21), hydrolyze cellodextrins and cellobiose to generate glucose (Hasunuma *et al.,* 2013) as shown in **Figure 2.3.**

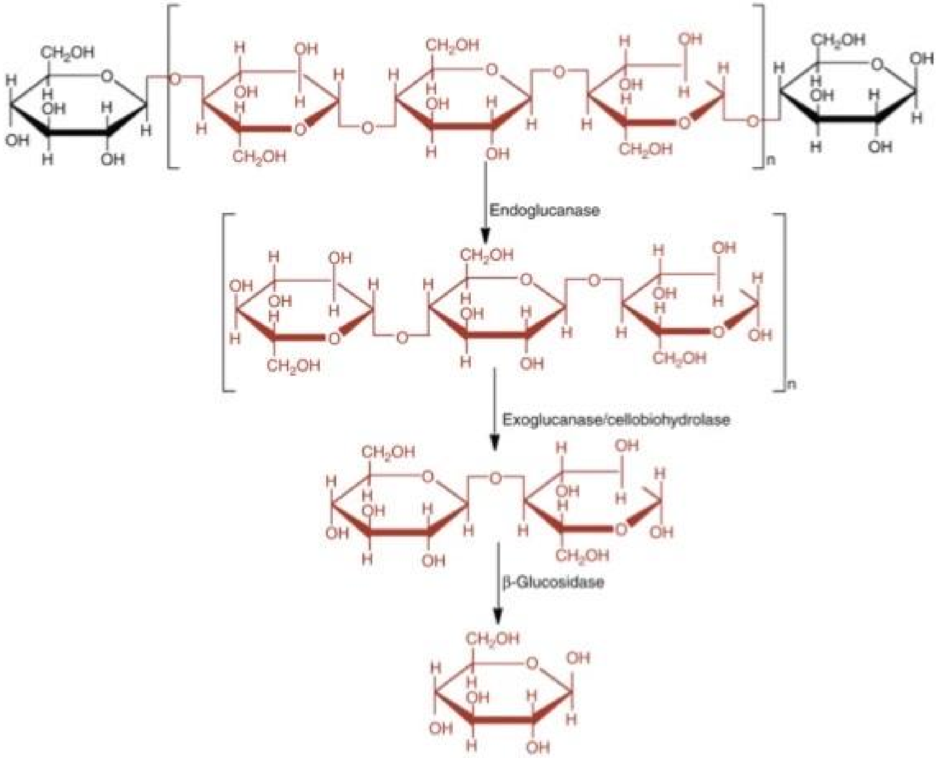


Figure 2.3 Degradation pathway by cellulases enzyme from cellulose to glucose (Khan *et al.,* 2016)

2.3 Microorganisms

Microorganisms are small living things in the world. They play a big role in the nutrient biogeochemical cycle. Humans use this living thing for the bioremediation process in industrial or agriculture processes (Singh *et al.,* 2007).

The microorganisms can be classified into three domains; Bacteria, Archaea, and Eukarya (Sattley and Madigan, 2015), by the comparison of ribosomal ribonucleic acid (rRNA) to study in the evolutionary and relationship of the organisms.

2.3.1 Bacteria and Archaea

These two domains are classified based on phylogenetic distinction, morphology, and biochemical and physiological traits. The unavailable of membrane in organelle and less compartmentalization makes the difference to Eukarya. Cells of them are existed in three major forms including coccus (sphericalshape), bacillus (rod-shape) and spirillum (spiral shape) and the less common form such as spirochete (tightly coiled) as shown in The difference between archaea and bacteria in rRNA which archaea have three RNA polymerases, but bacteria have only one. Bacteria can be classified into two groups by cell wall structure: Gram-positive and Gram-negative. Gram-positives have a thick impermeable wall composed of peptidoglycan and secondary polymer. Gram negatives have a thin peptidoglycan layer called inner membrane and another lipoprotein layer called outer membrane. These two groups can be identified by Gram staining (Beveridge, 2001).

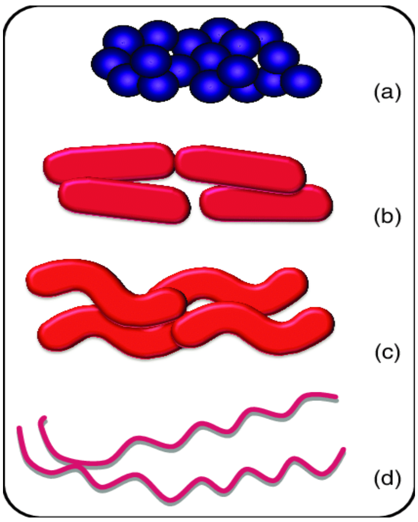


Figure 2.4 Morphological form of bacteria cells. (a) coccus; (b) bacillus; (c) spirillum; (d) spirochete. (Sattley and Madigan, 2015)

2.3.2 Eukarya

In the opposite from bacteria, Eukarya contains of membrane-bound organelles and include a defined nucleus such as fungi, protozoa, algae.

2.3.2.1 Fungi

Fungi are similar to plants which they have cell walls and nonmotile. In contrast, the difference is Fungi does not have chlorophyll and does not have photosynthesis process. In the soil ecosystem, the major role of fungi is decomposer and the nutrient recycler. Fungi can be di vided into two group by its form: fungi that consist of hyphae or a filament that can form into mycelia called moulds and unicellular, oval shaped fungi called yeast.

2.3.2.2 Protozoa

A unicellular organism that can reproduce either sexual (conjugation) and asexual (budding, spore formation, or mitotic fission) depends on species. Protozoa can be divided into three main groups by their morphology and movements: flagellates, amoebae, and ciliates. They can be (Warren and Esteban, 2019).

2.3.2.3 Algae

Algae are also similar to plant as fungi. The difference that separates algae from fungi is they can be able to perform photosynthesis by the available of Chlorophyll A. Their main role is a primary producer and the base of the food chain in marine and freshwater environment. Humans use algae for biogas production, cosmetics, fertilizers, food and pharmaceuticals (Goswami *et al.,* 2015).

2.4 Microbial study

2.4.1 Culture medium

Microorganism needs various nutrients for their growth such as waster, nitrogen source, and energy source. Each microorganism needs different nutrient. Culture medium contains nutrients that capable for microbial growth such a water, energy source, protein source.

However, there are several types of medium to use in each case. Enrichment media is a media that consist of rich nutrient and energy source for microbial growth, its used for increasing the number of microbial consortia. Selective media is a media that modified by using suitable compound such as antibiotics or dyes to isolate for the growth of only selected microorganisms. Minimal media is a media that contains the minimum nutrient possible for microbial growth. It contains just a carbon source, salts, and water.

2.4.2 Quadrant streak plate technique

Quadrant streak plate technique is used for isolating the microorganism to single colony or separating the mix culture into single colony by using sterile loop. First, sterilized a wire loop by passing the flame. When the loop is cool, charged with the bacterial mixture and make a stroke as shown in **Figure 2.5**. In each stroke, a wire loop is sterilized (Harrigan and McCance, 1966).

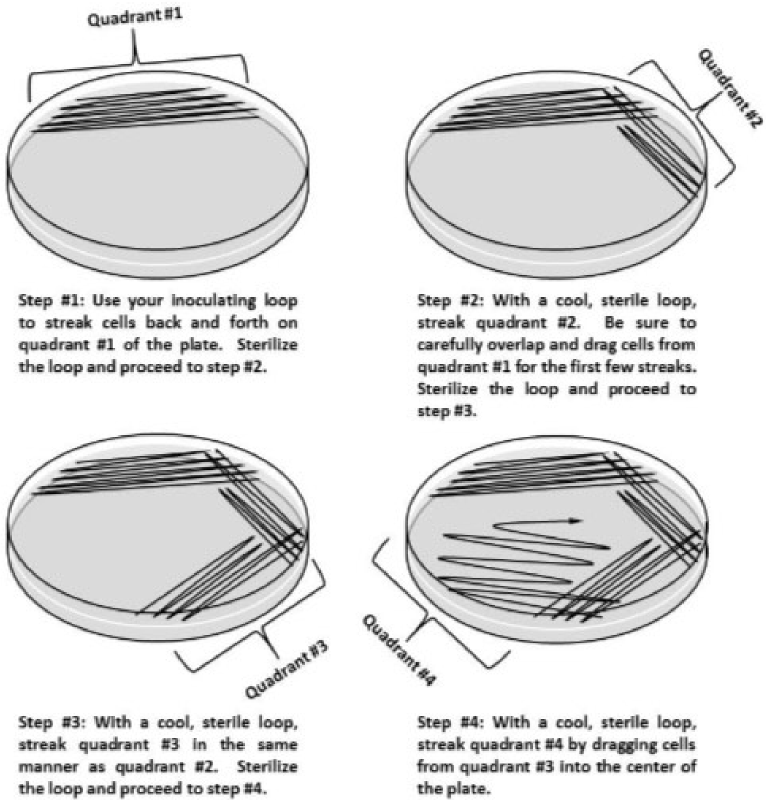


Figure 2.5 quadrant streaking plate direction (Burke, 2017)

2.4.3 Colony morphology study

To study characteristics of individual colonies can be observed on a single colony in petri dish. Different types of bacteria will have a different colony. Shapes and size can be categorized such as punctiform, round, irregul ar, filamentous, rhizoid. Margin of the colony can be described too such as entire, erose, undulate, lobate, filamentous, and rhizoid. Elevation can be observed by looking from a side view like flat, raised, convex, umbonate, wrinkled as shown in **Figure 2.6.**

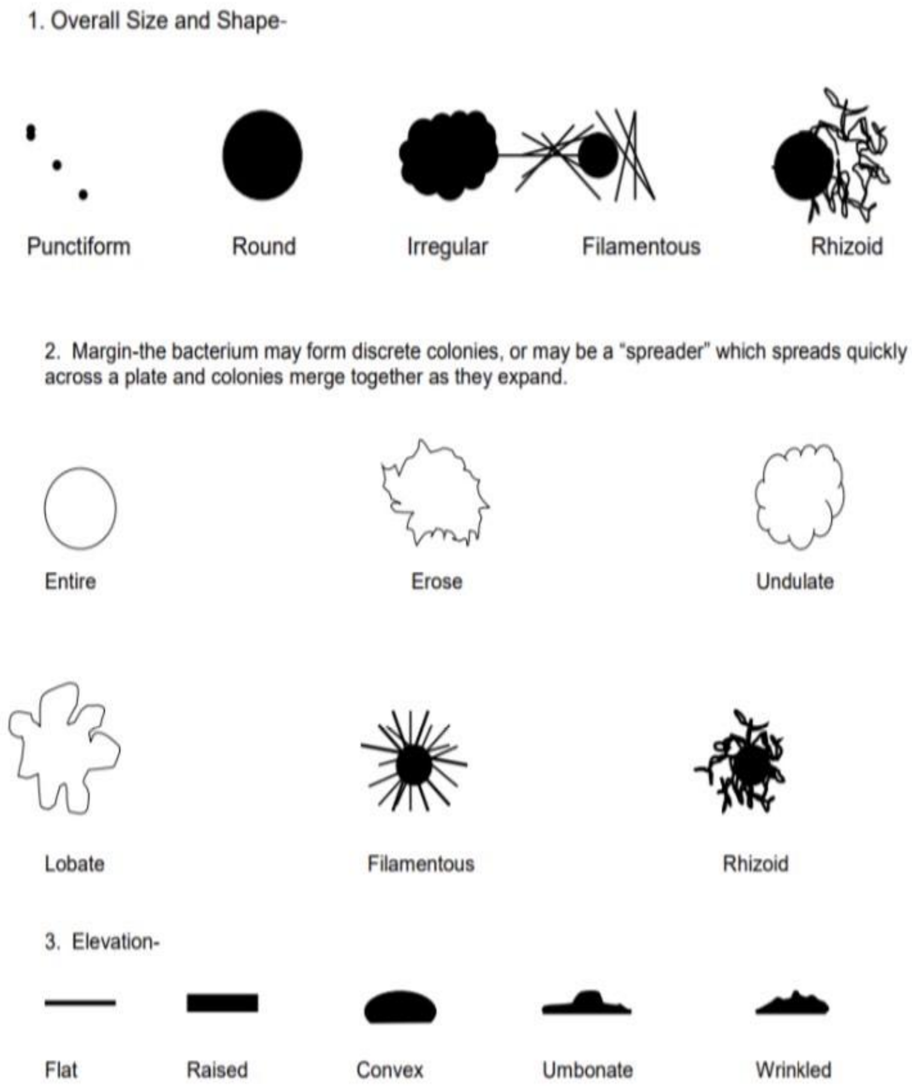


Figure 2.6 Characteristics of individual colony described by size and shapes, margin, and elevation (Burke, 2017)

2.4.4 Gram's iodine staining method

Gram's iodine staining method is use for screening for cellulase producing microorganism. The reagent that use for this technique contains iodine, KI and distilled water. Gram's iodine formed a bluish-black color with cellulose but does not form with hydrolyzed cellulose, giving a clearance zone around cellulase producing microorganism. This technique is a new method that takes just 3 to 5 minutes to obtain the clearance zone, compare with the standard method that use 0.1% Congo-red followed by 1 M NaCl and takes 30 – 40 minutes to obtain the hydrolysis zone (Kasana *et al.,* 2008).

2.4.5 Enzyme assay; Total cellulase assay

There are two ways to measure cellulase activity. First, measure the individual activities of component enzyme. Second, measure the total complex activity (Sharrock, 1988). Total cellulase assay are the measurement of endoglucanase, exoglucanase, and β-D-glucosidase by using insoluble and pure cellulosic substrates such as Whatman No.1 filter, cotton linter, microcrystalline cellulose, bacterial or algal cellulose.

From the International Union of Pure and Applied Chemistry (IUPAC), Filter Paper Assay (FPA) is the recommended method. This method is based on a fixed amount of 2 mg of glucose released from 50 mg filter paper in 60 minutes. The cellulase activity is described in terms of Filter Paper Unit (FPU) per millimeter of enzyme solution. The advantage of this method is the widely available of substrates and the susceptible to the cellulose degradation (Zhang *et al.,* 2009).

2.5 Bioremediation and Bioaugmentation

Remediation technologies were used to destroy the pollutants or transform them into harmless substance. Due to the acceptable waste treatment process, low -cost, low technology techniques, and can be carried to treat out on site, bioremediation is an option for remediation process. It is the process which organic wastes are degraded biologically by using living organism such as plants, microorganisms into less toxic substances (Viladi, 2001).

Microorganisms use redox reaction for energy production including respiration and other function for cell growth and reproduction. The main factor this process is required an energy source, electron acceptor, and nutrients to maintain the microbial growth. For the bioremediation process, there various factor to supports the biological function including contaminant concentration, contaminant bioavailability, site characteristics, redox potential and oxygen control, nutrients, moisture control, and temperature (Adam *et al.,* 2015). In addition, bioremediation can be used for accelerating the rate of biodegradation.

Bioaugmentation is one of the in-situ land treatment strategies by the addition of microorganisms into the contaminated site. The microorganism can be obtained from contaminated site or from the other source. Each species of microbes has a difference performance in degrading difference substances. The high efficiency degrading bacteria will be isolated and screened from on-site or off-site for enrichment and inoculate into the target site. Foreign microorganism from off-site or genetically modifying degrading efficiency depends on the ability to compete the native species and other environmental factors. To select the strain from the contaminated site for bioaugmentation will be confirmed that they can able for remediation in that site.

2.6 Rice straw management problems and alternative strategy

The major problem of rice agriculture is the rice straw waste after the cultivation. The crop residue needs to be removed for preparing the next harvest. Open field burning or rice straw burning is a widespread method to remove crop residue, control weeds and crop disease. The advantage of this method is fast, costless, and have a short timeframe to prepare for the next harvesting. But the main disadvantage is a large amount of CO_2 emission and other compounds such as CO, CH_4, NO_x, and SO_2 from the combustion which are toxic to environment and are human carcinogens. In addition, the disadvantage in the rice field is the loss of nutrients and energy that important for crops growth such as N, P, K, and S (Domínguez-Escribá and Porcar, 2010).

The alternative strategies to rice straw burning that are more environmentally friendly, less toxic, and decreasing the nutrient loss from the soil is soil incorporation or plough the rice straw into the soil to recycle the nutrients. There was a research studied the effect of rice straw incorporation (Saothongnoi *et al.,* 2014) concluded that the rice straw increased the soil fertility. The rice cultivation in soil with the rice straw achieved the higher soil organic matter and organic carbon than soil with rice straw ash and soil without rice straw and have the lowest bulk density which is helpful for soil preparation and enhance the air circulation in soil.

2.7 Related researches

The study of cellulolytic microorganism community has been of interests in agriculture countries such as Thailand for various applications such as enzyme production for biofuel production, and bioremediation.

Rachamontree (2017) screened the thermophilic and halophilic cellulase from saline soil in MahaSarakham for rice st raw degradation. Enzyme activity was measured by 3,5 dinitrosalicylic acid (DNS) method. Rice straw degradation was tested by using rice straw as carbon source for microbial growth and measured by DNS method. Bacterial strain was then identified by 16s rRNA gene sequencing. The highest enzyme activity of the isolated bacteria RMU41 was equal to 0.800 ± 0.020 U/ml, and the highest enzyme activity from rice straw degradation test was 0.246 ± 0.031 U/ml. The result from 16S rRNA gene sequencing has 100% homology to Streptomyces sp.

There is the study about identifying the cellulolytic bacteria in various environment in Nepal (Pandey *et al.,* 2019). The samples were collected in different sources; soil, animal guts, and hot spring. The isolates from the samples were identified by 16 S rRNA full sequencing and determine the cellulases activity by 3,5 dinitrosalicylic acid (DNS) method.

From the identification analysis shows that the samples are matched the sequence of Bacillus; Bacillus amyloliquefaciens, Bacillus nematocidal, Bacillus licheniformis and Paenibacillus genus; Paenibacillus sps.. The results from cellulase assay show the enzyme activity of the surface soil of 0.0666 FPU/ml.

Sirisena and Manamendra (1995) isolated the cellulose-degrading bacteria and characterized the cellulolytic bacteria from the decomposing rice straw. The different decomposing rice straw stages were sampled for isolation and identification by morphological and biochemical tests. From the results, Listeria sp. And Enterobacter sp. are available in the early stage of decomposition. After about one month, Pseudomonas sp. are available for enzyme production. Pseudomonas sp. produced the largest of hydrolysis zone by Congo-red staining test.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of microorganisms

Microorganisms were isolated and screened from unburned rice field soil at Kalasin province on 2 February 2021.

3.2 Raw Material and Sources

A soil sample and rice straw were collected from an unburned rice field in Kalasin. A soil sample was used for isolation and screening of the cellulolytic bacteria. Rice straw was used as a carbon source for biodegradation efficiency study. The soil texture and soil properties were studied by using feel method and observed the soil color (Arshad *et al.,* 1996).

3.3 Chemical and Reagents, and Special Instrument

3.3.1 Chemicals and Reagents

Chemical and reagent used in this study were analytical grade. Components, preparation, and application were described in **APPENDIX A.**

3.3.2 Special Instruments

1. Autoclave: HVA-85, Hirayama Japan
2. Hot air oven: D 06062, Model 700, Memmert, Germany
3. Incubator: KBW, BINDER, Germany
4. Shaking Incubator: 10X 400 Environmental Shaker, The United Kingdom
5. Laminar air flow

6. Precision Digital Scale Balance: 40SM-200A, Presica, Switzerland
7. Spectrophotometer: 1200, Labomed,inc., The United States of America
8. Vortex mixer
9. Vernier caliper

3.4 Culture media

3.4.1 Mineral Salt Medium

Mineral Salt Medium was a liquid medium that contains the minimum nutrient for bacteria isolation that contains inorganic salts, carbon source, and water.

3.4.2 Tryptic Soy Broth (TSB)

TSB was a general-purpose medium for enrichment and cultivation of various anaerobic microorganisms. It consists of casein and soybean as a protein source, Glucose as an energy source, and carboxymethylcellulose (CMC) as a carbon source.

3.4.3 CMC agar

CMC agar medium was used for Gram's iodine staining test which mainly contains carboxymethylcellulose (CMC) as a carbon source for cellulolytic bacteria.

3.4.4 CMC broth medium

CMC broth has a high portion of carboxymethylcellulose (CMC) as a carbon source used for culture enrichment before cellulase assay.

3.5 Samples and Cultivation Procedures

Bacterial sample from rice field soil was transferred into a mineral salt medium (Ali *et al.,* 2019; Gupta *et al.,* 2012) to prepare for isolation and growth of samples for 1 g and 9 ml, respectively. The soil sample was weighted for 1 g and applied into a broth 9 ml and incubated in a shaking incubator 100 rpm for 48 hours. The subculture was repeated into a new broth triplicately and incubated at the same condition.

To inhibit the micro be's activity, the incubated broth was stored at 4 ˚C. The enrichment was diluted serially and kept until proceeded.

3.5.1 Cellulolytic bacteria isolation and screening

Mineral salt medium broth that previously incubated was serially diluted in NaCl for 10^{-7} times. The 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} diluted broth culture was spreaded into TSB sterile agar media with 15 g/l agar -agar and 0.2 g/l Congo-red and incubated at 37˚C for 48 hours. The colonies that showed the discoloration wereselected, streaked into the TSB agar plate without Congo-red and incubated at 37˚C for 48 hours. They were individually picked and re-streaked into TSB agar without Congo-red until the pure cultures were obtained. The pure culture in each plate was picked and spotted into CMC agar (Kasana *et al.,* 2008) and incubated in the same condition for Gram's iodine taining and Hydrolysis capacity (HC) calculation. Gram's iodine will formed a bluish-black complex with cellulose but not formed with hydrolyzed cellulose, making a clearance zone around the colony. The isolates that obtained a high HC value was selected for cellulase assay and bioremediation test. The selected isolates were also transferred into agar slant for storage. Consequently, the isolates were investigated for colony morphology study.

3.6 Procedures of Chemical Analysis

3.6.1 Hydrolysis capacity measurement and calculation

From the Gram's iodine stained colonies that show the discoloration, the diameter of clearance zone was measured to calculate the Hydrolysis capacity (HC) by using Vernier caliper to estimate cellulase activity approximately (Hankin and Lester, 1977) and the colonies that have HC value greater than the third quartile (Q3) in statistic analysis was selected for enrichment and cellulase assay (Di Benedetto *et al.,* 2019). The zone of clearance and hydrolysis capacity is directly proportional to the production of cellulase (Pandey *et al.,* 2019). The following formula was used to calculate the hydrolysis capacity.

$$\text{Hydrolysis capacity} = \frac{\text{diameter of clearance zone}}{\text{colony diameter}}$$

3.6.2 Cellulase assay

The selected isolates from isolation and screening processes were selected and transport into the CMC broth medium for cellulase assay. CMC broth medium was centrifuged at 5000 rpm for 15 minutes at 4 ˚C. Cellulase activity wasdetermined by Filter paper (FPase) activity using the 3,5 dinitrosalicylic acid (DNS) method (Ghose, 1987) and using glucose as a standard for making a standard curve. Reagent preparation, and glucose standard were described in **APPENDIX A.** The process would be performed in steps as follows:

1. 1.0 ml of 0.05 N Na-citrate pH 4.8 was added into a 25 ml test tube.
2. 0.5 ml supernatant was added from media broth and diluted in citrate buffer.
3. Heated to 50 ˚C and add one Whatman No. 1 filter paper strip, 1.0 x 6.0 cm, make sure the paper does not wind up and then incubated 50 ˚C for 60 minutes
4. 3.0 ml DNS reagent was added into the test tube.
5. All samples, enzyme blank, glucose standard, and spectro zero were

   boiled for 5 minutes exactly in a water bath and transfer into a cold water bath.
6. 20 ml distilled water was added and mixed completely by inverting the tube so that the solution separates from the bottom of the tube.
7. After 20 minutes, measured against the spectro zero at 540 nm.
8. The cellulase activity from samples was determined by plotting the absorbance at 540 nm against the absolute amount of glucose.

   The glucose concentration was measured from absorbance in a glucose standard curve after subtraction of enzyme blank. The following formula was used to estimate the concentration of cellulase by calculating FPU (Filter paper unit).

$$\text{FPU} = \frac{0.37}{\text{Enzyme concentration to release 2.0 mg glucose}} \text{ unit ml}^{-1}$$

3.7 Biodegradation efficiency test

The selected isolates from the screening process were transferred into TSB broth without CMC. The biodegradation efficiency test was analyzed by gravimetric method to calculate the weight loss percentage. The analysis procedures in serial steps were as follows:

1. Dried the rice straw at 105 ˚C, cut into 3 cm pieces and weighed.
2. Added 3 pieces of cut rice strawinto 7 TSB broth for each isolate. Incubated at 37 ˚C for 2 weeks
3. On days 1, 3, 5, 6, 9, 11, 14, obtained the rice straw in the broth in each isolate and dried at 105 ˚C and weighed.
4. Determine the biodegradation efficiency by plotting the weight of rice straw versus the numbers of days in the biodegradation process.
5. The broth that did not inoculated was used as a control.

CHAPTER 4

RESULTS

4.1 Sample collection

Rice field soil sample was collected from rice field in Kalasin province, on 2 February 2021. The weather of soil sampling on that day was hot and cloudy. Measure the ambient temperature was 32 ˚C with 40% humidity. The soil temperature was 26.0 ˚C and pH 7. About the soil texture, it was dark brown. The top soil was dry because due to the drought situation. However, soil and the difference of this soil from other field in this area is that there has been no rice straw burning. The farmers used only plowing method and let the rice straw degraded in the soil. The data from Land Development as shown in **Figure 4.1** soil properties are a set of soils formed from the source material, sediments, and then transported over the deposition of coarse-grained materials supported by rock and salt layers. The topsoil is sandy loam or sandy soil. The lower soil is a dense soil layer with sodium salt accumulation. Having a sandy loamy soil texture or loamy clay. They are light brown to gray. Found a mix of brown, yellow or red dotted dots. Rice straw was collected from rice field for biodegradation test used as a carbon source.

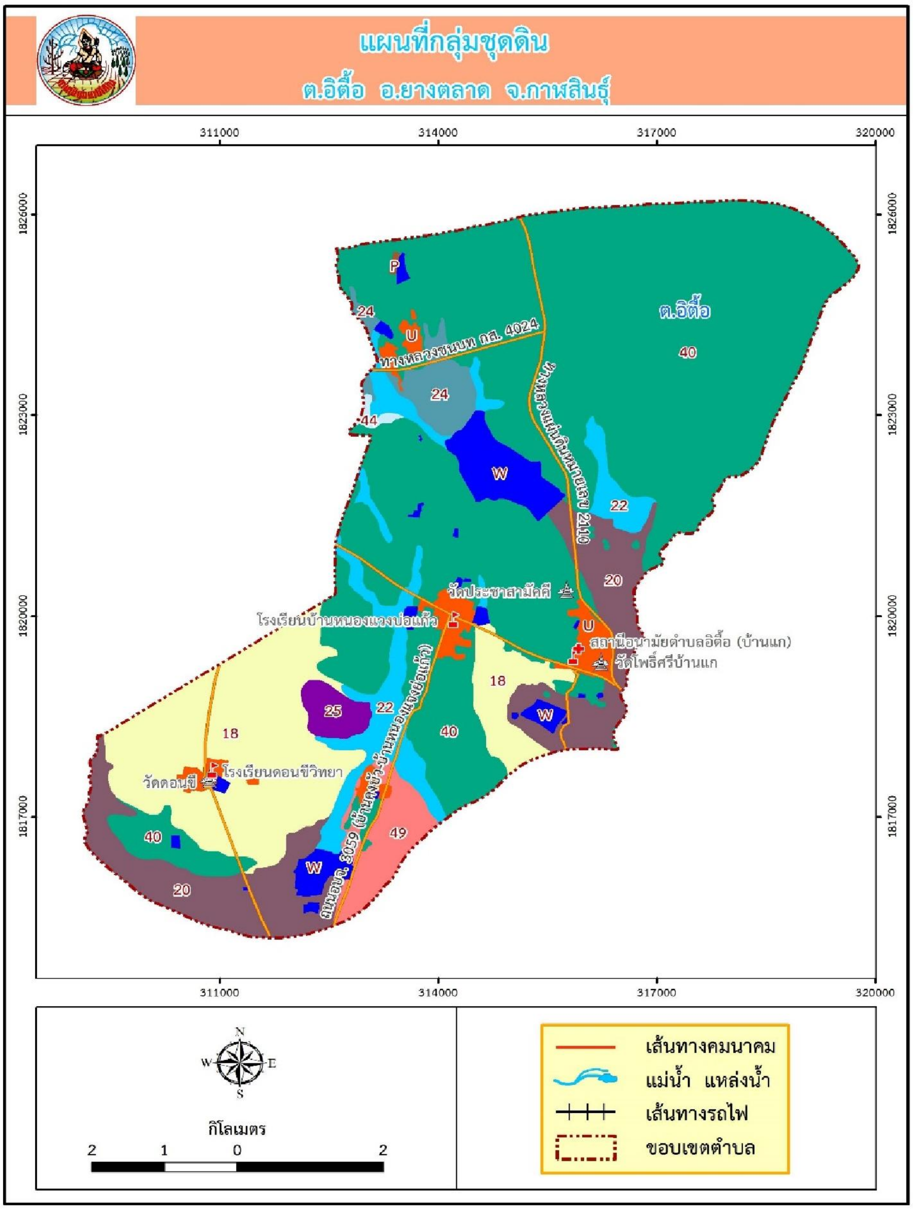


Figure 4.1 Kalasin province soil type map (Land Development Department, 2015)

4.2 Cellulolytic bacteria isolation and screening

Rice field soil sample was weighed for 1 g and applied to 9 ml sterilize Mineral Salt Medium broth then incubated for 48 hours. Repeat this step three times at least with the same condition. After the incubation, the biomass from the growth of microbes are available in bottom of the test tube. This result can be confirmed that the microbes can grow in the medium. $NaNO_3$ in the medium is used for amino acid polymer or protein synthesis.

The inorganic salt contains sodium, potassium, and calcium ions helps toregulate the bacteria membrane. The carbon source for the microbes is carboxymethylcellulose (CMC). $MgSO_4$ supplies the Mg^{2+} for DNA replication of the culture.

The incubated sample was serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} concentration. The 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilution was spread into TSB agar media with Congored and incubated at 37˚C for 48 hours. The mixed culture either cellulolytic or non-cellulolytic bacteria were grown in TSB Congo red agar as shown in **Figure 4.2**. The colonies that are cellulolytic bacteria were hydrolyze the Congo red and show the clear zone around their colony. The colony that tend to show the discoloration was streaked randomly into TSB agar plate without Congo-red for 60 colonies per plate. After the incubation of the streaked plate, the single colonies were obtained as shown in **Figure 4.3** and named as SB 1 to SB 60. The pure colonies from the previous step were picked and spotted into CMC agar for 48 plates to test the cellulose degrading bacteria by Gram's iodine staining.

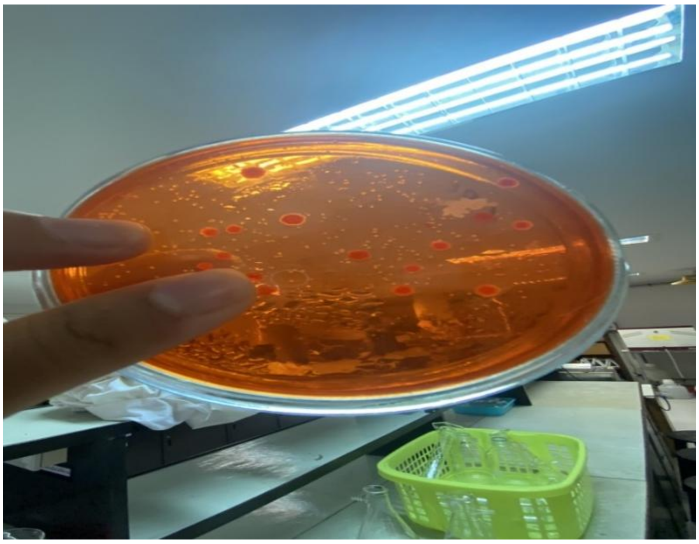


Figure 4.2 The mixed culture in TSB Congo-red agar from soil samples

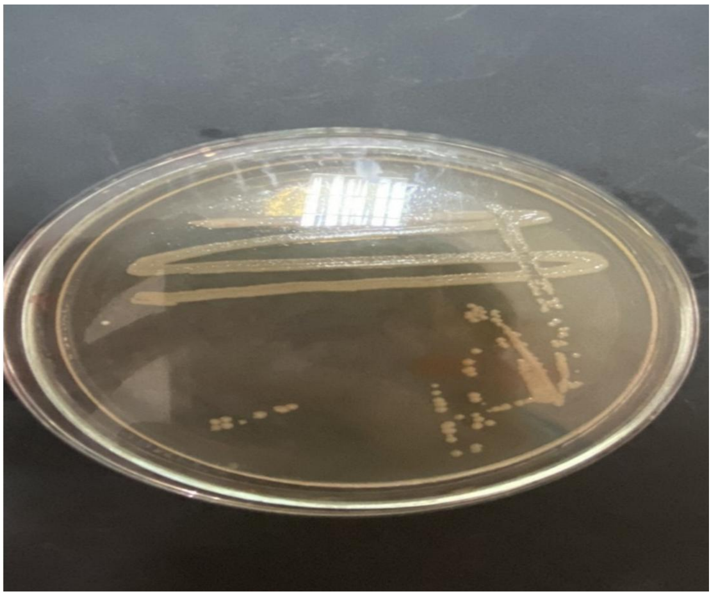


Figure 4.3 The single colony streaked from screening process in TSB agar

4.3 Hydrolysis capacity measurement and calculation

The colony that hydrolyze CMC was not formed complex structure with iodine and shown the zone of clearance as shown in **Figure 4.4** and the CMC was formed a complex with iodine makes the iodine change from brown to purple color. The results from Gram's iodine staining test 40 colonies out of 48 colonies that show the discoloration were measured the clear zone diameter and colony diameter for Hydrolysis capacity calculationas shown in **Table 4.1**. The high HC value can be predicted that the isolates have a high potential for cellulose degradation. For the selection of the high HC value, the data was ordered and analyzed by statistic. The isolates that obtained HC value greater than a third quartile was selected as a high cellulose degradation potential. From statistical analysis, SB 24 obtained the highest HC value equal to 17.86, the third quartile (Q3) was equal to 6.1725 and the colonies that have HC value greater than Q3 as following: SB 13, SB 19, SB 22, SB 24, SB 29, SB 33, SB 39, SB 50, SB 52, SB 58 was selected for cellulase assay and biodegradation test and the box plot graph of HC value was represented **in Figure 4.5**.

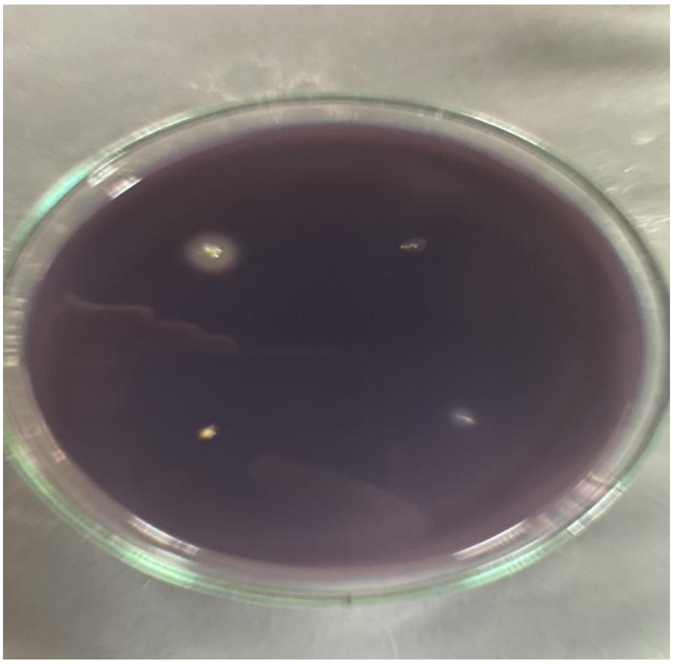
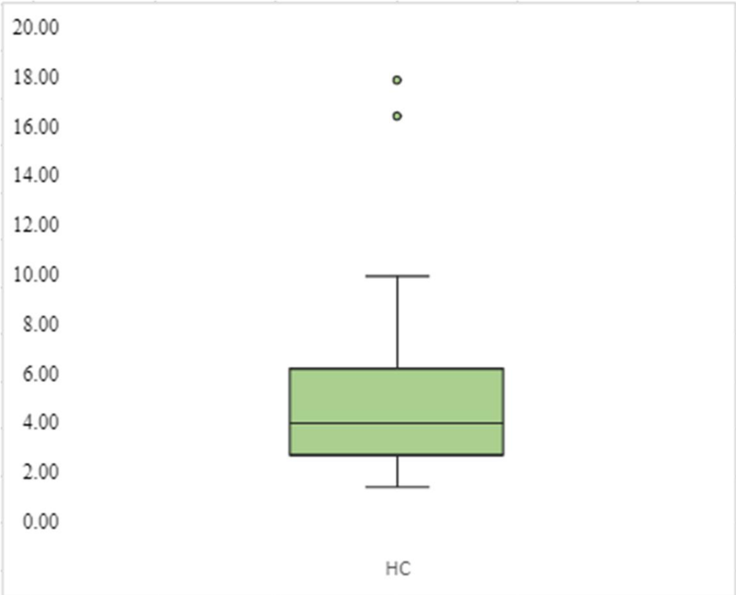


Figure 4.4 Gram's iodine staining test result from spotted isolates showing the clear zone by the hydrolysis of cellulolytic bacteria

Table 4.1 Hydrolysis capacity from Gram's iodine staining

Isolate	**Hydrolysis Capacity**	**SB 16**	2.48	**SB 35**	4.54
		SB 17	3.24	**SB 38**	2.57
SB 1	2.26	**SB 18**	3.54	**SB 39**	8.95
SB 2	4.36	**SB 19**	9.91	**SB 40**	5.04
SB 3	1.70	**SB 20**	4.32	**SB 41**	3.47
SB 4	2.08	**SB 22**	8.34	**SB 44**	5.97
SB 5	2.42	**SB 24**	17.86	**SB 47**	3.19
SB 6	3.61	**SB 25**	5.71	**SB 49**	4.56
SB 7	2.19	**SB 26**	4.95	**SB 50**	9.55
SB 9	3.46	**SB 27**	3.21	**SB 52**	9.21
SB 10	1.85	**SB 29**	16.40	**SB 55**	3.33
SB 11	1.90	**SB 31**	3.68	**SB 58**	6.42
SB 13	9.07	**SB 32**	5.48	**SB 59**	2.95
SB 14	4.96	**SB 33**	6.24	**SB 60**	1.38



Population size: 40
Median: 4.00
Min: 1.38
Max: 17.86
Q1: 2.6650
Q3: 6.1725
Outlier: 17.86

Figure 4.5 Box plot graph analysis of Hydrolysis Capacity from Gram's iodine staining

4.4 Colony morphology study

All for the isolates in agar plates were observed for colony morphology. From the selected isolates, the colony shape was found as round and punctiform shape. Margin was observed as entire. The elevation of the colony was found as convex and flat. All colony color was yellow.

Table 4.2 illustrated the selected isolates from Gram's staining morphology.

Isolate	Colony morphology		
	Shape	**Margin**	**Elevation**
SB 13	Round	Entire	Flat
SB 19	Round	Entire	Convex
SB 22	Round	Entire	Flat
SB 24	Round	Entire	Flat
SB 29	Round	Entire	Flat
SB 33	Punctiform	Entire	Convex
SB 39	Round	Entire	Flat
SB 50	Round	Entire	Flat
SB 52	Round	Entire	Flat
SB 58	Round	Entire	Flat

4.5 Cellulase assay and biodegradation efficiency test

Cellulase assay and biodegradation test were not be tested because the university temporary closure affected by Covid-19 pandemic situation.

CHAPTER 5

DISCUSSION & CONCLUSION

In the study of the growth, enzyme activity, and biodegradation potential of cellulose using rice straw as a carbon source, the cellulolytic bacteria were available by isolating the microorganism from the rice field soil in Kalasin province, Thailand.

Ten strains of cellulolytic bacteria, SB 13, SB 19, SB 22, SB 24, SB 29, SB 33, SB 39, SB 50, SB 52 and SB 58 were isolated, defined as a high cellulose-degrading potential, and choose for biodegradation test. From the Gram's iodine staining, SB 24 obtained the most HC value due to the large clear zone from the hydrolysis of the microbes to cellulose.

The selected strain could be confirmed as cellulolytic bacteria due to the colony morphology. From the colony morphology study, the isolates are mostly circular shape, entire margin, and flat or convex elevation. A small size, smooth or rough appearance, circular or irregular shape, have a defined margin are the characteristic of bacteria colony morphology which differs from a fungal colony that has a large size and large hypha around the colony, fuzzy appearance, and filamentous or rhizoid margin (Shil *et al.,* 2014; Goyari *et al.,* 2014). The culture medium also could be confirmed the bacterial growth. Ali (2019) and Gupta (2011) used Mineral Salt Medium for the isolation of cellulolytic bacteria. Therefore, it can be concluded that the isolated strains are bacteria. Various research used Trypticase Soy Broth or TSB for culturing bacteria because the high nutrition of the medium and their carbon and nitrogen sources that are capable for bacteria culturing (Tripathi *et al.,* 2016; Panda *et al.,* 2020).

From the soil sample collection site, the topsoil is sandy loam or sandy soil. The lower soil is a dense soil layer with sodium salt accumulation. Having a sandy loamy soil texture or loamy clay. They are light brown to gray. Found a mix of brown, yellow or red dotted dots.

The advice from the Land Development Department for soil conditioning in rice agriculture is to add organic fertilizer for adjusting the soil physical and chemical properties. The plowing the soil with the rice straw in a suitable humidity and let them stay for 3 -4 weeks is another method for improving the soil abundant. The rice field that was used for this study used the plowing method for preparing the field, makes the soil contact the oxygen from the atmosphere and enhance the microorganism activity, biodegradation rate, and improve the soil organic matter and soil abundance. So, can be predicted that the soil in this field was more abundant than the burned rice field.

The consortia were isolated from a soil sample into Mineral Salt Medium for isolation and enrichment. After the incubation, there was a precipitate in the enrichment tube which confirms that the microbes are available in the tube. The soil sample was screened for cellulolytic bacteria by spread into TSB Cong -red agar amended with carboxymethylcellulose as a carbon source and Tryptone as a protein source. The discoloration of Congo red around the colony shows the activity of cellulolytic bacteria. In the practical experiment, the discoloration was not clear enough to detect the cellulolytic bacteria because of the TSB agar medium used for enrichment, caused too much growth in the agar plate. Gupta (2012) suggested the confirmation of cellulolytic bacteria by streaking on the cellulose Congo red agar which contains inorganic salts, cellulose, gelatin, and Congo red. From the ingredients of the mentioned media, it was less rich in protein source and energy and more specialize to bacteria which TSB agar can be culture either bacteria or fungi. In some researches, washing Congo-red medium by 10% HCl is an alternative solution to enhance the visuality of the clearance zone (Panday *et al.,* 2018).

After streaking each colony into TSB agar to obtain a single colony for 60 isolates, each of the isolates was picked for spot colony for Gram's iodine staining for identifying cellulolytic bacteria. Gram's iodine was formed a bluish-black complex with cellulose but not formed with hydrolyzed cellulose, making a clearance zone around the colony (Kasana *et al.,* 2008). This staining method is an alternative method for detecting cellulolytic bacteria which is more rapid than Congo red method which takes a 30-40 minutes to obtain the clear zone, while the Gram's iodine can be done within 3-5 minutes. From the Gram's iodine staining test results, the non-hydrolyze zone was obtained a light purple color which is not dark enough to see the clear zone accurately.

The clearance zone from Gram's iodine staining from each isolate was measured for Hydrolysis capacity (HC) calculation. HC was estimated as a diameter of the clearance zone from cellulolytic activity. This estimation can be predicted the cellulase degrading capacity before cellulase assay or can be concluded as the higher HC, the greater cellulase degrading capacity from cellulolytic bacteria. The colonies that have high HC values were selected for cellulase assay and biodegradation tests by using the statistic. The HC values that was greater than the third quartile (6.1725) were selected for the next process. The isolates SB 13, SB 19, SB 22, SB 24, SB 29, SB 33, SB 39, SB 50, SB 52 and SB 58 can be concluded that they have a high cellulolytic ability.

There was a study about the relationship between soil and microbes. Microorganism in the soil uptakes large organic substance such as cellulose and hydrolyze to obtain the energy and used for their growth. Microbial population and their activities will transform the nutrients to available form and improve the soil fertility (Leaungvutiviroj *et al.,* 2010). This can be concluded that if the screened active cellulolytic bacterial were applied into the rice field, it will biodegrade the rice straw more efficiently and increase the soil fertility too.

Due to the available of Ferric oxide in the soil, can be predicted that the microbes that can uptake iron are also available in the soil. Arnold *et al.,* (1986) have studied about reductive dissolution of Ferric oxides by Pseudomonas sp. which is available in the soil. Ramasamy and Verachtert (1979) can isolate Pseudomonas sp. and degrade 60% of native cellulose in vitro in 5 days due to the availability of cellulases. The optimal pH for Pseudomonas sp. growth is approximately 6 – 8 pH and 7 pH obtain the highest growth (Singh *et al.,* 2015) and they can grow in range of 4 – 42 ˚C with and optimal temperature above 20 ˚C (Chakravarty and Gregory., 2015). Therefore, it can be predicted that the isolated strain can be Pseudomonas sp.

In conclusion, this is the study of cellulase potential by cellulolytic bacteria in the rice field. The nine isolates from the total of 60 isolates consisted of SB 13, SB 19, SB 22, SB 24, SB 29, SB 33, SB 39, SB 50, SB 52 and SB 58 are applied for rice straw treatment in biodegradation process by using Gram’s iodine for screening the cellulolytic bacteria. From this study, the screening method can be used to investigate the high rice straw degradation efficiency for rice straw waste management and also improve the soil abundant.

The cellulase assay and biodegradation test cannot be done because of the Covid-19 situation. However, is study can be confirmed that the soil in the rice field has a cellulose-degrading bacterium. So, further study can use the soil from the rice field for biodegradation and for measuring the cellulase activity. Moreover, the study to characterize 35the bacteria species such as morphological and biochemical study or 16 S rRNA gene sequencing are important for further study as well. However, the biodegradation efficiency comparison of the burned and plowing soil can be investigated in further study.

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APPENDICES

APPENDIX A – Reagents

Formulation

1. Gram Iodine

KI	2.0	g
Iodine	1.0	g
Distilled water	300	ml

2. DNS reagent

Distilled water	1416	ml
3,5-Dinitrosalicylic acid	10.6	g
NaOH	19.8	g
Rochelle salts (Na-K tartrate)	306	g
Phenol (melt at 50 ˚C)	7.6	ml
Na metabisulfite	8.3	g

3. Glucose standard

Glucose	0.2 – 5.0	mg
Distilled water	0.5	ml

Preparation

1. Gram's iodine preparation

The component was dissolved in distilled water and contained in the amber glass bottle.

2. DNS reagent

Distilled water, 3,5-dinitrosalicylic acid, and NaOH was dissolved completely, and then add Rochelle salts, phenol, and Na metabisulfite.

3. Glucose standard

Glucose was dissolved distilled water in different concentrations.

APPENDIX B – Media

Formulation

1. TSB agar amended with Congo red and CMC

   Formula per liter

Pancreatic Digest of Casein	17.0	g
Papaic Digest of Soybean	3.0	g
Dextrose	2.5	g
Sodium chloride	5.0	g
Dipotassium phosphate	2.5	g
CMC	5	g
Congo red	0.2	g
Agar	15	g

2. Mineral salt medium

   Formulation per liter

$NaNO_3$	2.5	g
KH_2PO_4	2	g
$MgSO_4$	0.2	g
NaCl	0.2	g
$CaCl_2 \cdot 6H_2O$	0.1	g
CMC	5	g

Final pH 7.0

3. CMC agar

Formulation per liter

$NaNO_3$	2	g
KH_2PO_4	1	g
$MgSO_4$	0.5	g
KCl	0.5	g
CMC	2	g
Peptone	0.2	g
Agar	17	g

4. CMC broth medium

Formulation per liter

K_2HPO_4	0.5	g
KH_2PO_4	0.5	g
$(NH_4)_2SO_4$	1.0	g
$MgSO_4 \cdot 7H_2O$	1.0	g
$CaCl_2$	0.1	g
NaCl	6	g
CMC	10	g

Preparation

All components were mixed and dissolved in glass beaker by heating on hot plate stirrer until they are completely dissolved. The dissolved solution was poured into the Erlenmeyer flask with cotton ball and aluminum foil cover and sterilized at 121°C for 15 minutes in an autoclave.

CURRICULUM VITAE

Mr. Rawit Aimraksa was born in Kalasin on the 8th of May 1999. He was raised in Kalasin and studied at Kalasin from kindergarten to high school. He achieved the degree of high school from Kalasinpittayasan School and started to study at Chulalongkorn University in 2017. In his university life, he was an activity student. He was a member of KorKor Band as a guitar player. The main hobby is riding a big bike and going to many places in Thailand on vacation.