Evaluation of Microbial Biodegradation of Rice Straw in Paddy Fields

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Abstract : The aim of this study was to isolate and select cellulolytic microbial from decay wood, livestock manure and composted rice straw for digesting and crumbling rice straw in paddy field. All of the isolated microorganism were tested for their ability to produce cellulase by staining with congo red on carboxyl methyl cellulose (CMC) agar and then determined the reduced sugar by dinitrosalicylic acid (DNS) method in CMC broth. 49 Bacteria isolates and 22 fungal isolates from 48 isolates showed clear zone with congo red method. They were selected to determine for cellulase production in CMC broth. The result showed that the 14 bacteria isolates and 3 isolates of fungi were detected from cellulase production and used as starter in molasses for digesting rice straw in paddy fields. The highest amount of reducing sugars detected in fungal starter was 814.43 µg/ml, which is higher than that of the bacteria starter (449.64 µg/ml) and bacteria mixed with fungal (180.02 µg/ml). In the field test, 14 isolates of bacteria and 3 isolate of fungi were used as starter culture inoculum in molasses solution, mixture of water and molasses at the ratio of 100 ml of starter mixture : 100 ml of molasses : 10 l of drinking water were incubated for 10 days with occasionally shaking, then were spread in paddy field at the ratio of 100 l per rai of paddy fields. The experimental area was 3 rais. Results of the experiment showed that the farmer could cultivate paddy fields in 10 days because rice straw was digested. The hardness of soil was reduced and the farmer could prepare the fields for growing rice more easily when compared with the fields that did not used microbial biodegradation. Thus, the advantages of an experiment were to reduce more than 20% of cultivated time, tiredness and energy consumption.

Keywords : Cellulolytic microbial, Rice straw degradation

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1. Introduction

Rice is the main crop of Thailand and large amount of rice straw is accumulated as a byproduct from rice cultivation, as rice straw makes up about 50% of the dry weight of the rice plants. Farmers do not incorporate rice straw in the crop fields because of its slow degradation rate, disease infestation, unstable nutrients, and reduced yield caused by the short-term negative effect of nitrogen immobilization. They usually dispose it through open field burning. As a consequence, carbon dioxide, carbon monoxide, methane, nitrous oxide, and sulphur dioxide are emitted into the atmosphere (Gadde *et al*., 2009). This process also emits harmful air pollutants (Kausar *et al*., 2010). Attention has been focused on nonhazardous, environment friendly and sustainable techniques for safe disposal of rice straw in a short period of time. Microbial composting is an effective environmentally sound alternative for the recycling of rice straw into compost. It promotes sustainable agriculture and environmental protection, improving the soil's physical, chemical, and biological properties, which ultimately results in better plant growth and yield. Suvarnabhumi rice research project aims to find research problems in Tambon Wat Dao, Amphoe Song Phi Nong, Suphan buri province, to share their learning with village philosophers and integrate research with community activities seminar in Tambon Wat Dao. The research problems from village philosophers need the degradation of rice straw in paddy fields. Development of rice production is expected which it can help farmers reduce production cost and to restore ecology systems, as in the past. Village philosophers have provided information to researchers that in the past, they had trouble getting rid of rice straw. Commonly practiced method is to burn the rice straw, resulting in air pollution. The effect on the environment and destroy the fertility of soil, thus modifying the method of decomposing rice straw instead. Therefore, it is an important reason for the proposed project in the development of cellulolytic microorganisms to degradation of rice straw in paddy fields. In order to further the knowledge in the community and knowledge science to help develop careers, improve quality of lives of the farmers in the community. The main objectives of this research were to isolate and screen of cellulolytic microorganisms from decay wood, livestock manure and composting rice straw to produce and evaluate community products for digesting and crumbling rice straw in paddy field.

2. Materials and Methods

2.1 Samples for isolation of cellulolytic microorganisms

Isolating and selecting of cellulolytic microorganisms from decay wood, livestock manure and composting rice straw to produce community products for digesting and crumbling rice straw in paddy field total 10 samples from various sources.

2.2 Isolating and screening of cellulolytic microorganisms

2.2.1 Isolating and screening of cellulolytic bacteria. (Kasana *et al.,* (2008); Hart *et al*., (2002); Kausar *et al*., (2010))

1 gram samples were mixed with 99 ml of buffered peptone water to dilute. Make serial dilution until 10^{-6}. 0.1 ml were spread on CMC agar plate and then incubated at room temperature for 24-48 h, then evaluate ability to produce cellulase by congo red method, the medium were flooded with an aqueous solution of 1% congo red for 15 min The congo red solution was then poured off and plates were further treated by flooding with 1 M NaCl for 15 min and poured off. Degradation of cellulose was visualized as a clearing zone around the bacteria colony. The diameters of the clearing zone around the colony were record. The isolates were purified on CMC agar to obtain pure cultures and storage in CMC broth supplemented with 30% glycerol at -40° C.

2.2.2 Isolating and screening of cellulolytic fungi.

1 gram samples were put in sterile petri dish, then 9 centimeter diameter filter paper overlaid onto samples. Filter paper was soaked with solution of PDB +0.5% CMC medium. Incubated at room temperature for 3-7 days. The single colony was transferred aseptically onto PDA+0.5% CMC media to obtain the pure culture. A 6 mm mycelia disc from 7 days old PDA culture was placed at the center of the plate and incubated at room temperature for 3 days. At day 3, the medium were flooded with an aqueous solution of 1% congo red for 15 min, the congo red solution was then poured off and plates were further treated by flooding with 1 M NaCl for 15 min and poured off. Degradation of cellulose was visualized as a clearing zone around the fungal colony. The diameters of the clearing zone around the colony were recorded.

2.3 Enzyme activity assay

The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using dinitrosalicylic acid (DNS) method (Miller, 1959). The isolated bacteria was inoculums in CMC broth, fungi isolated were inoculums in PDB+0.5% CMC, then incubated at 37 °C for 72 h with shaking speed 140 rpm. Culture broth was centrifugation at 6,000 rpm at room temperature for 10 min to obtain culture supernatants which were later measured cellulase activity by DNS method. The optical density was read at 520 nm by a spectrophotometer. Microorganisms isolate that produce highest cellulase activity was selected for evaluation of rice straw degradation and identification.

2.4 Evaluation of microbial biodegradation rice straw *in-vitro*

First, rice straw was cut to size 1 cm, 1 g (1%) of rice straw mixed in CMC broth volume 100 ml in flask 250 ml. Secondly, autoclave at 121 °C for 15 min. Thirdly, inoculums with 17 cellulolytic microorganisms that produce highest enzyme activity were including fungi, bacteria and mixed bacteria - fungi respectively, finally incubated at 37 °C for 48 h (shake, speed 140 rpm), then measuring enzyme activity by reducing sugar.

2.5 Evaluation of microbial biodegradation rice straw in paddy field

17 cellulolytic microorganisms were inoculums in molasses solution (molasses: water in a ratio of 1:100). Incubated at room temperature for 10 days and then evaluate performance degradation rice straw in farmer's rice field.

2.6 Identification of cellulolytic microorganisms

Bacteria isolated were presumptively identified based on morphological and biochemical characterizations. The parameters investigated included colonial morphology, gram stains and utilization of sugars, these were compared with Bergey's manual of systematic bacteria. (Holt *et al*., 1994)

Identification of fungi, which relies on morphological characteristics by slide culture technique was viewed with microscopes, sizes, shapes, arrangement of conidiophores, and conidiospores were observed and identified by compared with manual identification of fungi. (Gilman, 1957; Hawksworth *et al.,* 1995; Raper, 1965)

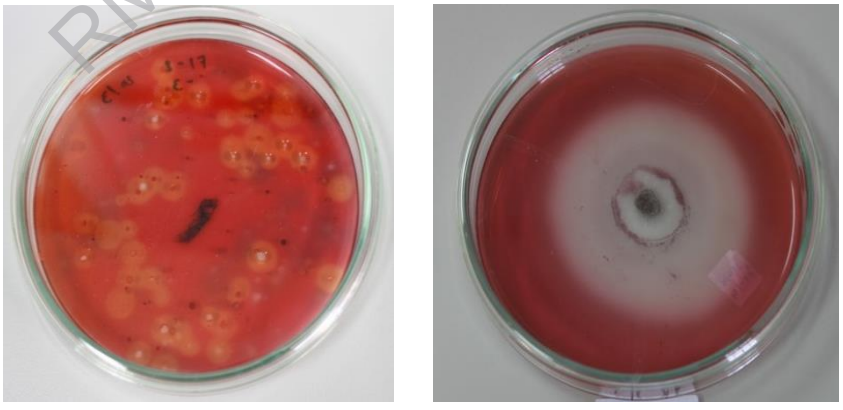
3. Results and Discussion

3.1 Isolating and screening of cellulolytic bacteria

Total 49 bacteria isolated including 22 gram-positive, 27 gram-negative, 38 rod, 11 cocci were isolated from 10 samples of decay wood, livestock manure and composting rice straw. That showed positive results with size of clear zone ranging 5 to 29 mm from congo red test method (Fig. 1). The highest is BDM1041, BDM1051, BDM1042 and BDM1043 which has clear zone was 29, 29, 28 and 28 mm (data not showed) respectively. Upon further evaluate quantitative of cellulose degrading enzyme in liquid medium.

3.2 Isolating of and screening cellulolytic fungi

Total 48 fungi isolated were isolated from 10 samples of decay wood, livestock manure and composting rice straw. When evaluating cellulose hydrolysis by measuring the diameter of clear zone in congo red test, found that 22 isolated showed positive results with size of clear zone ranging 18 to 90 mm from congo red test method (Figure 1). The highest is F1F-5, S1F-8 and W3F-2 which has clear zone was 90, 80 and 65 mm, respectively (data not showed). Upon further evaluate quantitative of cellulose degrading enzyme in liquid medium.



Bacteria **Fungi**

Figure 1: The ability to produce cellulase of bacteria and fungi isolated on CMC agar by congo red method.

In this study was used congo red method to detect cellulose hydrolysis because it was very convenient to read results quickly and dye durability. (Theather and Wood, 1982) Separated by a popular culture based on CMC agar and then stained with congo red also use other substances instead (Lamb and Loy, 2005). It has developed methods by Kasana *et al*., (2008) used gram's iodine staining instead congo red. Samples were isolated microorganisms that can produce cellulase to find amount and type of microorganisms of different samples in each area. Prasertsan (1992) determine the ability to produce cellulase by congo red method an initial inspection, which must have been the operation of the enzymes by microorganisms in the next experiment.

3.3 Enzyme activity in liquid medium

Determining reducing sugar by DNS method, 49 bacteria isolates and 22 fungal isolates from 48 fungi isolates showed clear zone with congo red test, and selected to determine cellulase production in CMC broth. The result showed that 14 bacteria isolates and 3 isolates of fungi were detected highest cellulase production. 14 bacterial isolates including F1-1, F1-3, F1-5, F1-6, F1-7, F1-8, F1-10, F3-1, F3-2, S1-2, S1-4, S2-3, S2-4, S2-6, that isolate of S2-3, F1-8 and F1-1 showed highest reducing sugar are 328.10, 304.26 and 297.30 µg/ml, respectively (Table 1). 3 fungal isolates including W2F-1D, W3F-7D and S1F-11D, reducing sugar are 316.97, 243.85 and 165.64 µg/ml, respectively (Table 1).

Table 1: Cellulase activity of crude enzyme and identification of cellulolytic microorganisms.

No.	Isolate	Clear zone (mm)	Reducing sugar (µg/ml)	Identified as
Bacteria				
1	F3-1	16	297.30	*Salmonella paratypi*
2	F3-2	11	214.01	*Bacillus cereus*
3	F1-1	22	280.02	*Bacillus subtilis*
4	F1-3	18	293.34	*Bacillus subtilis*
5	F1-5	21	233.02	*Bacillus subtilis*
6	F1-6	23	304.26	*Bacillus subtilis*
7	F1-7	21	238.49	*Bacillus subtilis*
8	F1-8	20	222.87	*Bacillus subtilis*
9	F1-10	5	270.87	*Bacillus subtilis*
10	S2-3	16	196.38	*Bacillus subtilis*
11	S24	7	222.97	*Bacillus subtilis*
12	S2-6	7	328.10	*Bacillus subtilis*
13	S1-2	3	195.59	*Bacillus subtilis*
14	S1-4	18	221.99	*Proteus mirabilis*
Fungi				
1	S1-11D	49	316.97	*Penicillium* spp.
2	W2-1D	22	243.85	*Chaetomium* sp.
3	W3F-7D	40	165.64	*Aspergillus terreus*

Which shows that the ability to produce cellulase on solid medium by gram's iodine is only preliminary, as tested with congo red, it is required to evaluate the operation of enzymes associated with other methods. As tested in liquid CMC broth by the DNS method. (Prasertsan *et. al.,* 1992) However, the production of microbial enzymes depends on several factors including amount of microorganisms, temperature, pH, carbon source, inducer or inhibitor, size of fermenter and aeration (Ali *et al*., 1991). Thus, the ability of the cellulase fungal in liquid CMC broth depends on several factors mentioned above, which are different in each type of microorganisms. There are many reports show that *Aspergillus niger* can produce cellulase the highest (Dashtban *et al*., 2009). *Bacillus* sp. showed a potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds (Krairitthichai and Thongwai, 2008). In the future, were should develop the products in many ways such as in form of power, pill and other which we can use easily.

3.4 Evaluation of the cellulolytic microorganisms isolated for *in-vitro* biodegradation of rice straw

17 cellulolytic microorganisms that produce highest enzyme activity were divided in 3 groups, including bacteria, fungi and mixed of fungi-bacteria, respectively. Result showed that the highest amount of enzyme activity (reducing sugars) detected in fungal starter were 814.43 µg/ml, and bacteria starter were 449.64 µg /ml higher than bacteria mixed with fungal (180.02 µg/ml) (Table 2)**.**

Table 2: Reducing sugar of degradation rice straw by cellulolytic microorganism.

No.	Isolate	Reducing sugar (µg/ml)
1	14 Bacteria isolates	449.64
2	3 Fungi isolates	814.43
3	14 Bacteria+3 Fungi isolates	180.02

Fungi were more effective producing cellulose than other microorganisms, but may be grow slower than other types of microorganisms (Alexander, 1977). When mixed with bacterial-fungi we found that some bacteria may be produced substances that inhibit growth of fungi, or competing in food sources due to exhausted, fungi grow more slowly than bacteria, or may be due to effects of glucose and cellobios caused by decomposition inhibit the production of cellulose.

The concentration of carbon source effects the production of cellulase as well. The much or too little amount of carbon source in food will effect on growth of bacteria and cannot produce cellulase. The inoculum *Bacillus* sp. that produced cellulase in 1% CMC medium as carbon source, found that the microbial produce cellulase was highest. Likewise, reported by Gokhale *et al*. (1991)

found that *A. niger* NCM 1207 produce cellulase are highest when cultured in 1% glucose. However, reported by Krairitthichai and Thongwai, (2008) found that when cultured *B. subtilis* CMU4-4 and *B. coagulans* TI-5 in different carbon sources, found that 2 isolates bacterial were highest enzyme specific activity when culture in 0.2% CMC. In this experiment, were use 1% rice straw as a carbon source and evaluate digestion of rice straw in laboratory before use in rice field.

3.5 Evaluation of cellulolytic microorganisms isolates for biodegradation of rice straw in paddy field

In the paddy field test were used 14 isolates of bacteria (F1-1, F1-3, F1-5, F1-6, F1-7, F1-8, F1-10, F3-1, F3-2, S1-2, S1-4, S2-3, S2-4, S2-6.) and 3 isolates of fungi (W2F-1D, W3F-7D, S1F-11D) inoculum in mixture of water and molasses with ratio of 100 ml of starter mixture : 100 ml of molasses : 10 l of drinking water, incubated for 10 days with occasionally shaking. Result found that mycelial pellet growth on surface of molasses solution, which can evaluate degradation of rice straw in the field, then spreaded them with ratio of 100 l per rai of paddy field. The area to perform the experiment was 3 rais and the control treatment performed by pumping pure water flood on the field area of 0.25 rai. The result of an experiment found that, the farmer can cultivate paddy fields in 10 days because rice straw was digested. The hardness of soil was reduced and the farmer could prepare rice fields for growing rice easily when compared with unused microbial biodegradation area. Thus, the advantages of an experiment were to reduce cultivated times around 20%, tiredness and energy consumption.

3.6 Identification of cellulolytic microorganism

Identification of cellulolytic microorganisms by monitoring morphological, including gram's stain, shape and arrangement of cells and biochemical properties. Results show that bacteria identified as *Salmonella paratyphi*, *Bacillus cereus*, *B. subtilis* and *Proteus mirabilis*. Fungi identified as *Penicillium* spp, *Chaetomium* sp. and *Aspergillus terreus* (Table 1) similarly reported by Krairitthichai and Thongwai, (2008) showed that isolated and screening of cellulolytic bacteria found that identified as *Bacillus* sp.

4. Conclusion

A total 97 microorganisms including 49 bacteria and 48 fungi were isolated and selected of cellulolytic microbial from decay wood, livestock manure and composting rice straw for produce community products for digesting and crumbling rice straw in paddy field by congo red method. 14 bacteria and 3 fungi isolated show ability to produce cellulase determined by DNS method. 17 cellulolytic microorganisms identified as *Salmonella paratyphi*, *Bacillus cereus*, *B. subtilis*, *Proteus mirabilis*, *Penicillium* spp, *Chaetomium* sp. and *Aspergillus terreus*, were used as starter culture in molasses solution for digesting rice straw in farmer's field which highest amounts of reducing sugars. We found that, farmer could cultivate paddy fields in 10 days, because rice straw was digested. The hardness of soil was reduced and the farmer could prepare the fields for growing rice more easily. Thus, the advantages of an experiment were to reduce cultivated times tiredness and energy

consumption. Cellulolytic isolated microorganisms could potential to be developed as cellulolytic consortium for rapid and efficient composting of rice straw into a value-added product of agro waste materials.

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