

Screening of Thermophilic and Halophilic Cellulase – Producing Bacteria from Saline Soil Samples

Phakamas Rachamontree^{*}

*Program in Biology, Faculty of Science and Technology, Rajabhat Maha Sarakham University,
Maha Sarakham, 44000, Thailand*

Abstract

Maha Sarakham Province is an agricultural area with primarily rice cultivation cause large amounts of rice straw after the harvest season. The application of cellulase - producing bacteria for rice straw degradation is more attention because it is an environmentally friendly. This area is mostly tropical drought and soil salinity hence some such extremotolerant bacteria can thrive in these conditions as desired. Thus, this research aimed to isolate the cellulase - producing thermophilic and halophilic bacteria. Seventy saline soil samples were collected from rice fields in Maha Sarakham Province, Thailand. A total of one hundred and six isolated bacterial isolates were obtained from soil salinity. The cellulase - producing activities was preliminary examined using the formation of clear zone observation on CMC agar. The results revealed that nine generated isolations was 25-105 mm of clear zone radius. The cellulase production of each isolate was estimated by enzyme activity after various cultured condition in medium broth. The effect of salt concentration on cellulase production was conducted in lactose broth containing 1% CMC with varying concentrations of sodium chloride (3-10%). The results revealed that there were no significant differences in enzymatic activity. The highest enzyme activity was found in RMU41 with a value of 0.826 ± 0.007 U/mL at 50 °C (salt concentration of 10%) and found the cellulase activity of 0.246 ± 0.031 U/mL when cultured in lactose broth supplementation with rice straw as a carbon source in 2.5% concentration. The 16S rDNA sequence analysis was indicated that the RMU41 strain was highly related with *Streptomyces thermogriseus* and *Streptomyces thermovulgaris* of 99.79% similarity.

Keywords: Thermophilic bacteria, Halophilic bacteria, Cellulase and Enzyme activity

^{*} Corresponding author: E-mail: kungphaka@gmail.com

การคัดเลือกแบคทีเรียชอบร้อนและชอบเค็มที่สามารถผลิตเอนไซม์เซลลูเลสได้จากตัวอย่างดินเค็ม

ผกามาศ ราชมนตรี*

สาขาวิชาชีววิทยา คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏมหาสารคาม อ.เมือง จ.มหาสารคาม 44000

บทคัดย่อ

จังหวัดมหาสารคามเป็นพื้นที่การเกษตรที่มีการเพาะปลูกข้าวเป็นหลักจึงมีฟางข้าวหลังฤดูเก็บเกี่ยวปริมาณมาก การประยุกต์ใช้แบคทีเรียที่สามารถผลิตเอนไซม์เซลลูเลสเพื่อกำจัดฟางข้าวจึงได้รับความสนใจเนื่องจากเป็นมิตรกับสิ่งแวดล้อม แต่เนื่องจากพื้นที่ส่วนมากเป็นเขตร้อนแห้งแล้งและดินเค็มจึงต้องใช้แบคทีเรียที่สามารถเจริญในสภาวะดังกล่าวได้ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อคัดเลือกแบคทีเรียชอบร้อนและชอบเค็มที่มีความสามารถในการผลิตเอนไซม์เซลลูเลส โดยเก็บตัวอย่างดินเค็มในเขตจังหวัดมหาสารคาม 70 ตัวอย่าง ซึ่งสามารถแยกแบคทีเรียได้ 106 ไอโซเลท จากนั้นทดสอบการผลิตเอนไซม์เซลลูเลสเบื้องต้นโดยสังเกตโซนไฮรอปโคโลนีบนอาหารเลี้ยงเชื้อแข็ง CMC ผลการทดลองพบ 9 ไอโซเลท ที่สามารถสร้างรัศมีโซนไฮรอปขนาด 25-105 มิลลิเมตร นำแต่ละไอโซเลทมาศึกษาความสามารถในการผลิตเอนไซม์เซลลูเลสโดยวัดค่ากิจกรรมเอนไซม์หลังการเพาะเลี้ยงในอาหารเหลวที่สภาวะต่างๆ การศึกษาผลกระทบของความเข้มข้นเกลือ (3-10 เปอร์เซ็นต์) ต่อการผลิตเอนไซม์เซลลูเลสในอาหารเลี้ยงเชื้อ Lactose broth ที่เติม CMC เข้มข้น 1 เปอร์เซ็นต์ ผลการทดลองพบว่าค่ากิจกรรมเอนไซม์ไม่แตกต่างกันอย่างมีนัยสำคัญ โดยพบไอโซเลท RMU41 มีค่ากิจกรรมเอนไซม์สูงที่สุดเท่ากับ 0.826 ± 0.001 ยูนิตต่อมิลลิลิตร ที่อุณหภูมิ 50 องศาเซลเซียส และความเข้มข้นเกลือเท่ากับ 10 เปอร์เซ็นต์ นอกจากนี้ไอโซเลท RMU41 ที่เพาะเลี้ยงใน Lactose broth ที่เติมฟางข้าวเข้มข้น 2.5 เปอร์เซ็นต์ เป็นแหล่งคาร์บอนให้ค่ากิจกรรมเอนไซม์เท่ากับ 0.246 ± 0.031 ยูนิตต่อมิลลิลิตร เมื่อวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ 16S rDNA ของไอโซเลท RMU41 พบว่ามีความใกล้เคียงกับ *Streptomyces thermogriseus* และ *Streptomyces thermovulgaris* 99.79 เปอร์เซ็นต์

คำสำคัญ: แบคทีเรียชอบร้อน, แบคทีเรียชอบเค็ม, เอนไซม์เซลลูเลส และกิจกรรมเอนไซม์

* ผู้เขียนให้ติดต่อ: E-mail: kungphaka@gmail.com

Introduction

Thailand is predominantly an agricultural country, especially rice cultivation by total acreage of 25.70 million acres. After the harvest, leaving stumps or rice straw is eliminated to the utilization of land area for the next cultivation. Large amounts of rice straw are produced every year around 25.45 million tons by the most quantity of 13.70 million tons per year in the Northeast (Land Development Department, n.d.), even farmers are bringing rice straw to make other useful applications (as animal feed, fertilizer, etc.) but they remain a lot of rice straw. The farmer is getting rid of rice straw by burning, which is a time - saving method, but this causes environmental pollution and destruction of soil fertility. The biological method for rice straw degradation such as enzyme digestion is more attention because of environmentally friendly. Rice straw is renewable lignocellulose, which contains three major polymer groups: cellulose of 32.15%, hemicellulose of 28%, and lignin of 19.64% (Shawky *et al.*, 2011). Cellulose, a major cell-wall constituent of higher plant, is a linear polysaccharide consisting of β -1,4-linked D-glucose residues. They are commonly degraded by the cellulase enzyme which consists of three major components, i.e., endoglucanases, exoglucanases and β -glucosidases (Lugani *et al.*, 2015). Therefore, the application of cellulase - producing microorganism for rice straw degradation have been interested. In addition, the using exogenous cellulase not only to accelerate rice straw decomposition, but also increase soil fertility (Han and He, 2010).

Cellulase is generally considered to be performed by a variety of microorganisms,

including fungi and bacteria (Sheng *et al.*, 2016). However, cellulases from bacteria are widely exploited because of high growth rate and inhabit a wide variety of environmental and industrial niches that are extreme environments such as thermophilic or psychrophilic, alkaliphilic or acidiphilic and halophilic strains (Sadhu and Maiti, 2013). Moreover, cellulase is widely applied, due to its potential applicability in various processes such as enzymatic clarification, biogas production, bioethanol production, triphasic biomethanation, agricultural and plant waste management, chiral separation and ligand binding studies, starch processing, textile industry etc. (Rekadwad *et al.*, 2015; Mohanta, 2014). Maha Sarakham Province is an agricultural area with primarily rice cultivation by rice acreage of 818,671 acres (Office of Agricultural Economics, n.d.) that produce large amounts of rice straw after the harvest season. More than 95% of the total area cover by potash and rock salt (Department of Mineral Resources, 2009) cause the problem of soil salinity. The present study focused on thermophilic and halophilic bacteria, due to they could survive and grow at high temperature and salinity condition in this area. A group of facultative thermophilic bacteria grow at high temperature with the optimum temperature of about 50°C. Halophilic bacteria are able to grow optimally in media containing NaCl concentration of 3-15% (Yu and Li, 2015). Therefore, the objectives of this study were to isolated and screened the thermophilic and halophilic cellulase - producing bacteria from soil samples obtained from rice fields in Maha Sarakham province, Thailand that has reported issues of soil salinity problems.

Materials and Methods

1. Sample collection

Soil samples were collected from the top 0-15 cm (Han and He, 2010) of rice fields in 6 districts (Phayakkhaphum Phisai, Chiang Yuen, Kantharawichai, Borabue, Wapi Pathum, and Na Dun) in Maha Sarakham Province, Thailand that has reported issues of soil salinity problem in isolation of cellulase - producing bacteria. Samples were collected in sterile bottles and stored at 4°C until used.

2. Isolation of thermophilic bacteria

Twenty - five grams of soil samples were diluted in 225 ml of butterfield's phosphate buffer by ten-fold serial dilution. After that 100 µl of diluted samples were spread on carboxymethyl cellulose (CMC) agar contained (per liter) 0.2 g of peptone, 2.0 g of NaNO₃, 1.0 g of MgSO₄·7H₂O, 1.0 g of K₂HPO₄, 0.5 g of KCl, 2.0 g of carboxymethyl cellulose and 1.7 g of agar (Kasana *et al.*, 2008). After incubation at 50°C (optimum temperature of facultative thermophilic bacteria) for 48 hours, the morphologically different colonies were purified by streak technique on CMC agar and preserved at 4°C until used.

3. Screening of cellulase - producing bacteria

Each purified isolate was cultured in Lactose broth (LB) consisted of (per liter) 3.0 g of beef extract, 5.0 g of peptone, 5.0 g of lactose as inoculum. Five microliters of inoculum were spotted on CMC agar and incubated at 50°C for 48 hours. After that plates were flooded with 0.1% Congo red for 15-20 minutes and then destained with 1 M NaCl for 15-20 minutes (Kasana *et al.*, 2008). The preliminary cellulase - producing activities were estimated by clearing zone around

the colonies and hydrolysis capacity (HC value) as shown in equation (1) (Lu *et al.*, 2005).

$$HC = \frac{\text{diameter of clearing zone (cm)}}{\text{diameter of colony (cm)}} \dots\dots(1)$$

4. Examination of halophilic cellulase - producing bacteria

The isolated bacteria were inoculated into 50 ml of LB medium containing 1% CMC as a carbon source (Yang *et al.*, 2014) and supplement with different concentrations of NaCl vary from 3 to 10% (w/v). The culture was incubated at 50°C using shaken mode, for 48 hours at 220 rpm. The enzyme activity was examined by using cell-free supernatant after centrifugation at 10,000 rpm under 10°C for 10 minutes.

The enzyme activity was measured the releasing of reducing sugar from the using of 1% CMC as substrate compared to the glucose standard curve as a control. The suitable diluted cell - free supernatant 0.5 ml was mixed with 0.5 ml of 1% CMC in 0.05 M citrate buffer (pH 4.8) and incubated at 50°C for 30 minutes. The reaction was added 2 ml of Dinitrosalicylic acid (DNS) and boiled for 15 minutes, then stop reacting by cooling in ice (Krairitthichai, 2009). The amount of reducing sugar released by the hydrolysis of CMC was measured at 540 nm by the DNS method (Miller, 1959). One unit of cellulase activity was defined as the amount of enzyme to release 1 µmol of reducing sugar per minute, showed as units per milliliter (U/mL).

5. Examination of rice straw degradation

The isolated bacteria was inoculated into 50 ml of basal medium containing (per liter) 5.0 g of (NH₄)₂SO₄, 10.0 g of KH₂PO₄, 5.0 g of K₂HPO₄, 0.1 g of MgSO₄, 0.2 g of NaCl, and 10.0 g of yeast extract that supplement with different concentration of

rice straw varies from 0.5 to 2.5% (w/v) (Lugani *et al.*, 2015) as carbon source. The culture was incubated at 50°C and centrifuged at 10,000 rpm, 10°C for 10 minutes. The cell - free supernatant was examined for enzyme activity by DNS method.

6. Identification of bacterial strain

Only one isolated bacteria was showed the highest enzyme activity was selected as a cellulase - producing bacteria for identification of strain. A genus and species of the selected isolate was identified based on the similarity of 16S rDNA gene sequence. The genomic DNA of the selected isolate was extracted by using Genomic DNA mini Kit (Geneaid Biotech Ltd., Taiwan) and used as template for PCR reaction. A PCR product for sequencing was conducted by using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA).

The nucleotide sequence from all primers was assembled by the Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) program. The identification of phylogenetic neighbors was initially proceeded with the BLASTN program with the database of published prokaryotic type strains.

7. Statistical analysis

The data were analyzed by one-way analysis of variance (one - way ANOVA) using the enzyme activity as the factor. Means were compared by *post hoc* Duncan's test and statistical significance was defined as $p < 0.01$.

Results and Discussion

1. Isolation and screening of thermophilic cellulase - producing bacteria

Seventy soil samples were collected from rice fields in six districts i.e. Phayakkhaphum Phisai, Chiang Yuen, Kantharawichai, Borabue, Wapi Pathum, and Na Dun. Soil samples were diluted and spread plate. The results were showed 106 isolates on CMC agar with different morphology after incubating at 50°C for 48 hours. Congo red assay on CMC agar was used to primary screen cellulase - producing capacity of bacteria. Results reveal nine isolates that appeared radius of clearing zone >10 mm range of 25-105 mm (Fig. 1) around the colony and high hydrolysis capacity (HC) value (Table 1). Table 1 was showed radius clearing zone and HC value of selected isolates that were parameters explained the ability of theses isolates to produce cellulolytic enzymes by using CMC as substrate. The results were surprising, isolate RMU30_1, RMU30_2, RMU36 and RMU37 revealed a low radius of the clearing zone, but high in hydrolysis capacity, in contrast to isolate RMU41 generated a high radius of the clearing zone but at lowest in hydrolysis capacity. These variation results probably due to some inconstancy experimental parameters such as pH change, temperature, incubation time during growth period and enzyme production process (Ahmad *et al.*, 2013).

2. Examination of halophilic cellulase - producing bacteria

Nine isolations that generated large clearing zone by Congo red assay on CMC agar were selected to examine the enzyme activity at high temperature. Data obtained from this study was tested by one-way ANOVA by *post hoc* Duncan's test. The results could be divided into five groups; the highest enzyme activity of 0.800 ± 0.020 U/mL was obtained in bacterial isolate RMU41 (Fig. 2) that is significantly different ($p < 0.01$) with other

isolates follow by isolate RMU20_4 (0.774 ± 0.071 U/mL), isolate RMU25 and RMU30_2 (0.658 ± 0.005 to 0.661 ± 0.022 U/mL), isolate RMU43 (0.585 ± 0.085 U/mL), and the lowest enzyme activities were obtained in isolate RMU29, RMU30_1, RMU36 and RMU37 (0.459 ± 0.024 to 0.552 ± 0.100 U/mL).

Table 1 Radius clearing zone and HC value of selected isolate

Isolated number	Radius of clearing zone (mm)	HC value
RMU20_4	30	3.67
RMU25	105	5.25
RMU29	80	1.69
RMU30_1	25	5.80
RMU30_2	25	6.60
RMU36	25	4.20
RMU37	25	5.80
RMU41	100	1.35
RMU43	70	1.93

The Experiment was tested the influence of salt concentration on cellulase production. The isolated bacteria RMU41 that generated a high of enzyme activity was selected for examination of the influence of salt concentration on cellulase production using DNS method. The results were revealed that the isolate RMU41 was showed salt tolerance up to 10% NaCl concentration (w/v) at 50°C with the highest enzyme activity of 0.826 ± 0.007 U/mL, which no significant differences ($p > 0.01$) (Table 2) as compared to other salt concentration. Singh *et al.* (2010) documented that the salt - tolerant alkaliphilic actinomycetes from the saline habitats could be produced extracellular enzymes including proteases,

amylases, cellulases and lipases at higher salt and alkaline pH condition.

3. Examination of rice straw degradation

The isolated bacteria RMU41 was cultured in basal medium supplement with rice straw as a carbon source in different concentrations (0.5-2.5%, w/v) at 50°C to estimate the enzyme activity. The highest enzyme activity of 0.246 ± 0.031 U/mL was found in rice straw concentration of 2.5% (Fig. 3), which is significant differences ($p < 0.01$) as compared with the other rice straw concentrations. Saratale *et al.* (2012) who reported that *Streptomyces* sp. MDS grown in Dubos medium and used a natural agricultural residue (rice straw) and wood straw at an initial concentration of 10 g/L as a carbon source for cellulase (exoglucanase) production at 30°C which enzyme activities were observed of 8.51 and 1.35 U/mL, respectively.

4. Identification of bacterial strain

The morphological observation further demonstrated that RMU41 is a rod shaped Gram-positive bacterium. To identify the RMU41, a fragment of 16S rDNA was generated by PCR reaction and sequenced as described in the method. The sequencing result has 100% homology to 16S rDNA fragment of *Streptomyces* sp. Isolate RMU41 shared, particularly high similarity percentage of nucleotide sequence (99.79%) with *Streptomyces thermogriseus* and *Streptomyces thermovulgaris* was observed and then called RMU41 as *Streptomyces* sp. RMU41. According to Mohanta (2014), reported the cellulase production by the actinomycetes on mesophilic condition that found cellulase activities ranged from 0.501 to 1.381 U/mL. While *Streptomyces* sp. RMU41 produced the enzyme activities ranged from 0.645-0.826 U/mL at

thermophilic and halophilic conditions. According to the previous studies reported that *Streptomyces* sp. DSK59 achieved a maximum endoglucanase at 42.5-45°C and pH range of 6.25-6.75 (Budihal *et al.*, 2016) and several of *Streptomyces* sp. has the optimal temperature for cellulase activity of 40-55°C (McCarthy, 1987). Moreover, the application in many aspects of cellulase has been studied such as food, animal feed, textile and laundry, agriculture, etc. In an agriculture applications for enhancing growth of

crops, seed germination, rapid plant growth and flowering and controlling plant diseases (Behera *et al.*, 2016). In this research found *Streptomyces* sp. RMU41 can produce the cellulase activity of 0.246 ± 0.031 U/mL with used rice straw as carbon source. According to Abdulla and El-Shatoury (2007) who reported that Actinomyces four generas (*Kibdelosporangium*, *Micromonospora*, *Streptomyces* and *Nocardioideis*) were generated cellulolytic enzyme could efficiently degrade rice straw.

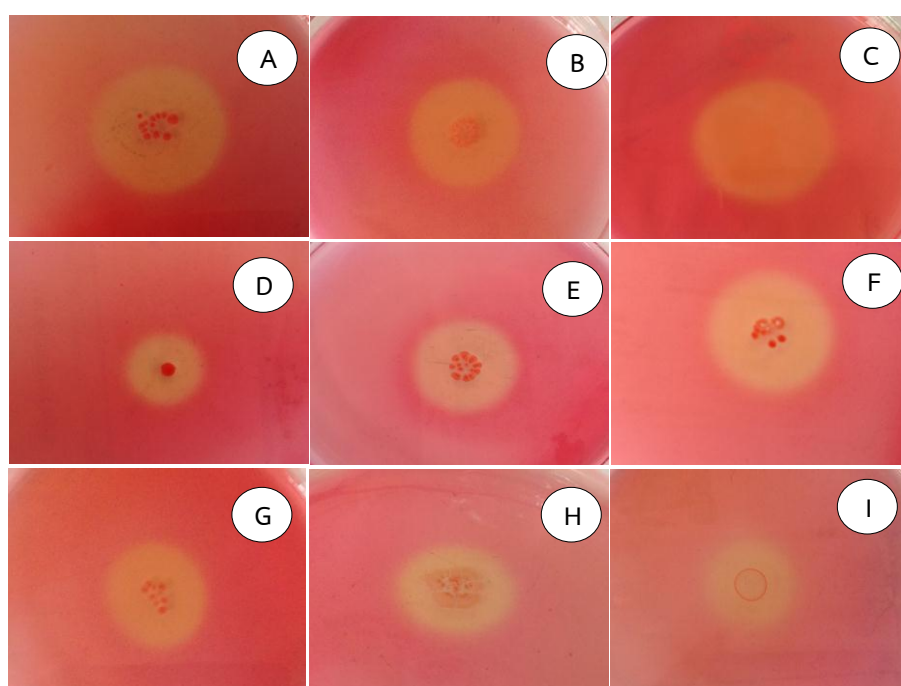


Fig. 1 Clearing zone by Congo red assay on CMC agar of isolates (A) RMU20_4 (B) RMU25 (C) RMU29 (D) RMU30_1 (E) RMU30_2 (F) RMU36 (G) RMU37 (H) RMU41 and (I) RMU43

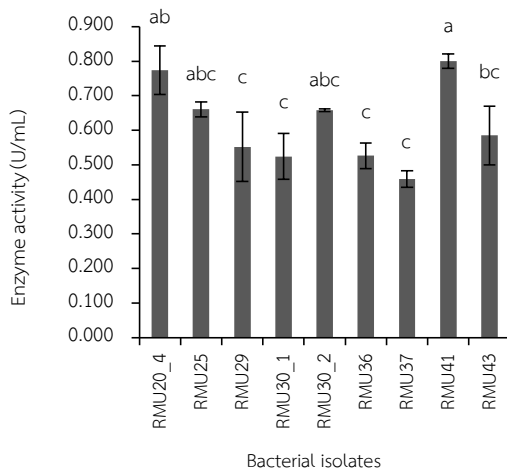


Fig. 2 Enzyme activities of nine isolates at 50°C. Means with the same letters are not significantly different (Duncan's test, $p < 0.01$). Bars represented standard deviations.

Table 2 Enzyme activities of NaCl concentrations variation by bacterial isolate RMU41

NaCl concentrations (%, w/v)	Enzyme activity (U/mL)
3	0.754 ^{ab} ± 0.028
4	0.645 ^b ± 0.091
5	0.723 ^{ab} ± 0.023
6	0.792 ^b ± 0.036
7	0.814 ^b ± 0.011
8	0.808 ^b ± 0.002
9	0.792 ^b ± 0.000
10	0.826 ^b ± 0.007

^{a,b} Mean values (±standard deviation) in the same column by different letters are significantly different ($p < 0.01$).

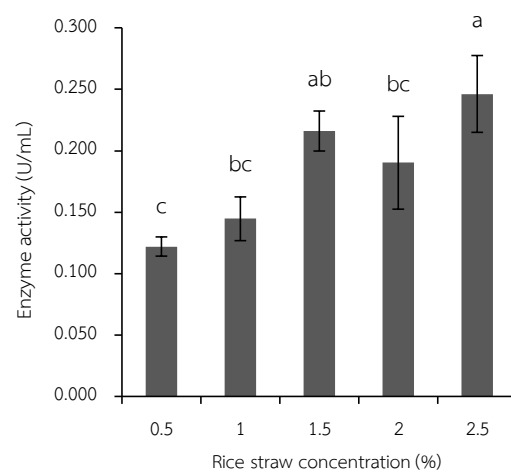


Fig. 3 Enzyme activities of bacterial isolate RMU41 at 50°C of varying the rice straw concentrations (0.5-2.5%) as carbon source. Means with the same letters are not significantly different (Duncan's test, $p < 0.01$). Bars represented standard deviations

Conclusion

This study purposed to isolate and screened the thermophilic and halophilic cellulase - producing bacteria from soil samples obtained from rice fields in Maha Sarakham Province, Thailand that has reported issues of a soil salinity problem for further application in these extreme condition areas. Totally 106 isolations were isolated from these samples and 9 isolates have a high hydrolysis capacity of the Congo red screening method. Nine isolated candidates were investigated for the influence of salt concentration at high temperature (50°C) on enzyme activity. RMU41 is the isolated bacteria that observed the highest enzyme activity of 0.826 ± 0.007 U/mL at a salt concentration of 10% (w/v). It was identified as Actinomycetes that has similarity of 100% to *Streptomyces* sp. base on 16S rDNA gene sequence.

The present study found the cellulase - producing *Streptomyces* sp. RMU41 at high temperature and high salt concentration suggests a possible role of this microorganism for its thermotolerant and halotolerant capabilities in nature. Therefore, it could be optimized for production yield and activity for application in rice straw decomposition on stress condition and reduced environment pollution from rice straw burned.

Acknowledgements

1. Research and Development Institute, Rajabhat Maha Sarakham University for financial supporting this study
2. Department of Biology, Faculty of Science and Technology, Rajabhat Maha Sarakham University for laboratory supporting

References

- Abdulla, H.M. and El-Shatoury, S.A. 2007. *Actinomyces* in rice straw decomposition. Waste Management. 27: 850-853.
- Ahmad, B., Nigar, S., Sadaf Ali Shah, S., Bashir, S., Ali, J., Yousaf, S. and Bangash, J.A. 2013. Isolation and identification of cellulose degrading bacteria from municipal waste and their screening for potential antimicrobial activity. World Appl. Sci. J. 27(11): 1420–1426.
- Behera, B.C., Sethi, B.K., Mishra, R.R., Dutta, S.K. and Thatoi, H.N. 2016. Microbial cellulase-Diversity & biotechnology with reference to mangrove environment: a review. J. Gen. Eng. Biotechnol. 15(1): 197-210.
- Budihal, S.R., Agsar, D. and Patil, S.R. 2016. Enhanced production and application of acidothermophilic *Streptomyces* cellulase. Biores. Technol. 200: 706-712.
- Department of Mineral Resources. 2009. Identification zone for geological management and mineral resources Mahasarakham province [online]. [Accessed October 27, 2016]. Available from: URL: <http://www.dmr.go.th/download/digest/Mahasarakam.pdf>. (in Thai)
- Han, W. and He, M. 2010. The application of exogenous cellulase to improve soil fertility and plant growth due to acceleration of straw decomposition. Biores. Technol. 101: 3724–3731.
- Kasana, R.C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr. Microbiol. 57: 503–507.
- Krairitthichai, S. 2009. Isolation and screening of bacteria producing cellulase for leave decomposition. M.Sc. Thesis, Chiang Mai University, Thailand. (in Thai)
- Land Development Department. n.d. Straw tilling for soil improvement and increasing yield [online]. [Accessed October 27, 2016]. Available from: URL: http://www.ddd.go.th/menu_moc/POSTER/rice/rice.htm. (in Thai)
- Lu, W.J., Wang, H.T., Yang, S.J., Wang, Z.C. and Nie, Y.F. 2005. Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system. J. Gen. Appl. Microbiol. 51: 353–360.

- Lugani, Y., Singla, R. and Sooch, B.S. 2015. Optimization of cellulase production from newly isolated *Bacillus* sp. Y3. J. bioprocess Biotech. 5(11): 1-6.
- McCarthy, A.J. 1987. Lignocellulose-degrading actinomycetes. FEMS Microbiol. Rev. 46: 145-163.
- Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(4): 426-428.
- Mohanta, Y.K. 2014. Isolation of cellulose-degrading actinomycetes and evaluation of their cellulolytic potential. Bioeng. Biosci. 2(1): 1-5.
- Office of Agricultural Economics. n.d. Wet season rice: cultivated area, harvested area, yield, and yield per rai year 2016/2017 [online]. [Accessed October 27, 2016]. Available from: URL: <http://www.oae.go.th/download/prcai/DryCrop/majorrice/1-60.pdf>. (in Thai)
- Rekadwad, B.N., Pathan, P.K. and Khobragade, C.N. 2015. Characterization of industrially important cellulase produced by *Actinomyces bovis* isolated from landfill site. J. Chem. Pharm. Res. 7(10): 214-219.
- Sadhu, S. and Maiti, T.K. 2013. Cellulase production by bacteria: a review. British Microbiol. Res. J. 3(3): 235-258.
- Saratale, G.D., Saratale, R.G. and Oha, S.E. 2012. Production and characterization of multiple cellulolytic enzymes by isolated *Streptomyces* sp. MDS. Biom. Bioen. 47: 302-315.
- Shawky, B.T., Mahmoud, M.G., Ghazy, E.A., Asker, M.M.S. and Ibrahim, G.S. 2011. Enzymatic hydrolysis of rice straw and corn stalks for monosugars production. J. Genet. Eng. Biotechnol. 9: 59-63.
- Sheng, T., Zhao, L., Gao, L.F., Liu, W.Z., Cui, M.H., Guo, Z.C., Ma, X.D., Ho, S.H. and Wang, A.J. 2016. Lignocellulosic saccharification by a newly isolated bacterium, *Ruminiclostridium thermocellum* M3 and cellular cellulase activities for high ratio of glucose to cellobiose. Biotechnol. Biofuels. 9(172): 1-11.
- Singh, S.P., Thumar, J.T., Gohel, S.D. and Purohit, M.K. 2010. Molecular diversity and enzymatic potential of salt-tolerant alkaliphilic actinomycetes. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology [online]. [Accessed December 10, 2016]. Available from: URL: <http://www.formatex.info/microbiology2/280-286.pdf>
- Yang, W., Meng, F., Peng, J., Han, P., Fang, F., Ma, L. and Cao, B. 2014. Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. Elec. J. Biotechnol. 17: 262-267.
- Yu, H.Y. and Li, X. 2015. Alkali-stable cellulase from a halophilic isolate, *Gracilibacillus* sp. SK1 and its application in lignocellulosic saccharification for ethanol production. Biom. Bioen. 81: 19-25.