

**THE USE OF AGRICULTURAL WASTES AS SUBSTRATES
FOR CELL GROWTH AND CARBOXYMETHYL
CELLULASE (CMCase) PRODUCTION BY *Bacillus subtilis*,
Escherichia coli AND *Rhizobium* sp.**

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ABSTRACT

Bacillus subtilis ATCC 6633, *Escherichia coli* ATCC 25922 and *Rhizobium* sp. DASA23010 were examined for carboxymethyl cellulase (CMCase) production. CMCase produced from all strains was characterized as cell-bound enzyme. All strains could grow in medium containing CMC as a sole carbon source. The highest activities obtained from *B. subtilis*, *E. coli* and *Rhizobium* sp. were not significantly different. Comparing the growth in three agricultural wastes, maximum cell numbers were obtained from the media containing pineapple peel, vegetable residue and corncob, respectively. Corncob stimulated higher production of CMCase than other agricultural wastes.

KEYWORDS: Carboxymethyl cellulase, CMCase, *Bacillus subtilis*, *Escherichia coli*, *Rhizobium*, agricultural wastes

1. INTRODUCTION

Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes [1]. An important feature of cellulose, relatively unusual in the polysaccharide world, is its crystalline structure [2]. The crystalline structure and insoluble nature of cellulose represents a formidable challenge for enzymatic hydrolysis [3]. Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere [2].

The complete degradation of cellulose is made by a cellulolytic enzyme system. It has been established that there are three main types of enzymes found in the cellulase system that can degrade cellulose: exo- β -1,4-glucanase, endo- β -1,4-glucanase and β -glucosidase. The endoglucanases act internally on the chain of cellulose cleaving β -linked bond liberating non-reducing ends, and exoglucanases remove cellobiose from this non-reducing end of cellulose chain. Finally, β -glucosidase completes the saccharification by splitting cellobiose and small cello-oligosaccharides to glucose molecule [4].

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Carboxymethyl cellulase (endo- β -1,4-glucanase EC 3.2.1.4) is one of the component enzymes of the cellulase system which hydrolyzes 1,4- β -D-glucosidic bonds within the cellulose molecules [5]. Carboxymethyl cellulase (CMCase) activity has been found in a variety of fungi and bacteria such as *Trichoderma longibrachiatum* [3], *Aspergillus niger* [6], *Aspergillus japonicus* [7], *Trichoderma reesei* [8], *Paenibacillus* [9], *Sinorhizobium fredii* [10], *Bacillus cereus* [11], *Myxobacter* [12], *Bacillus circulans* [13], *Cellulomonas flavigena* [14], *Bacillus megaterium* [15], *Rhizobium leguminosarum* [16], *Clostridium thermocellum* [17-18], *Thermomonospora fusca* [19] and *Cellulomonas uda* [20]. Different agricultural wastes have been studied for CMCase production of cellulolytic microorganisms. *Thermoascus aurantiacus* produced the highest levels of CMCase in corncob, grasses and corn straw [4]. *Trichoderma viride* showed CMCase activity in mixed substrates of rice straw and wheat bran [21].

In this work, CMCase of the selected bacteria including *B. subtilis*, *E. coli* and *Rhizobium* sp. was characterized. Growth of bacteria and production of CMCase were studied by using carboxymethyl cellulose (CMC) as a sole carbon source and different agricultural wastes as substrates. The information can be used for the application of agricultural wastes as plentiful and cheap sources for microbial growth aiming at the production of enzyme. Thus the disposal of agricultural waste will be reduced.

2. MATERIALS AND METHODS

2.1 Microorganisms and culture condition

B. subtilis ATCC 6633 and *E. coli* ATCC 25922 were obtained from National Institute of Health, Ministry of Public Health, Thailand. A collection of root-nodule bacteria was obtained from Soil Microbiology Research Group, Division of Soil Science, Department of Agriculture, Ministry of Agriculture and Cooperative. Unless indicated otherwise, *B. subtilis* and *E. coli* were cultured in NB or NA at 37°C. Root-nodule bacteria were cultured in Yeast Mannitol Agar (YMA) or Yeast Mannitol Broth (YMB) [22] at 28°C.

2.2 Sequence analysis of partial 16S rDNA

To identify genus of root-nodule bacterium, the 16S rDNA gene was analyzed as described elsewhere [23]. The purified PCR product was sequenced by Macrogen, Korea. The nucleotide sequence was aligned using BLASTN (<http://www.ncbi.nlm.nih.gov>).

2.3 Plate assay

Bacteria collected were screened for CMCase production by point-inoculation on CMC agar plates [24]. After colonies were formed, plate assays were done as described elsewhere [24]. CMCase production was observed as clearing (reduction of staining) of congo red at the position of colonies. Index values were defined as diameter of clearings (cm.) divided by diameter of colonies (cm.).

2.4 CMCase assay

To determine whether CMCase produced from each strain is extracellular enzyme or cell-bound enzyme, bacteria were allowed to grow in CMC broth [24] until they reached an OD₆₀₀ of 1.0. Cell cultures were precipitated by centrifugation. Cell pellets were washed, resuspended in distilled water and disrupted by ultrasound at 50 amplitude for 10 min. Cell-free supernatant and disrupted cell suspension were assayed for CMCase activity. CMCase activities were assayed during different time intervals by incubating samples with CMC at 37°C for 20 min. Reactions were terminated by heating in water bath at 60°C for 25 min, followed by cooling within 5 min. in

cold water bath. Reducing sugars in samples before incubating with CMC and reducing sugars after incubating with CMC were measured by the method of Bergmeyer [25]. The absorbance at 710 nm was measured. The concentration of reducing sugars was determined by comparison with a glucose standard curve. One unit of CMCase activity is defined as μmole glucose equivalent liberated per min. under assay conditions.

2.5 Growth of selected bacteria in CMC broth

Each strain of bacteria was cultured and used as inoculum. The total cell count in inoculum was examined by the standard plate count method. Cell culture was precipitated by centrifugation, washed, resuspended in sterilized water and inoculated into CMC broth [24]. The initial cell number of each strain was 1.00×10^6 CFU/ml. The cell number was measured during different time intervals by the standard plate count method. CMCase activity was measured during each interval as described above.

2.6 CMCase production during the growth of selected bacteria in agricultural wastes

Growth and CMCase production of each strain were assayed as described above except that agricultural wastes were substituted instead of CMC in medium at the equal concentration (10 g/l of medium). All agricultural wastes were ground into powder using a blender. Agricultural wastes used in this study included corncobs, pineapple peels and vegetable residues. The amounts of reducing sugar in media containing each agricultural waste were measured by the method of Bergmeyer [25].

2.7 Statistical analyses

Experimental data were compared by the SPSS program version 13.0 (SPSS Inc., Chicago, IL).

3. RESULTS AND DISCUSSION

3.1 Plate assay

CMC degradation was observed on CMC agar plates inoculated with *B. subtilis*, *E. coli* and root-nodule bacteria DASA23010. The clearing occurred only below colonies, suggesting that the enzyme remain attached to colonies. The index values obtained from *B. subtilis*, *E. coli* and DASA23010 were 1.07, 1.00 and 1.00, respectively. The clearing on CMC agar plates after staining was shown in Figure 1.

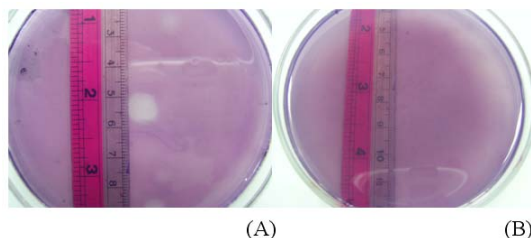


Figure 1 Clearing on CMC agar plates after staining indicates CMC degradation (A) as compared with control without CMC degradation (B).

3.2 Sequence analysis of partial 16S rDNA

The 16S rDNA sequence of root-nodule bacteria DASA23010 indicates high homology (87%-89%) with members of *Rhizobium/Agrobacterium* group. The morphological characteristics were tested with carbol-fuchsin staining, growth in NA medium and YM medium containing 25 µg/ml congo red. The strain DASA23010 was identified as *Rhizobium* sp. based on the 16S rDNA sequence and the morphological characteristics. The 16S rDNA sequence of *Rhizobium* sp. DASA23010 has been deposited in GenBank under accession number FJ755842.

3.3 CMCase assay

CMCase activity was measured in cell-free supernatant (extracellular enzyme) and disrupted cell pellets (cell-bound enzyme). Extracellular CMCase activity was calculated as mU/ml supernatant and cell-bound CMCase activity was calculated as mU/mg cell. CMCase activities of *B. subtilis*, *E. coli* and *Rhizobium* sp. are shown in Figures 2-4, respectively. CMCase activity was observed in disrupted cell pellets of all strains. Maximum production of CMCase was detected during the exponential phase. The maximum CMCase activities in *B. subtilis*, *E. coli* and *Rhizobium* sp. were 40.4 ± 0.9 , 41.1 ± 0.0 , and 40.2 ± 0.4 mU/mg cell, respectively. The highest activities obtained from all strains were not significantly different. There was no CMCase activity in cell-free supernatant from all strains. Therefore CMCase produced from all three strains was characterized as cell-bound enzyme. This is consistent with the results obtained from plate assays in which clearing occurred only below colonies, suggesting that the enzyme remain attached to the bacteria [16]. The CMCase activity in *C. thermocellum* and three cellulolytic isolates has been reported to be paralleled to cell growth [18]. Cell-bound CMCase has been reported in various organisms such as *R. leguminosarum* [16, 26] and *T. reesei* [8]. While extracellular CMCase has been reported in *Bacillus* sp. [27].

3.4 Growth of selected bacteria in CMC broth

Growth in CMC broth was observed in all strains. The results indicate that these bacteria could utilize CMC as a sole carbon source. The maximum cell numbers of *B. subtilis*, *E. coli* and *Rhizobium* sp. were $1.30 \times 10^9 \pm 2.00 \times 10^8$, $1.86 \times 10^9 \pm 4.73 \times 10^7$ and $2.10 \times 10^9 \pm 1.00 \times 10^8$ CFU/ml, respectively. The growth curves of *B. subtilis*, *E. coli* and *Rhizobium* sp. are shown in Figures 2-4, respectively.

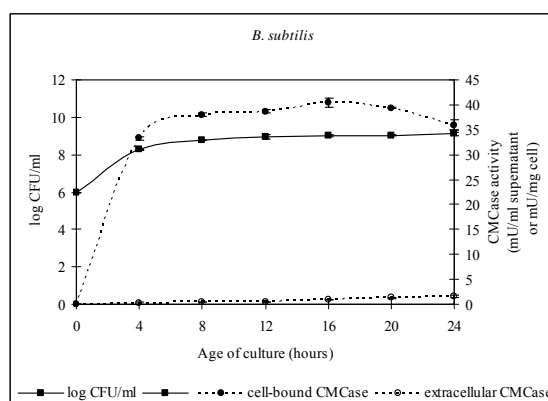


Figure 2 Growth curve and CMCase activity of *B. subtilis* in CMC broth. The values shown are the mean value of 3 replicates. Error bars indicate standard deviation.

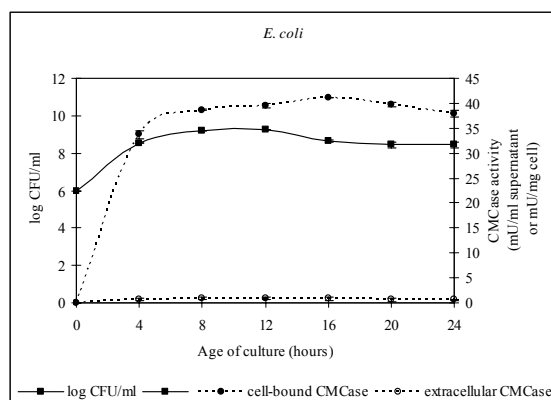


Figure 3 Growth curve and CMCase activity of *E. coli* in CMC broth. The values shown are the mean values of 3 replicates. Error bars indicate standard deviation.

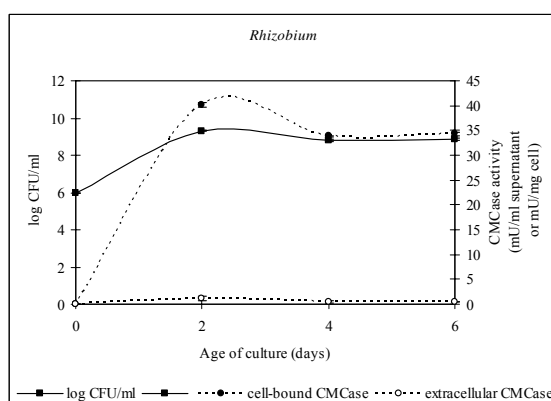


Figure 4 Growth curve and CMCase activity of *Rhizobium* sp. in CMC broth. The values shown are the mean values of 3 replicates. Error bars indicate standard deviation.

3.5 CMCase production during the growth of selected bacteria in agricultural wastes

Growth of each organism was determined in different agricultural wastes. The similar tendency was observed in all organisms. Growth and cell-bound CMCase activity of *B. subtilis*, *E. coli* and *Rhizobium* sp. in agricultural wastes are shown in Figures 5-7, respectively. Maximum cell numbers were obtained from the medium containing pineapple peels. The maximum cell numbers of *B. subtilis*, *E. coli* and *Rhizobium* sp. were $6.50 \times 10^8 \pm 2.00 \times 10^7$, $2.88 \times 10^9 \pm 2.52 \times 10^7$ and $2.11 \times 10^9 \pm 3.51 \times 10^7$ CFU/ml, respectively while minimum cell numbers were obtained from the medium containing corncob. Contrary to cell growth, the highest activity of *B. subtilis* and *Rhizobium* sp. was obtained from cells grown in corncob whereas the highest activity of *E. coli* was obtained from cells grown in corncob and pineapple peels. The highest activities of *B. subtilis*, *E. coli* and *Rhizobium* sp. were 15.6 ± 0.2 , 3.6 ± 0.1 and 12.7 ± 0.1 mU/mg cell, respectively. Maximum production of CMCase was detected during the stationary phase while CMCase production was very low during the lag phase to the mid-log phase of growth. Even though

corncob could stimulate the highest enzyme activity in all organisms, their growth in corn cob was slightly lower than that in vegetable residues. One explanation is that when these agricultural wastes were used as substrate, the difference in cell growth is affected by various factors such as the presence of activator or inhibitor, vitamins or growth factors, especially sugar content in these substrates. Sugar content affected CMCase production because bacteria could utilize sugar residues in agricultural wastes as carbon source. Thus it is possible that the production of cellulolytic enzyme including CMCase in agricultural wastes would be suppressed. The initial amounts of reducing sugars in the media containing pineapple peels, corn cobs and vegetable residues were 340.35 ± 7.06 , 209.58 ± 2.51 and 129.10 ± 0.80 $\mu\text{g/ml}$, respectively. The results of cell growth in agricultural wastes showed the efficacy of applying these agricultural wastes as plentiful and cheap media for biomass production that will benefit the utilization of these abundant agricultural wastes. In addition, *B. subtilis* and *Rhizobium* sp. are well known for their plant-growth promoting ability. These agricultural wastes can also be employed as carriers and/or nutrient source for biofertilizer production with these plant-growth promoting rhizobacteria.

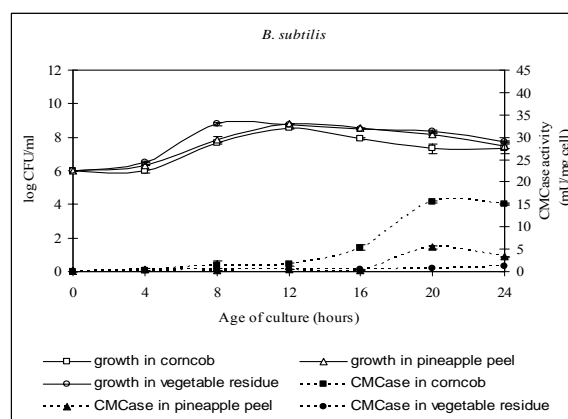


Figure 5 Growth curve and CMCase activity of *B. subtilis* in agricultural wastes. The values shown are the mean values of 3 replicates. Error bars indicate standard deviation.

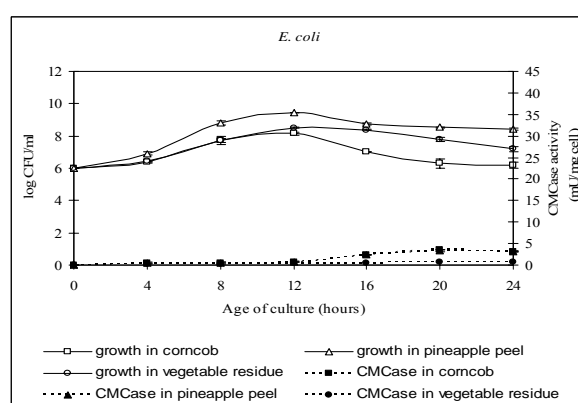


Figure 6 Growth curve and CMCase activity of *E. coli* in agricultural wastes. The values shown are the mean values of 3 replicates. Error bars indicate standard deviation.

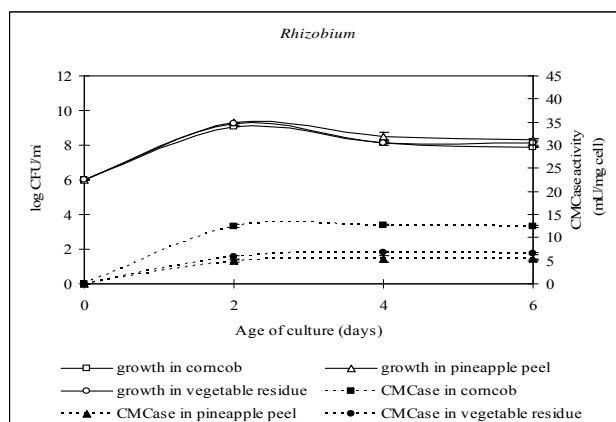


Figure 7 Growth curve and CMCase activity of *Rhizobium* sp. in agricultural wastes. The values shown are the mean values of 3 replicates. Error bars indicate standard deviation.

4. CONCLUSIONS

B. subtilis, *E. coli* and *Rhizobium* sp. produced cell-bound CMCase and utilized CMC as a sole carbon source. Agricultural wastes, including corncob, pineapple peels and vegetable residues, could be degraded and used as cheap sources for microbial growth.

5. ACKNOWLEDGEMENTS

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