CHAPTER IV
RESULTS AND DISCUSSION

4.1 Nomenclature and Classification of Termites

Termites used in the experiments are classified in two types—lower termites and higher termites.

The lower termites are

Class *Insectra*

Order *Isoptera*

Family *Rhinotermitidae*

Subfamily *Rhinotermitidae*

Genus *Schedorhinotermes sp.*

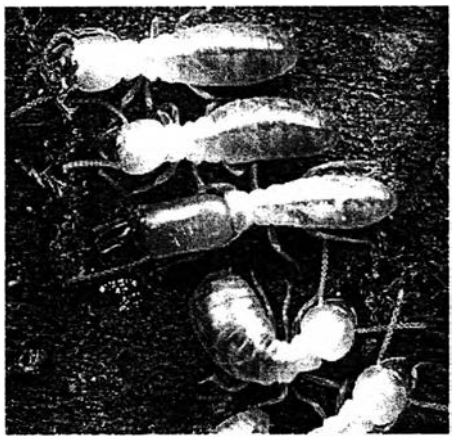


Figure 4.1 Lower termites, *Schedorhinotermes sp.*(tolweb.org).

The higher termites are

Class *Insectra*

Order *Isoptera*

Family *Termitidae*

Subfamily *Termitidae*

Genus *Microcerotermes sp.*

Figure 4.2 Higher termites, *Microcerotermes sp.*(safeguardtb.com.au).

4.2 Screening of Cellulose-Degrading Bacteria

4.2.1 Isolation of Cellulase-Producing Bacteria from Termites

The pure isolates from both dead and living lower termites, *Schedorhinotermes sp.*, are 7 and 8 isolates, respectively, as shown in Table 4.1

Table 4.1 Number of pure isolates and number of clear-zone producing isolates from *Schedorhinotermes sp.*

Condition of lower termite	Number of pure isolates	Number of clear-zone* producing isolates
Dead termite	7	0
Living termite	8	0
Total	15	0

Clear-zone* = Clear zone around colony on carboxymethyl cellulose agar, CMC agar

Bacterial isolates from *Microcerotermes sp.*, higher termite, are isolated using 3 conditions: aerobic; aerobic with anaerobic pretreatment step; and 2 steps anaerobic. The number of pure isolates from each condition is shown in Table 4.2.

Table 4.2 Number of pure isolates and number of clear-zone producing isolates from *Microcerotermes sp.*

Isolation condition	Number of pure isolates	Number of clear-zone* producing isolates
Aerobic	13	7
2 steps anaerobic	20	19
Aerobic with anaerobic pretreatment step	24	21
Total	57	47

Clear-zone* = Clear zone around colony on 65 modified DSMZ agar medium 3

In the preliminary step, all pure isolates from both types of termites are classified as the cellulase-producing bacteria by detecting the clear-zone, which appears around the colony, after flooding with 0.1 wt./vol.% congo red solution and

0.1 M NaCl. The residual CMC is absorbed by congo red solution, but degraded CMC is not absorbed and appears as a clear-zone (Teather and Wood, 1982).

Table 4.1 shows the preliminary results from the lower termites. All pure isolates from the lower termites do not show any clear-zone. Thus, the pure isolates from these termites could not degrade the cellulose. These results confirm the results from literature, which supports that the lower termites do not use the bacteria for degrading the cellulose, but use protozoa to degrade cellulose to glucose (Inoue *et al.*, 1997; Nakashima *et al.*, 2002; Wheeler *et al.*, 2007).

The number of clear-zone producing isolates from the higher termites is shown in Table 4.2. In each condition of isolation, some pure isolates have the potential to degrade CMC and produce the clear-zone. These results can again be confirmed by previous literatures (Schafer *et al.*, 1996; Bakalidou, 2002; and Wenzel *et al.*, 2002).

4.3 Determination of Hydrolysis Capacity Value (HC value)

The cellulase-producing bacteria from higher termites are determined for the HC value by calculating the ratio of the diameter of the appeared clear-zone and that of the bacterial colony. Figures 4.3 and 4.4 show the colony of isolate strain F 002 before and after detecting the clear-zone.

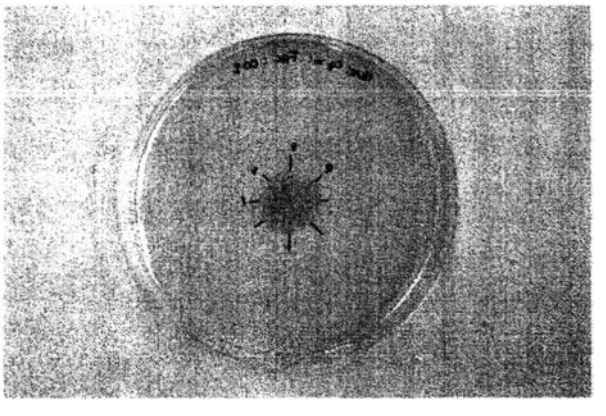


Figure 4.3 Bacterial colony of isolate strain F 002 on 65 modified DSMZ agar medium 3 before detecting the clear-zone.

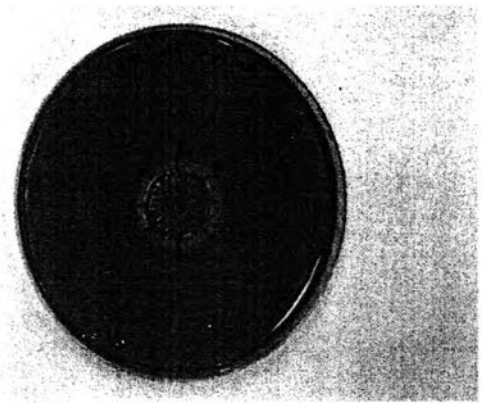


Figure 4.4 Clear-zone around the bacterial colony of isolate strain F 002 on 65 modified DSMZ agar medium 3 after flooding by 0.1 wt./vol.% congo red solution and 0.1 M NaCl.

The HC values of the cellulase-producing bacteria from higher termites, which are classified by isolation conditions, are shown in Tables 4.3–4.5.

Table 4.3 HC values of cellulase-producing bacteria isolated by the aerobic condition

Number of isolates	HC value
A 002	**2.74**
A 005	1.71
A 007	1.23
A 009	2.38
A 011	1.28
A 012	2.52
A 013	1.59

The highest HC value of this condition is 2.74 from isolate strain A 002.

Table 4.4 HC values of cellulase-producing bacteria isolated by 2 steps anaerobic condition

Number of isolates	HC value
M 001	2.46
M 002	2.38
M 003	1.75
M 004	1.79
M 005	1.27
M 006	2.07
M 007	2.32
M 008	2.31
M 010	2.91
M 011	1.78
M 012	1.60
M 013	1.78
M 014	1.82
M 015	**3.67**
M 016	2.14
M 017	3.52
M 018	2.53
M 019	2.47
M 020	1.66

The highest HC value of this condition is 3.67 from isolate strain M 015.

Table 4.5 HC values of cellulase-producing bacteria isolated by aerobic with anaerobic pretreatment step condition

Number of isolates	HC value
F 001	2.35
F 002	1.55
F 003	2.05
F 004	1.86
F 005	1.78
F 006	2.19
F 007	1.43
F 008	2.12
F 009	2.42
F 012	1.89
F 013	2.00
F 015	2.37
F 016	2.40
F 017	2.52
F 018	**2.53**
F 019	1.50
F 020	2.09
F 021	1.50
F 022	2.09
F 023	1.81
F 024	2.25

The highest HC value of this condition is 2.53 from isolate strain F 018.

The HC values are related to the activity of endoglucanase enzyme, which controls the cellulose hydrolysis rate in the primary hydrolysis step (Zhang *et al.*, 2006). Thus, the HC value is used as a tool for determining the high potential cellulase-producing bacteria.

From the HC value determination, the pure isolates: strain A 002; M 015; and F 018; have the highest HC values. Thus, these isolates are considered as the effective isolates, which will be used to determine the cellulase enzyme activities, their tolerance to the presence to ionic liquid, and genus identification of the bacteria. The effective isolates from each condition are summarized in Table 4.6.

Table 4.6 Effective isolates classified by the isolation conditions

Isolation condition	Effective isolate
Aerobic	A 002
2 steps anaerobic	M 015
Aerobic with anaerobic pretreatment step	F 018

Figures 4.5–4.7 show the three effective isolates (strain A 002, M 015, and F 018) on 65 modified DSMZ agar medium 3.

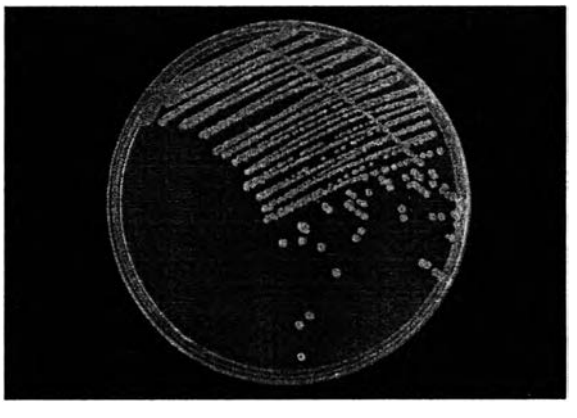


Figure 4.5 Isolate strain A 002.

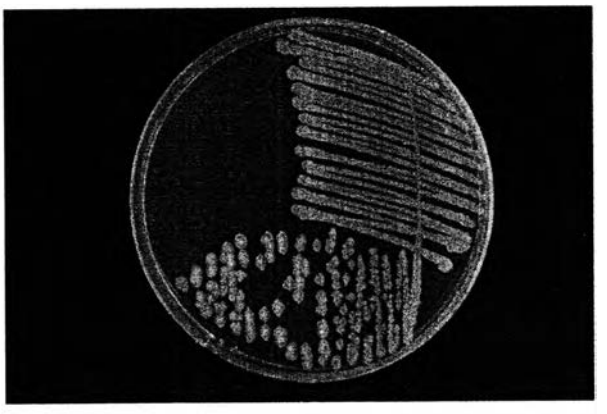


Figure 4.6 Isolate strain M 015.

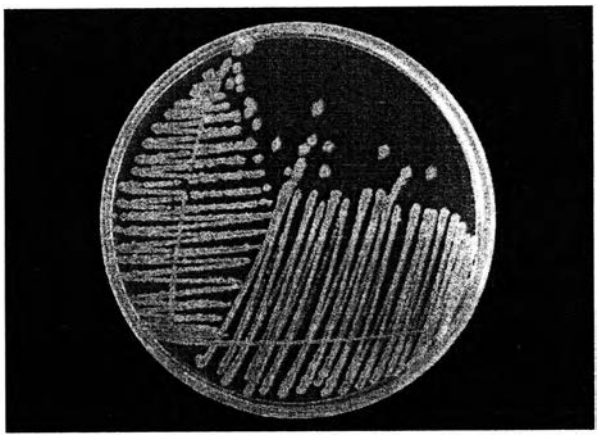


Figure 4.7 Isolate strain F 018.

4.4 Specific Cellulase Enzyme Activity Assay

The effective isolates—strain A 002, M 015, and F 018—are determined for their specific cellulase enzyme activities by culturing the isolates in the production mediums (65 modified DSMZ broth medium 3) for 24 hours at various temperatures (37, 40, 45, and 50°C).

4.4.1 Effect of Temperature on Specific Cellulase Enzyme Activity of Cellulase-Producing Bacteria

4.4.1.1 Strain A 002

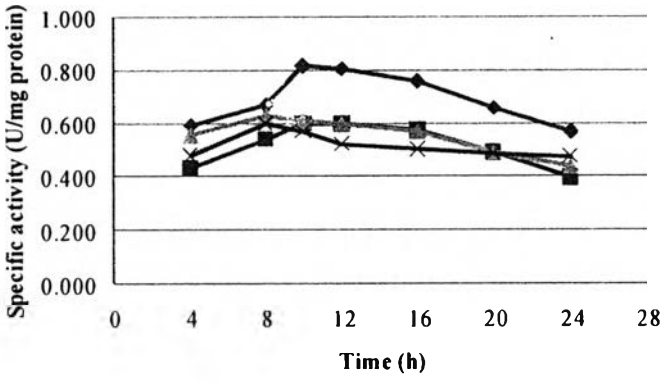
Specific cellulase activities of strain A 002 are cultured at various temperatures and shown in Figure 4.8.

The specific endoglucanase activity profiles of each temperature increase until reaching the maximum values and gradually decrease with time. The highest specific endoglucanase activities at 37, 40, 45, and 50°C are 0.814, 0.598, 0.625, and 0.598 U/mg protein, respectively.

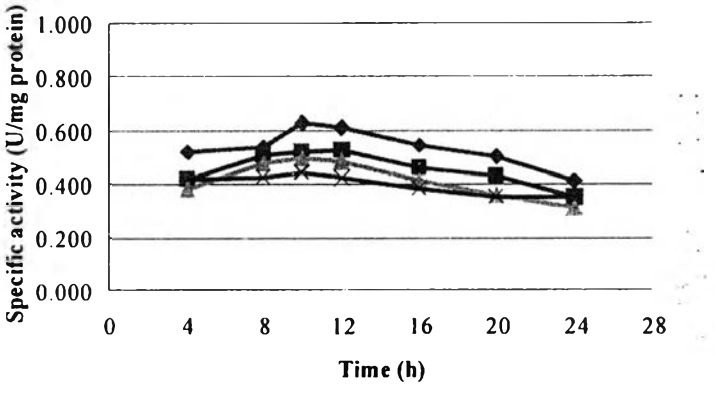
The specific exoglucanase activity profile of this strain at 37°C has the similar trend to the specific endoglucanase activity profile. At 40 and 45°C, the profiles also gradually increase from 4 hours and reach the maximum value before gradual decreasing with time as 37°C, but the activities are lower. At 50°C, the profile is nearly constant in the first 12 hours and slightly decreases with time. The highest specific exoglucanase activities at 37, 40, 45, and 50°C are 0.626, 0.529, 0.497, and 0.445 U/mg protein, respectively.

The specific β-glucosidase activity profiles at each temperature are also similar to the specific exoglucanase activity profiles. The highest specific β-glucosidase activities at 37, 40, 45, and 50°C are 0.620, 0.555, 0.523, and 0.421 U/mg protein, respectively.

(a)



(b)



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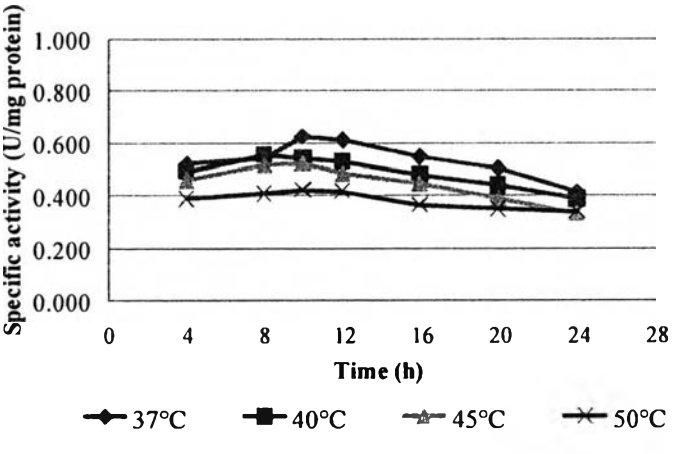


Figure 4.8 Specific cellulase enzyme activity of strain A 002 at pH 7.2 at various temperatures (37, 40, 45, and 50°C): (a) specific endoglucanase activity, (b) specific exoglucanase activity, and (c) specific β-glucosidase activity.

4.4.1.2 Strain M 015

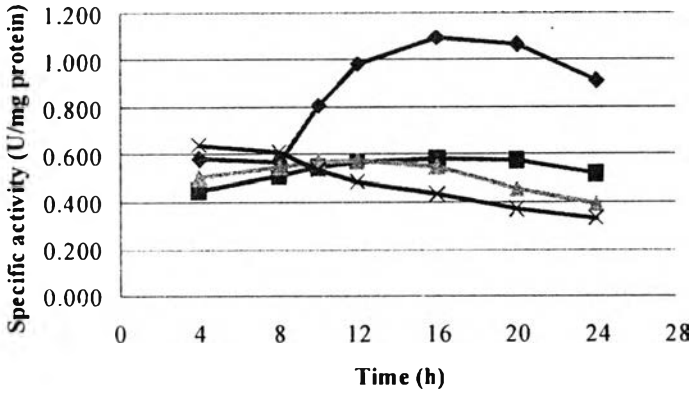
Specific cellulase activities of strain M 015 at different temperatures are shown in Figure 4.9.

Different profiles of the specific endoglucanase activity of this strain are observed. At 37°C, a slight decrease in the specific activity is observed in the first 8 hours. The profile then sharply increases to the maximum value at 16 hours and gradually drops with time. At 40°C, the increase in the profile is observed within 16 hours, and after that there is a gradual decrease. At 45°C, the profile increases within 12 hours and gradually decreases after reaching the highest specific activity. The profile at 50°C is different from the other profiles. At this condition, the highest activity is observed at 4 hours, and after that the specific activities decrease with time. The highest specific endoglucanase activities at 37, 40, 45, and 50°C are 1.098, 0.581, 0.577, and 0.642 U/mg protein, respectively.

The specific exoglucanase activity profile at 37°C drops before a gradual increase to the highest activity. At 40 and 45°C, the similar profiles are observed, and at 50°C, the specific activity profile is gradual drop with time. The highest specific exoglucanase activities at 37, 40, 45, and 50°C are 0.532, 0.458, 0.454, and 0.457 U/mg protein, respectively.

The specific β-glucosidase activity profile at 37°C is different from the other temperatures. At 37°C, a slight decrease is observed in the first 8 hours before reaching the maximum value, but the those at the other temperatures are nearly constant before a gradual drop with time. The specific β-glucosidase activities at 37, 40, 45, and 50°C are 0.516, 0.479, 0.475, and 0.423 U/mg protein, respectively.

(a)



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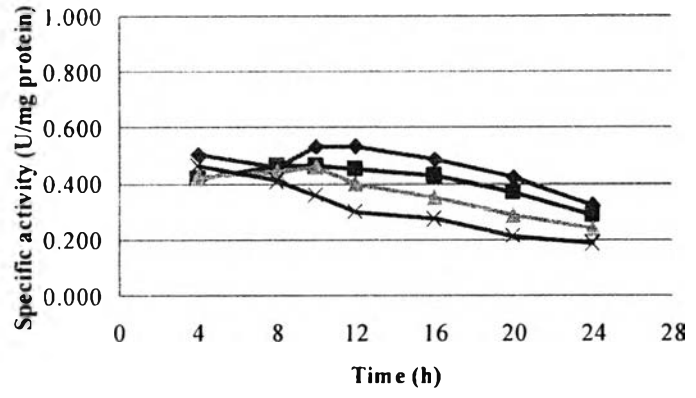

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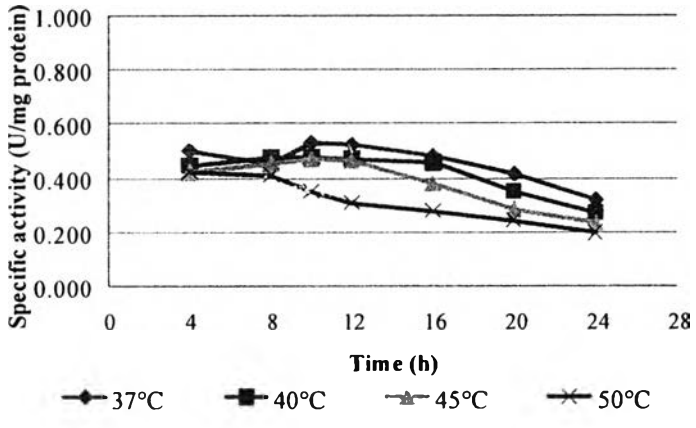


Figure 4.9 Specific cellulase enzyme activity of strain M 015 at pH 7.2 at various temperatures (37, 40, 45, and 50°C): (a) specific endoglucanase activity, (b) specific exoglucanase activity, and (c) specific β-glucosidase activity.

4.4.1.3 Strain F 018

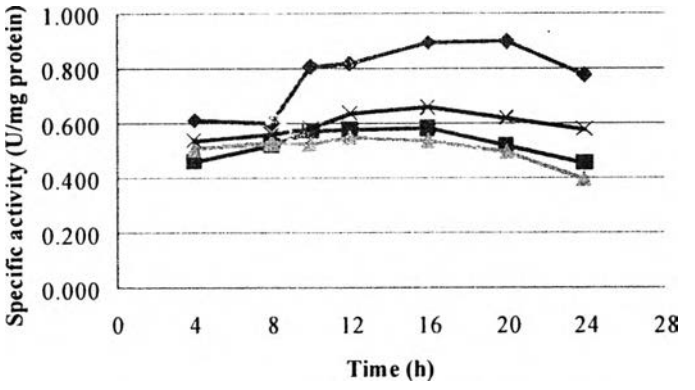
Specific cellulase activities of strain F 018 at different temperatures are shown in Figure 4.10.

The specific endoglucanase activities remain relatively constant in the first 8 hours at 40 to 50°C, and they gradually increase until reaching the maximum value. In contrast, at 37°C, the profile sharply increases to the peak and drops after 20 hours. The highest specific endoglucanase activities at 37, 40, 45, and 50°C are 0.900, 0.582, 0.551, and 0.639 U/mg protein, respectively

The specific exoglucanase activity at 37°C drops before a sharp increase to the highest value as the strain M 015. At 40 and 45°C, the similar profiles are observed, and at 50°C, the specific activity profile is nearly constant after 10 hours. The highest specific exoglucanase activities at 37, 40, 45, and 50°C are 0.684, 0.557, 0.501, and 0.519 U/mg protein, respectively.

The specific β-glucosidase activity profiles are different at the studied temperatures. At 40°C, the sharp increase within 10 hours is observed before reaching the maximum value. However, in the case of 37°C, the profile drops from 4 to 8 hours before a sharp increase to the maximum value. At 45 and 50°C, the profiles slightly increase within the first 12 hours before dropping with time. The highest β-glucosidase activities at 37, 40, 45, and 50°C are 0.673, 0.606, 0.540, and 0.473 U/mg protein, respectively.

(a)



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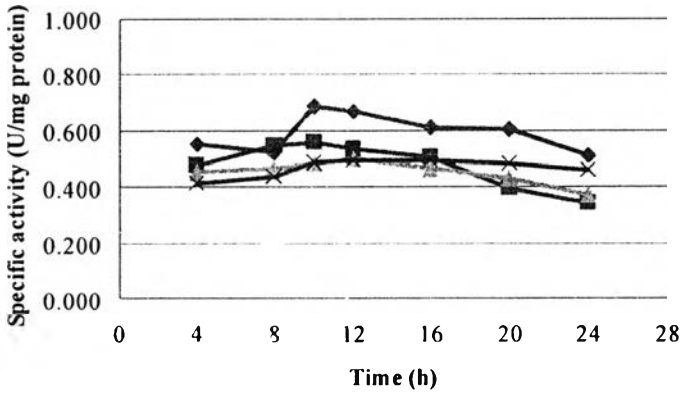

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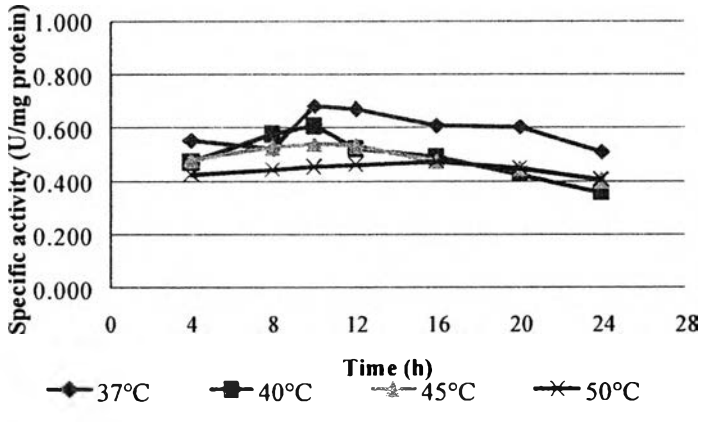

Figure 4.10 Specific cellulase enzyme activity of strain F 018 at pH 7.2 at various temperatures (37, 40, 45, and 50°C): (a) specific endoglucanase activity, (b) specific exoglucanase activity, and (c) specific β-glucosidase activity.

4.4.2 Comparison of Specific Cellulase Enzyme Activity between Three Effective Isolates at the Optimum Temperature

The optimum temperature, where all isolates have the highest specific cellulase enzyme activity, is 37°C. At this temperature, the cellulase enzyme activities of all isolates are compared to determine a strain that has the highest specific endoglucanase activity, specific exoglucanase activity, and specific β-glucosidase activity.

4.4.2.1 Specific Endoglucanase Activity

The specific endoglucanase activity profiles of the effective isolates at the optimum temperature are shown in Figure 4.11. At 37°C, strain M 015 has the highest specific endoglucanase activity as compared to strain F 018 and strain A 002. The specific endoglucanase activity of strain M 015 slightly decreases from 4 to 8 hours. After that, it sharply increases until reaching the maximum value, 1.098 U/mg protein, at 16 hours and then sharply decreases.

The specific endoglucanase activity profile of strain F 018 is similar to that of strain M 015. The specific endoglucanase activity profile of strain F 018 slightly drops from 4 to 8 hours. Then, it gradually increases until reaching the maximum value, 0.900 U/mg protein, at 20 hours and then gradually drops with time.

The specific endoglucanase activity profile of strain A 002 is different from that of strain M 015 and strain F 018. The profile sharply increases and reaches the maximum value at 10 hours about 0.814 U/mg protein before decreasing with time.

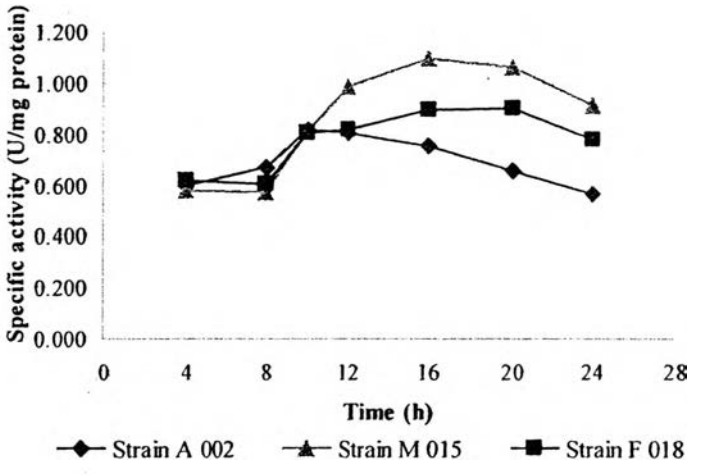

Figure 4.11 Comparison of specific endoglucanase activity between strain A 002, M 015, and F 018 at 37°C, and pH 7.2 for 24 hours.

4.4.2.2 Specific Exoglucanase Activity

The specific exoglucanase activity profiles of all strains at the optimum temperature are shown in Figure 4.12. The similar profiles of all effective strains are observed. The specific exoglucanase profiles of strain F 018 and M 015 slightly decrease from 4 to 8 hours. In contrast, strain A 002 slightly increases. All strains reach the maximum specific activity at 10 hours, and the activities slowly decline until 24 hours. The maximum specific activity values of strain F 018, A 002, and M 015 are 0.684, 0.626, and 0.532 U/mg protein, respectively.

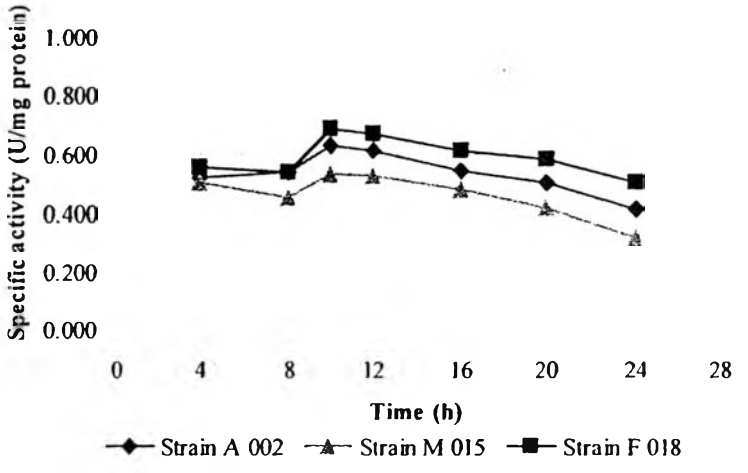

Figure 4.12 Comparison of specific exoglucanase activity between strain A 002, M 015, and F 018 at 37°C and pH 7.2 for 24 hours.

4.4.2.3 Specific β-glucosidase Activity

The specific β-glucosidase activity profiles of all strains at the optimum temperature are shown in Figure 4.13. The shapes of the specific β-glucosidase activity profiles of all strains are similar to the specific exoglucanase activity profiles, as shown in Figure 4.12. For the first 8 hours, the specific β-glucosidase activity of all strains drops, but it rapidly increases to the maximum value. The maximum values of strain A 002 and M 015 are 0.620 and 0.504 U/mg protein, respectively, at 10 hours, but the highest activity of strain F 018 is 0.673 U/mg protein at 12 hours.

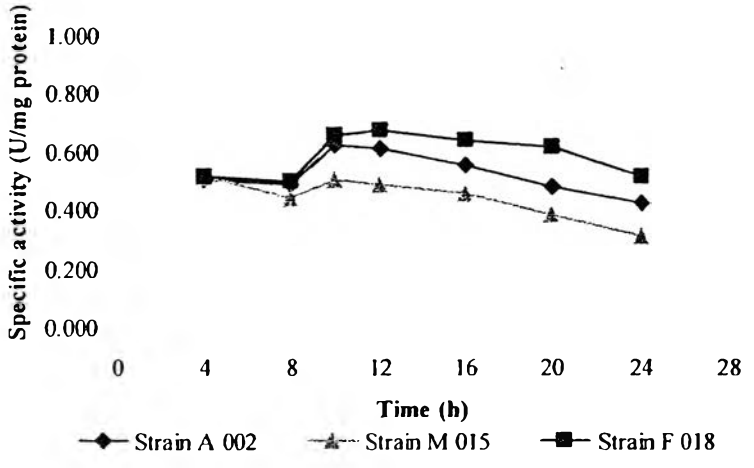

Figure 4.13 Comparison of specific β-glucosidase activity between strain A 002, M 015, and F 018 at 37°C for 24 hours.

4.5 Determination of the Specific Growth Rate (μ) and Tolerance of Cellulase-Producing Bacteria in the Presence of [BMIM]Cl

4.5.1 Tolerance of the Cellulase-Producing Bacteria in the Presence of [BMIM]Cl

The tolerance studies of the 3 effective isolates were investigated by tracking the bacterial growths in 65 modified DSMZ broth medium 2, which contains various concentrations of [BMIM]Cl (0, 0.1, 0.5, 1.0 , 5.0, and 10.0 vol.%) within 24 hours. The bacterial growth detection of each strain was performed by measuring the optical density at 550 nm and plots them as the growth curve of each strain.

Figures 4.14–4.16 show the growth curves of strain A 002, M 015, and F 018, which are the growth at the optimum temperature with various concentrations of [BMIM]Cl.

For strain A 002, it can tolerate [BMIM]Cl in the range of 0.1 to 1.0 vol.%, but it is dead when the concentration of [BMIM]Cl exceeds 5.0 vol.%. The growth retardation is observed in the lag phase when this strain is cultured in [BMIM]Cl at 0.5 and 1.0 vol.%. However, at 0.1 vol.%, no change in the lag phase is observed as compared to the culture, which did not contain [BMIM]Cl. The lag

phases of this strain, which are inoculated in 0.1, 0.5, and 1.0 vol.%, are 2, 6, and 11 hours, respectively.

For strain M 015, this strain also tolerates [BMIM]Cl at 0.1 to 1.0 vol.% and dies when the concentration of [BMIM]Cl exceeds 5.0 vol.% as strain A 002. However, this strain can rapidly adapt itself in the presence of the ionic liquid as no growth retardations are observed at any concentrations. The lag phases of this strain in the range of 0.1 to 1.0 vol.% are 2 hours.

The tolerance profiles of strain F 018 are similar to the strain M 015. This strain can tolerate the [BMIM]Cl in the range of 0.1 to 1.0 vol.% without retardation in the lag phases and it also dies when the concentrations of [BMIM]Cl is over 5.0 vol.%. The lag phases of this strain in the range of 0.1 to 1.0 vol.% are 2 hours.

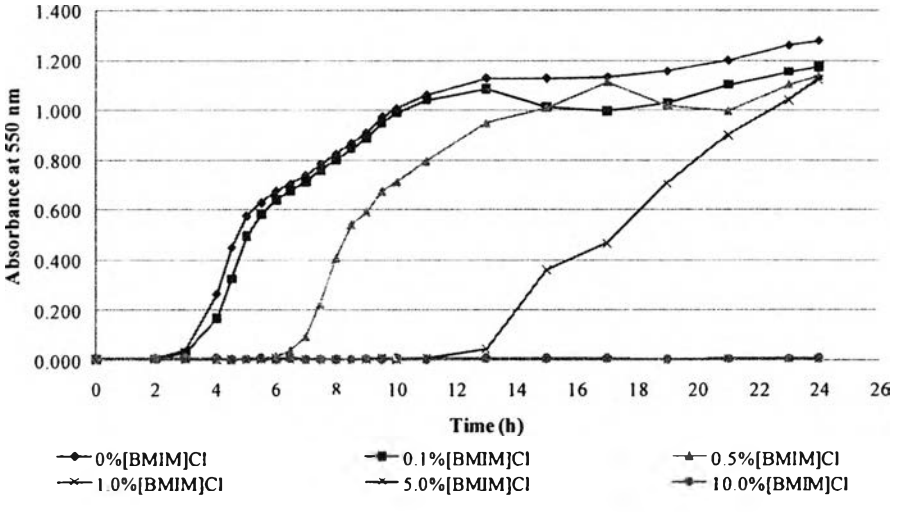

Figure 4.14 Growth curves at 37°C and pH 7.2 of strain A 002 at various concentrations of [BMIM]Cl.

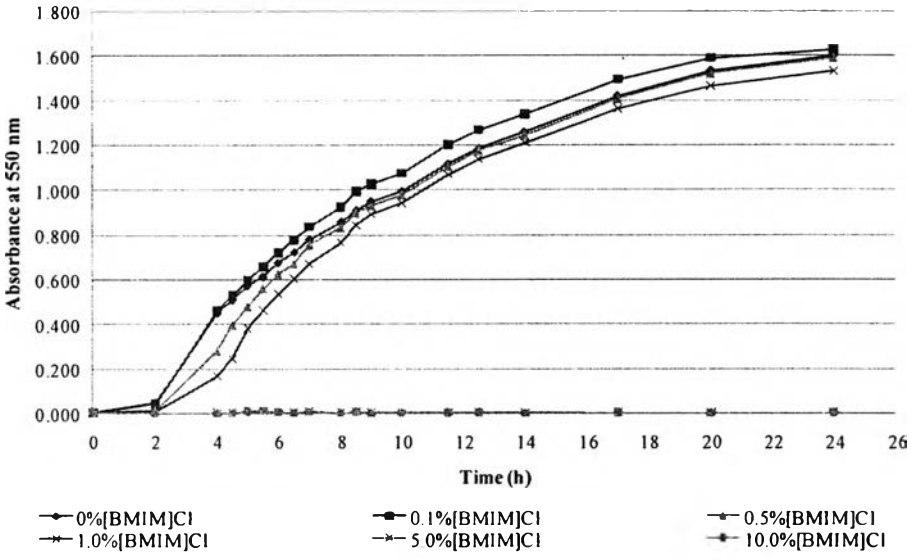

Figure 4.15 Growth curves at 37°C and pH 7.2 of strain M 015 at various concentrations of [BMIM]Cl.

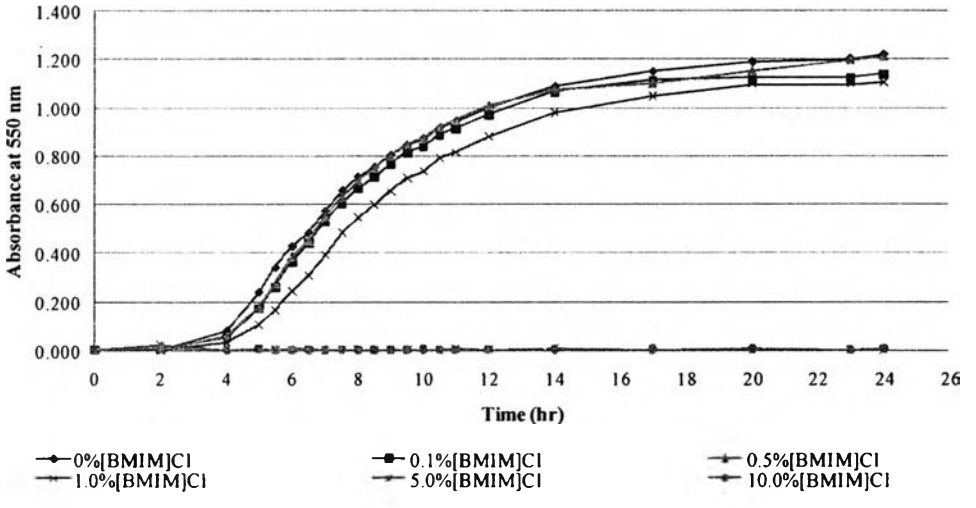

Figure 4.16 Growth curves at 37°C and pH 7.2 of strain F 018 at various concentrations of [BMIM]Cl.

4.5.2 Specific Growth Rate of Cellulase-Producing Bacteria in the Presence of [BMIM]Cl

From the tolerance studies, the results show that all strains can tolerate [BMIM]Cl in the range of 0.1 to 1.0 vol.% with some growth retardations in strain A 002, but no growth retardations are observed for strain M 015 and F 018. Moreover, the effect of these range of concentrations on the specific growth rate in the exponential phase of each strain was also investigated.

Figure 4.17 shows the specific growth rate profiles of each strain with the concentration of [BMIM]Cl in the range of 0.1 to 1.0 vol.%. The results show that the presence of [BMIM]Cl affects the specific growth rate of all strains. Without [BMIM]Cl, the specific growth rate of strain F 018 is the highest among three strains.

For strain F 018, [BMIM]Cl can enhance the specific growth rate when the concentrations are less than 0.5 vol.%, which is the optimum concentration. At this concentration, the specific growth rate is higher than the control without [BMIM]Cl about 1.032 folds. The sharp decrease in the specific growth rate is observed when the concentration is higher than 0.5 vol.%.

For strain M 015, the specific growth rate profile is similar to that of strain F 018. With 0.5 vol.% [BMIM]Cl, the specific growth rate of this strain is higher than the control about 1.144 folds. The slight decrease in the specific growth rates is observed when the concentration is increased. However, at 1.0 vol.% [BMIM]Cl, the specific growth rate is still higher than the specific growth rate of the control.

For strain A 002, [BMIM]Cl also enhances the specific growth rate of this strain as the other strains, but the optimum concentration is changed from 0.5 to 0.1 vol.%. The enhanced value is 1.044 folds compared with the control. In addition, the relatively constant of the specific growth rate is also observed when the concentration exceeds 0.1 vol.%.

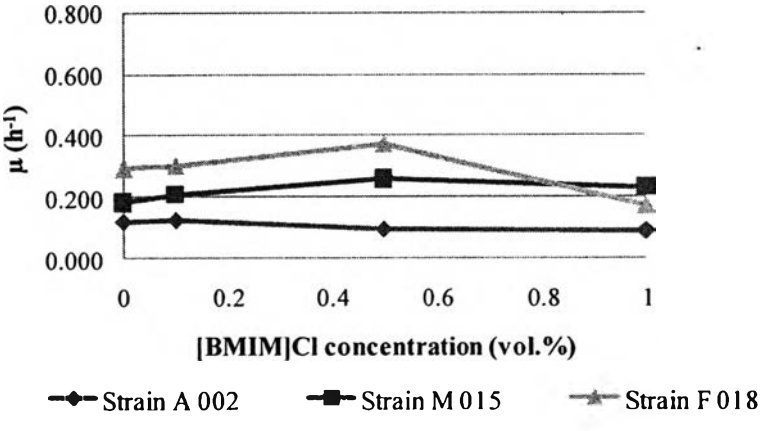

Figure 4.17 Relationships between specific growth rate (μ) of strain A 002, M 015, and F 018 with various concentrations of [BMIM]Cl.

4.6 Nomenclature and Classification of Bacteria from Determinative Bacteriology

4.6.1 Preliminary Identification by Microbiological Methods

The effective isolates—strain A 002, M 015, and F 018—are preliminary identified for their colonial appearance, pigmentation, cell shape, Gram's staining, spore forming, the oxidase test, and catalase test. The results are shown in Table 4.7.

Table 4.7 Preliminary identification of strain A 002, M 015, and F 018 by microbiological methods

Strain	Colonial Appearance	Pigmentation	Cell shape	Gram's staining	Spore forming	Oxidase test	Catalase test
A 002	Circular, flat, entire, rough,and membranous	Light brown cream	Rod	+	+	-	+
M 015	Spindle, raised, entire, glistering, and opaque	Light brown cream	Rod	+	+	-	+
F 018	Spindle, flat, filamentous, glistering, and opaque	Light green cream	Rod	+	+	-	+

4.6.2 Preliminary Identification by Biochemical Methods

The effective isolates—strain A 002, strain M 015, and strain F 018—are preliminarily identified for their genus and species by detecting the carbohydrate fermentations in the API 50 CHB kit. These isolates were inoculated in API 50 CHB medium, which contained an indicator, and then were cultured in different types of carbohydrates for 24 hours. The color of the indicator is changed when the carbohydrates are fermented to acids. In addition, these isolates are also tested with the biochemical tests of API 20 E kit for 24 hours. During the incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of a reagent. The biochemical characteristics from the API 50 CHB kit and API 20 E kit are used to identify the strain by identification software from Biomérieux, France. Tables 4.8 and 4.9 show biochemical characteristics of the three effective isolates.

The preliminary identification results from the Biomérieux software show that isolate strain A 002 is *Bacillus subtilis* or *Bacillus amyloliquefaction* with 99.9% identity; strain M 015 is *Bacillus subtilis* or *Bacillus amyloliquefaction* with 99.9% identity; and strain F 018 is *Brevibacillus non reactive* with 94.9% identity.

Table 4.8 API 50 CHB biochemical characteristics of three effective isolates

Carbohydrates	Strain A 002	Strain M 015	Strain F 018
Control	-	-	-
Glycerol	+	+	-
Erythritol	-	-	-
D-arabinose	-	-	-
L-arabinose	-	+	+
D-ribose	+	+	+
D-xylose	-	-	-
L-xylose	-	-	-
D-adonitol	-	-	-
Methyl-ßD-xylopyranoside	-	-	-
D-galactose	-	-	-
D-glucose	+	+	-
D-fructose	+	+	-
D-mannose	+	+	-
L-sorbose	-	-	-
L-rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	+	+	+
D-mannitol	+	+	-
D-sorbitol	+	+	-
Methyl-αD-mannopyranoside	-	-	-
N-acetylglucosamine	-	-	-
Amygdalin	-	+	-
Arbutin	+	+	-
Methyl-αD-glucopyranoside	-	+	-

(+) Color of indicator is changed due to carbohydrate fermentation.

(-) No change in color of indicator

Table 4.8 (Continued)

Carbohydrates	Strain A 002	Strain M 015	Strain F 018
Esculin ferric citrate	+	+	-
Salicin	+	+	-
D-cellobiose	-	+	-
D-maltose	-	+	-
D-lactose (bovine origin)	-	-	-
D-melibiose	-	-	-
D-saccharose (sucrose)	-	+	-
D-trehalose	-	+	-
Inulin	+	+	-
D-melezitose	-	-	-
D-raffinose	+	-	-
Amidon (starch)	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentiobiose	+	-	-
D-turanose	-	-	-
D-lyxose	-	-	-
D-tagatose	-	-	-
D-fucose	-	-	-
L-fucose	-	-	-
D-arabitol	-	-	-
L-arabitol	-	-	-
Potassium gluconate	-	-	-
Potassium 2-ketogluconate	-	-	-
Potassium 5-ketogluconate	-	-	-

(+) Color of indicator is changed due to carbohydrate fermentation.

(-) No change in color of indicator

Table 4.9 API 20 E biochemical characteristics of three effective isolates

Active ingredients	Strain A 002	Strain M 015	Strain F 018
2-Nitrophenyl-ßD-galactopyranoside	+	+	+
L-arginine	-	-	-
L-lysine	-	-	-
L-ornithine	-	-	-
Trisodium citrate	+	+	-
Sodium thiosulfate	-	-	-
Urea	-	-	-
L-tryptophane	-	-	-
L-tryptophane	-	-	-
Sodium pyruvate	+	+	+
Gelatin (bovine origin)	+	+	+

(+) Change in color

(-) No change in color

4.6.3 DNA Base Composition

DNA of the three effective strains is extracted by using the DNA Simax Kit (Beijing SBS Genetech Co., Ltd., China). The polymerase chain reaction (PCR) products of all isolates are purified and sequenced the 16S rDNA by Macrogen Inc. (Souel, South Korea). The 16S rDNA sequencing results of all isolated are compared with the 16S rDNA gene sequences, which are available in the BLAST database software.

4.6.3.1 16S rDNA of Strain A 002

The nucleotides of strain A 002 have the important characteristics as follows,

Locus: FJ613581, 1177 bp DNA linear

Definition: Bacillus subtilis

Accession: FJ613581

Reference: 2 (bases 1 to 1177)

Authors: Rhee,Y.H., Vendan,R.T. and Lee,S.H.

Title: Direct submission

Journal: Submitted (08-JAN-2009) Microbiology, Chungnam National University, 79 Daehangno, Yuseong-gu, Daejeon 305–764, Korea.

Origin:

1 atgcgggtgc tatacatgca agtcgagcgg acagatggga gcttgctccc tgatgttagc

61 ggcggacggg tgagtaacac gtgggtaacc tgcctgtaag actgggataa ctccgggaaa

121 ccggggctaa taccggatgg ttgtttgaac cgcatggttc aaacataaaa ggtggcttcg

181 gctaccactt acagatggac ccgcggcgca ttagctagtt ggtgaggtaa cggctcacca

241 aggcaacgat gcgtagccga cctgagaggg tgatcggcca cactgggact gagacacggc

301 ccagactcct acgggaggca gcagtaggga atcttccgca atggacgaaa gtctgacgga

361 gcaacgccgc gtgagtgatg aaggttttcg gatcgtaaag ctctgttgtt agggaagaac

421 aagtaccgtt cgaatagggc ggtaccttga cggtacctaa ccagaaagcc acggctaact

481 acgtgccagc agccgcggta atacgtaggt ggcaagcgtt gtccggaatt attgggcgta

541 aagggctcgc aggcggtttc ttaagtctga tgtgaaagcc cccggctcaa ccggggaggg

601 tcattggaaa ctggggaact tgagtgcaga agaggagagt ggaattccac gtgtagcggt

661 gaaatgcgta gagatgtgga ggaacaccag tggcgaaggc gactctctgg tctgtaactg

721 acgctgagga gcgaaagcgt ggggagcgaa caggattaga taccctggta gtccacgccg

781 taaacgatga gtgctaagtg ttaggggtt tccgcccctt agtgctgcag ctaacgcatt

841 aagcactccg cctggggagt acggtcgcaa gactgaaact caaaggaatt gacgggggcc

901 cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg cgaagaacct taccaggtct

Nucleotide sequence of strain A 002 is:

GTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAA
ACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAA
AGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAA
GAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA

AGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAA
GTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTG
GGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGT
CTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTT
TCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGCCCGCACAAGCG
GTGGAGCATGGTGGTTTAATTCGAAGCAACGCGAAGA

4.6.3.2 16S rDNA of Strain M 015

The nucleotides of strain M 015 have the important characteristics as follows,

Locus: EF656456, 1515 bp DNA linear

Definition: Bacillus subtilis

Accession: EF656456

Reference: 2 (bases 1 to 1515)

Authors: Yao,L., Wang,Q., Fu,X. and Mei,R

Title: Direct Submission

Journal: Submitted (05-JUN-2007) Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, Yuanmingyuan West RD2, Haidian District, Beijing 100094, China

Origin:

1 agagtttgat cctggctcag gacgaacgct ggcggcgtgc ctaatactgc agtcgagcgg
61 acagatggga gcttgctccc tgatgttagc ggcggacggg tgagtaacac gtgggtaacc
121 tgcctgtaag actgggataa ctccgggaaa ccggggctaa taccggatgg ttgtttgaac
181 cgcatggttc aaacataaaa ggtggcttcg gctaccactt acagatggac ccgcggcgca
241 ttagctagtt ggtgaggtaa cggctcacca aggcaacgat gcgtagccga cctgagaggg
301 tgatcggcca cactgggact gagacacggc ccagactcctacgggaggca gcagtaggga
361 atcttccgca atggacgaaa gtctgacgga gcaacgccgc gtgagtgatg aaggttttcg
421 gatcgtaaag ctctgttgtt agggaagaac aagtaccgtt cgaatagggc ggtaccttga
481 cggtacctaa ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt
541 ggcaagcgtt gtccggaatt attgggcgta aagggctcgc aggcggtttc ttaagtctga

601 tgtgaaagcc cccggctcaa ccggggaggg tcattggaaa ctggggaact tgagtgcaga
661 agaggagagt ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccag
721 tggcgaaggc gactctctgg tctgtaactg acgctgagga gcgaaagcgt ggggagcgaa
781 caggattaga taccctggta gtccacgccg taaacgatga gtgctaagtg ttaggggtt
841 tccgcccctt agtgctgcag ctaacgcatt aagcactccg cctggggagt acggtcgcaa
901 gactgaaact caaaggaatt gacgggggcc cgcacaagcggtggagcatg tggtttaatt
961 cgaagcaacg cgaagaacct taccaggtct tgacatcctc tgacaatcct agagatagga
1021 cgtccccttc gggggcagag tgacaggtgg tgcatggttg tcgtcagctc gtgtcgtgag
1081 atgttgggtt aagtcccgca acgagcgcaa cccttgatct tagttgccag cattcagttg
1141ggcactctaa ggtgactgcc ggtgacaaac cggaggaagg tggggatgac gtcaaatcat
1201 catgcccctt atgacctggg ctacacacgt gctacaatgg acagaacaaa gggcagcgaa
1261 accgcgaggt taagccaatc ccacaaatct gttctcagtt cggatcgcag tctgcaactc
1321 gactgcgtga agctggaatc gctagtaatc gcggatcagc atgccgcggt gaatacgttc
1381 ccgggccttg tacacaccgc ccgtcacacc acgagagttt gtaacacccg aagtcggtga
1441 ggtaaccttt taggagccag ccgccgaagg tgggacagat gattggggtg aagtcgtaac
1501 aaggtagccg tatcg

Nucleotide sequence of strain M 015 is:

AGTACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCG
GCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAA
CTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTC
AAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGC
ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGT
TGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTA
CCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGG
CGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCA
TTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGT
GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAA

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAA
CCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCC
TTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGC
CAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGC
CAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTG
CGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGG
GACAGATGATGGGGTGAAGTCG

4.6.3.3 16S rDNA of Strain F 018

The nucleotides of strain F 018 have the important characteristics as follows,

Locus: EF656456, 1515 bp DNA linear

Definition: Bacillus subtilis

Accession: EF656456

Reference: 2 (bases 1 to 1515)

Authors: Yao,L., Wang,Q., Fu,X. and Mei,R

Title: Direct Submission

Journal: Submitted (05-JUN-2007) Department of Plant Pathology, College of Agronomy and Biotechnology, China Agricultural University, Yuanmingyuan West RD2, Haidian District, Beijing 100094, China

Origin:

1 agagtttgat cctggctcag gacgaacgct ggcggcgtgc ctaatactgc agtcgagcgg
61 acagatggga gcttgctccc tgatgttagc ggcggacggg tgagtaacac gtgggtaacc
121 tgcctgtaag actgggataa ctccgggaaa ccggggctaa taccggatgg ttgtttgaac
181 cgcatggttc aaacataaaa ggtggcttcg gctaccactt acagatggac ccgcggcgca

241 ttagctagtt ggtgaggtaa cggctcacca aggcaacgat gcgtagccga cctgagaggg
301 tgatcggcca cactgggact gagacacggc ccagactcctacgggaggca gcagtaggga
361 atcttccgca atggacgaaa gtctgacgga gcaacgccgc gtgagtgatg aaggttttcg
421 gatcgtaaag ctctgttgtt agggaagaac aagtaccgtt cgaatagggc ggtaccttga
481 cggtacctaa ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt
541 ggcaagcgtt gtccggaatt attgggcgta aagggctcgc aggcggtttc ttaagtctga
601 tgtgaaagcc cccggctcaa ccggggaggg tcattggaaa ctggggaact tgagtgcaga
661 agaggagagt ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccag
721 tggcgaaggc gactctctgg tctgtaactg acgctgagga gcgaaagcgt ggggagcgaa
781 caggattaga taccctggta gtccacgccg taaacgatga gtgctaagtg ttaggggtt
841 tccgcccctt agtgctgcag ctaacgcatt aagcactccg cctggggagt acggtcgcaa
901 gactgaaact caaaggaatt gacgggggcc cgcacaagcggtggagcatg tggtttaatt
961 cgaagcaacg cgaagaacct taccaggtct tgacatcctc tgacaatcct agagatagga
1021 cgtccccttc gggggcagag tgacaggtgg tgcatggttg tcgtcagctc gtgtcgtgag
1081 atgttgggtt aagtcccgca acgagcgcaa cccttgatct tagttgccag cattcagttg
1141ggcactctaa ggtgactgcc ggtgacaaac cggaggaagg tggggatgac gtcaaatcat
1201 catgcccctt atgacctggg ctacacacgt gctacaatgg acagaacaaa gggcagcgaa
1261 accgcgaggt taagccaatc ccacaaatct gttctcagtt cggatcgcag tctgcaactc
1321 gactgcgtga agctggaatc gctagtaatc gcggatcagc atgccgcggt gaatacgttc
1381 ccgggccttg tacacaccgc ccgtcacacc acgagagttt gtaacacccg aagtcggtga
1441 ggtaaccttt taggagccag ccgccgaagg tgggacagat gattggggtg aagtcgtaac
1501 aaggtagccg tatcg

Nucleotide sequence of strain F 018 is:

ATACTGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACT
CCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCA
AACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCA
TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCG
ACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTT
GTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTAC

CTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGC
GGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCSGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTG
TAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGA
CTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT
TAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACzCATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTT
CGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCC
AGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA
AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCC
AATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC
GTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAAGGTGGG
ACAGATGATTGGGGTGAAGTCTNAGNCGAGTAAGC

The results of 16S rDNA sequencing lead to a conclusion that all effective isolates are *Bacillus subtilis*, which have different strains because of the different biochemical and microbiological characteristics.