ISOLATION, SCREENING AND THE INFLUENCE OF CULTIVATION FACTORS ON CELLULASE OF BACTERIA ISOLATED FROM TERMITES GUT

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ABSTRACT

From five termites guts samples collected in different places in Ha Noi and Vinh, 11 isolates which formed halo on agar plates with CMC were isolated. Among them strain G4 possessed the highest CMCase activity. Based on morphology and 16S rRNA gene sequences analysis, G4 was identified as *Bacillus subtilis* G4. The results from cultivation study revealed that M3 medium containing rice bran 1 %, soy flour 1 %, casein 1 % and 1 % NaCl was the best medium for cellulase production of *B. subtilis* G4. At optimal conditions for G4 which were inoculums ratio 1 %, cultivation temperature 37ºC, cultivation time 72 hours, shaking speed 150 rpm and initial pH medium 7, CMCase and FPase activity reached 3.36 U/ml and 0.35 U/ml, respectively.

Keywords: termites gut, bacteria, cellulase, CMCase, FPase.

1. INTRODUCTIONS

Cellulose is the most abundant renewable biomass on earth (100 billion tons of dry volumes/year). Cellulose is degraded by cellulase by hydrolyzing β – 1,4 linkages in cellulose chains. Three types of cellulases based on the type of catalysed reaction are endocellulase (CMCase), exocellulase and β-glucosidase (cellobiase). Cellulase is the most applied enzyme in the food industry, paper industry and textile industry ... to hydrolyze cellulose-rich materials for improving the extraction efficiency and product quality. However, the most important applications of cellulase is involving in the hydrolysis of raw materials such as straw, bagasse, pulp ... to form the simple sugars.

Termites are among the most important lignocellulose-digesting insects. There are various species of bacteria in termites' gut that are capable of degrading cellulose and hemicelluloses [1]. In addition, the sources of cellulolytic bacteria in the termite guts are expected to be widely used in the industry as well as mining gene sources for the production of recombinant enzymes.

In Vietnam, studies on cellulase production from bacteria isolated from termites gut are limited. Therefore, in this study the isolation and selection of bacterial strains of high cellulase biosynthesis from termites guts was carried out. Furthermore the influence of the culture conditions on cellulase biosynthesis of selected strain was studied for improving the cellulase production.

2. MATERIALS AND METHODS

2.1. Materials

Termites: Termite's samples were collected from different regions in Hanoi and Vinh regions.

Chemicals: 3,5 – Dinitrosalicylic acid (DNS), NaOH (England), Na-K tartarate (England), Na metabisulfite (England), Citric acid monohydrate (Germany) and the other chemicals of analytical grade from China.

2.2. Methods

*Handling of samples***.** The collected termite samples were surface sterilized with 70% ethanol to remove contamination, washed with sterile distilled water and then remove the head. The intestinals were ground in 0.9 % NaCl and then incubated either on the enrichment medium M1 **(**$NaNO_3$ 2.5 g; KH_2PO_4 2 g; $MgSO_4$ 0.2 g; NaCl 0.1 g; $CaCl.6H_2O$ 0.1 g, CMC 2.5 g in 1 litter water pH 7) at 37 ^oC for 7 days [2]; medium M2 **(**$NaNO_3$ 2.5 g; K_2HPO_4 1 g; $MgSO_4$ 0.5g; KCl 2 g; Peptone 2 g, CMC 5 g in 1 litter water at pH 7) at 37 ^oC for 48 h [3]; or medium M3**:** rice bran 20 g; casein 10 g; soybean powder 10 g; NaCl 10 g in 1 litter water, pH 7) at 37 ^oC for 36 h [4].

*Isolation and screening of Cellulolytic bacteria***.** Samples from enriched medium M1, M2, M3 were diluted and incubated on isolation medium (KH_2PO_4 0.5 g; $MgSO_4$ 0.25 g; Peptone 2 g, CMC 2.5 g; agar 18 g in 1 litter water at pH 7) at 37 ^oC for 48 h. Bacteria from single colonies were repeatedly grown on solid agar plates until a pure culture was obtained. The plates were flooded with 0.1 % Congo red for 20 min and washed with 1 M NaCl for 15 min. The clear zone formed by the isolates was indicated their cellulase activity [5]. Cellulose-degrading potential of the positive isolates was qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony. Furthermore the strains were cultured at 37°C at 150 rpm in LB media containing 0.5 % CMC. Broth culture after 24 h of incubation was centrifuged at 10000 rpm for 15 min at 4 ^oC. Supernatant was collected for further enzyme assays.

Enzyme essay. Endoglucanase (CMCase) activity was determined by incubating 0.5 mL of crude enzyme with 0.5 mL of 2 % CMC in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C for 30 min. The reducing sugar released was measured by the DNS method at 540 nm using glucose as standard. One unit of CMCase activity was defined as the amount of enzyme producing 1 μmol of reducing sugar (measured as glucose) per minute under the specified assay conditions [1].

FPase activity was determined by incubating 0.5 mL of supernatant with 1.0 mL of 0.05 M sodium citrate buffer (pH 4.8) containing Whatman No.1 filter paper strip - 1.0 × 6.0 cm (= 50 mg). The reducing sugar released was measured by the DNS method at 540 nm using glucose as standard. One unit of enzymatic activity is defined as the amount of enzyme that releases 1 μmol reducing sugars (measured as glucose) per mL per minute [1].

3. RESULTS AND DISCUSSION

3.1. Isolation, screening and idenfication of cellulolytic bacteria

From 5 termite's samples, 11 isolates which formed halo on agar plates with Congo red were obtained (Table 1). The result of hydrolysis capacity, CMCase and FPase activity of them was presented on Table 1.

Table 1. Hydrolytic capacity and cellulase activity of isolates/

Isolates	Hydrolytic capacity (D/d)	CMCase (U/ml)	FPase (U/ml)
CM2-4	2.4	0.097	0.027
CG2	0.8	0.025	0.020
CG4	2.4	0.034	0.023
CG4-1-2	2.3	0.047	0.024
D1-3	1.7	0.038	0.018
D1-7	1.4	0.037	0.023
D1-8	0.8	0.027	0.017
D1-12	3.8	0.064	0.023
T2-11	8.0	0.150	0.041
TM1-7-1	0.7	0.021	0.016
G4	**10**	**0.286**	**0.026**

D-diameter of halos around the colonies; d-diameter of colony

The results from Table 1 showed that the hydrolytic capacity of 11 isolated varied from very low value 0.7 to quite high 10. The two strains G4 and T2-11 possess highest value of hydrolytic capacity (HC), 8 and 10. The results of HC were corresponding to CMCase and FPase activities, where these two strains had the highest one. The G4 strain reached highest CMCase of 0.286 U/ml, which almost 10-fold higher than the lowest CMCase by strain TM1-7-1. Though G4 possesses only second high FPase activity 0.026 U/ml, compared to the highest 0.041 U/ml of strain T2-11. The G4 was chosen for further study.

Table 2. Biochemical test of G4.

Test	Result
Gram staining	+
Motility	+
Indole	-
Methyl red	-
Urease	+
Citrate utilization	-
Catalase	+
Starch hydrolysis	+
Sucrose fermentation	-

The G4 is rod shape bacilli, gram positive and spore forming (Figure 1C). Biochemical characteristics of G4 were presented on Table 2. Colonies's shape is circle, rounded edges and opaque white (Figure 1A). The partial 16S rDNA sequence analysis showed similarity of 100 % to *Bacillus subtilis* species (Table 3). Based on these data, G4 was assigned as *Bacillus subtilis* G4. Our results are in agreements with previous findings of Sharma *et al*., whereas *Bacillus* sp.

3B8 was among the isolated bacteria from termites gut. The CMCase of isolated *Bacillus* sp 3B8 was 0.12 ± 0.01 U/ml which was two times lower than our [4]. According to Sreena *et al*., five isolates showing significant zone of clearance were selected, among them three belonged to *Bacillus* and one to *Staphylococcus* and *Enterobacter* sp. [6]. Thus *Bacillus* sp. was quite common cellulolytic strain isolated from termites gut.

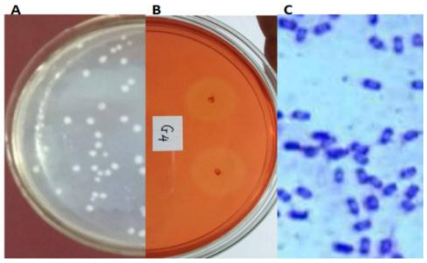

Figure 1. Morphology of G4. Colony of G4 (A); Halo zone formed by G4 on CMC agar plate (B) Gram staining of G4 (C).

Table 3. 16S rRNA Gene Sequence Analysis for Identification of G4

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis subsp. inaquosorum strain KCTC 13429 16S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	100%	KT989848.1
Bacillus amyloliquefaciens 16S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	100%	KU922934.1
Bacillus amyloliquefaciens strain B15, complete genome	1048	8119	100%	0.0	100%	CP014783.1
Bacillus subtilis subsp. subtilis strain D12-5, complete genome	1048	10439	100%	0.0	100%	CP014858.1
Bacillus sp. M29(2016) 16S ribosomal RNA gene, partial sequence	1048	1048	100%	0.0	100%	KU870670.1

3.2. Effect of culture medium and cultivation parameters on CMCase activity

3.2.1. Effect of culture medium

Bacillus subtilis G_4 was cultivated on LB, M1, M2 and M3 medium. After incubation at 37 °C for 48 hr, 150 rpm, CMCase activity was measured (Fig. 2). Maximum CMCase production was obtained at M3 medium, CMCase enzyme activity reached 1.54 ± 0.026 U/ml, two times higher than LB medium. Rice bran, one of component of M3 medium, comprises protein 18 %, fat 1.5 – 2 %, starch 25.5 %, fiber 19 % (including cellulose, lignin, inulin, pectin)… Rice bran was the best carbon source for CMCase acitivity of *Bacillus licheniformis* AU01 [7]. Rice bran was used as agro waste for cellulase production by *Bacillus* sp. in numerous studies [4, 8].

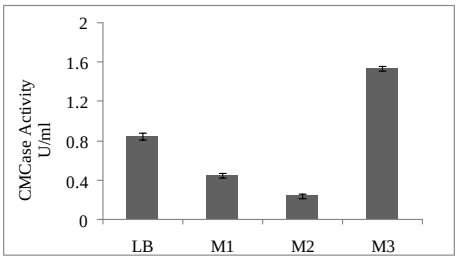

Figure 2. Effect of culture medium on CMCase activity.

3.2.2. Effect of inoculum ratio on CMCase activity

B. subtilis G4 was incubated in LB medium for 48 hrs (OD_{600} reached 3.0 – 3.2) with subsequently transferring to M3 medium at inoculums ratio 0.5; 1; 5; 10 % (v/v) and was cultivated for 48 hrs, 150 rpm, 37 ºC. The CMCase activity was determined (Fig. 3).

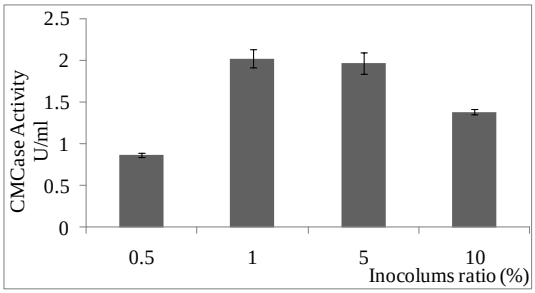

Figure 3. Effect of inoculums ratio on CMCase activity.

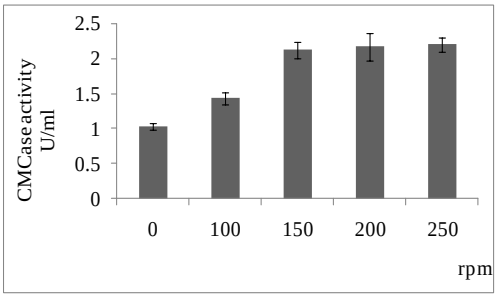

Figure 4. Effect of agitation on CMCase activity.

The inoculum ratio of 1 % gave the highest CMCase activity 2.03 ± 0.11 U/ml. Increasing or decreasing the inoculum rate beyond this value resulted in decline in CMCase activity (Fig. 3). Therefore the inoculum ratio 1 % was chosen for the next experiment.

3.2.3. Effect of agitation on CMCase activity

B. subtilis can be aerobic or facultative anaerobic. In this study the effects of agitation on CMCase activity were investigated. *B. subtilis* G4 was transferred in M3 medium at inoculum ratio 1 % and incubated at 37 ºC with different shaking rates 0, 100, 150 and 200 rpm for 48 hrs.

CMCase activity was recorded at different shaking rates (Fig. 4). Increase of shaking rate from 0 to 150 rpm resulted on 2-times higher CMCase activity 2.03 ± 0.11 U/ml. But further increase to 150, 200, 250 rpm brought insignificant increase of CMCase activity (2.12 ± 0.11U/ml , 2.17 ± 0.20 U/ml and 2.2 ± 0.11 U/ml). Our results are similar to that of Sreedevi *et al.*, who also found that *Bacillus* sp. BSS3 had the highest enzyme activity at agitation speed 150 rpm [9]. Therefore 150 rpm was used for the following experiment.

3.2.4. Effect of cultivation time on CMCase activity

CMCase activities were recorded at different cultivation time. The maximal CMCase production was obtained at 72 hrs of incubation, reached 2.88 ± 0.19 U/ml (Fig. 5). Maximum cellulase production was also achieved after 72 hours of incubation at 37 °C by *Bacillus pumilus* EWBCM1 [5].

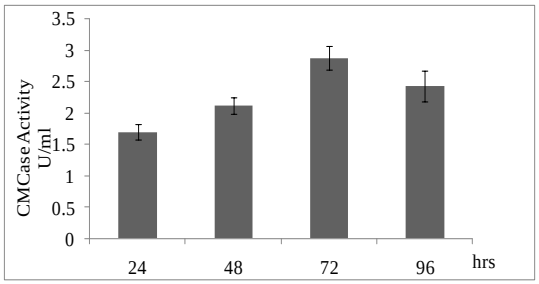

Figure 5. Effect of cultivation time on CMCase activity.

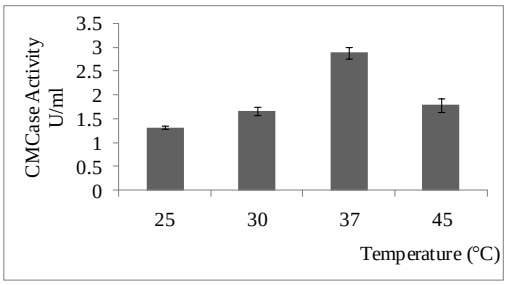

Figure 6. Effect of temperature on CMCase activity.

3.2.5. Effect of temperature on CMCase activity

B. subtilis G4 was cultivated in M3 medium, inoculum rate 1 %, 150 rpm, at temperatures of 25, 30, 37, 45 °C for 72 hrs.

CMCase enzyme production is temperature dependent. In our experiments, the *B. subtilis* G4 exhibited maximum enzyme production at 37 ºC; 2.88 ± 0.13 U/mL (Fig. 6). It is believed that the temperature influences the secretion of extracellular enzyme by possibly changing/or altering the physical properties of the cell membrane. *Bacillus* grew best at 37 °C in numerous reports [5, 6, 10].

3.2.6. Effects of rice bran concentration on CMCase activity

To study the effect of rice bran concentration, the medium M3 with different rice bran concentrations 1 g/l; 2.5 g/l; 5 g/l; 10 g/l; 15 g/l and 20 g/l was used. *B. subtilis* G4 was cultivated at 37 °C, 150 rpm for 72 hrs. Maximum CMCase reached 3.36 ± 0.08 U/ml with rice bran concentration 10 g/l (Fig. 7). It seems that higher rice bran concentration inhibited the cellulase production.

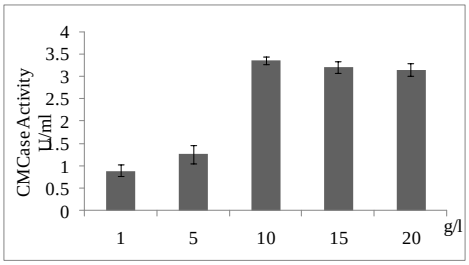

Figure 7. Effect of rice bran concentration on CMCase activity.

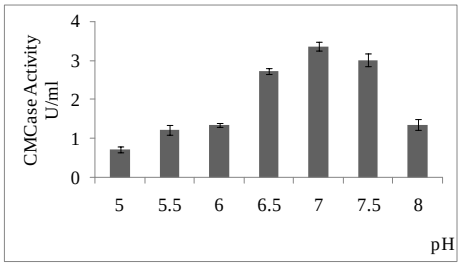

Figure 8. Effect of pH on CMCase activity.

3.2.7. Effect of pH on the CMCase activity

In this experiment, initial pH of M3 medium was adjusted to 5.5; 6; 6.5; 7; 7.5; 8 to evaluate the effect of pH on the activity of the CMCase.

The optimal pH for CMCase activity is found to be at pH 7 (3.36 ± 0.12 U/ml) for *B. subtilis* G4. Increasing or decreasing the pH beyond this resulted in declining in enzyme activity (Fig. 8). According to Sharma, *Bacillus*. sp S3B8 isolated from termites gut was also produced maximum CMCase at pH 7 [10].

Thus the appropriate condition for high CMCase activity by *B. subtilis* G4 were culture medium M3 containing 1 % rice bran, 1 % soybean meal, 1 % casein and 0.1 % NaCl at a inocunum ratio 1 %, culturing at temperature at 37 ºC, agitation rate 150 rpm, cultivation time 72 hours and initial pH medium 7. At this condition CMCase activity reached 3.36 U/ml and the FPase activity reached 0.35 U/ml.

4. CONCLUSIONS

By morphological, biochemical characteristics and 16s RNA sequence analysis, the isolate exhibited the highest CMCase and second high FPase activity among 11 isolates from termites gut was identified as *Bacillus subtilis* G4. The *Bacillus subtilis* G4 produced cellulase at condition pH 7, temperature 37 ºC, which were similar to *Bacillus* sp. from other sources. The CMCase and FPase activities of G4 after optimization were more than 11- and 13-fold increase, from 0.286 U/ml to 3.36 U/ml and 0.026 U/ml to 0.35 U/ml, respectively.

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TÓM TẮT

PHÂN LẬP, TUYỂN CHỌN VÀ CÁC YẾU TỐ ẢNH HƯỞNG ĐẾN KHẢ NĂNG SINH TỔNG HỢP CELLULASE CỦA VI KHUẨN PHÂN LẬP TỪ RUỘT MỐI

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Từ 5 mẫu ruột mối thu thập tại các điểm khác nhau ở Hà Nội và Vinh, 11 chủng có khả năng tạo vòng thủy phân trên môi trường thạch CMC đã được phân lập, trong đó chủng G4 cho hoạt độ CMCase cao nhất. Dựa vào đặc điểm hình thái, phân tích trình tự gen 16S rARN, vi khuẩn G4 thuộc loài *Bacillus subtilis*. Kết quả nghiên cứu ảnh hưởng điều kiện nuôi cấy đến sinh tổng hợp cellulase cho thấy trên môi trường M3 với hàm lượng cám gạo 1 %, bột đậu tương 1 %, casein 1 % và NaCl 1 % với tỉ lệ cấp giống 1 %, nhiệt độ nuôi cấy 37 ºC, chế độ lắc 150 vòng/phút, thời gian lên men 72 giờ và pH môi trường ban đầu nuôi cấy là 7 cho hoạt độ CMCase cao nhất đạt 3,36 U/ml và hoạt độ FPase đạt 0,35 U/ml.

Từ khóa: ruột mối, vi khuẩn, cellulase, CMCase, FPase.