

CELLULASE ACTIVITY OF *BACILLUS SUBTILIS* DSC.03 DEPENDS ON THE STRAIN CULTIVATION PARAMETERS

T. Q. TRUONG¹✉, K. D. NGUYEN²

¹Faculty of Agriculture – Fisheries, University of Cuu Long, Vinh Long, Vietnam;

²Faculty of Biotechnology, Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam;

✉e-mail: truongquoctat@mku.edu.vn

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Cellulase derived from cellulolytic bacteria plays an increasingly important role in biotechnology and is characterized by high consumption demand on the global enzyme market. Bacterial strains of the genus *Bacillus* have been reported to be capable of synthesizing cellulase, which breaks β -1,4-glycosidic bonds, releasing glucose as the end product. Nevertheless, enzyme activity and yield are affected by many factors, especially by bacterial strain and culture medium conditions. This study aimed to assess *Bacillus subtilis* DSC.03 cellulase activity depending on the strain cultivation parameters. *Bacillus subtilis* DSC.03 was isolated from durian peel compost samples. Carboxymethyl cellulase (CMC) activity was determined by reaction with 3,5-dinitrosalicylic acid in the crude enzyme extract of the bacterial strain. It was shown that cultivation time, temperature and substrate concentration have a significant impact on the CMC activity of DSC.03. The optimal parameters were as follows: 60 h at 40°C, 1% carboxymethyl cellulose and 2% inoculum. Under these conditions the strain exhibited a maximum CMC activity of 1.72 U/ml.

Keywords: *Bacillus subtilis* DSC.03, bacterial cellulase, carboxymethyl cellulase activity, culture conditions.

The application of biotechnology to convert cellulose-rich biomass sources from agricultural by-products is considered a sustainable and effective approach to developing a circular economy, increasing economic value, and minimizing environmental pollution [1]. This process is carried out effectively and safely using cellulolytic microorganisms to obtain the final hydrolysis product, glucose [2]. These microorganisms synthesize cellulase enzyme complexes (endoglucanase, exoglucanase, and β -glucosidase) [3]. This enzyme complex works in conjunction to break the β -1,4-glycosidic bonds in the cellulose structure, releasing glucose molecules [3, 4]. Cellulase is the enzyme with the highest consumption demand, accounting for about 20% of the global enzyme market [5]. Cellulase is increasingly used in many different fields, including bioethanol production; in medicine, cellulase is used to treat *Pseudomonas* biofilms, replacing antibiotics or strong chemical detergents; in the food and beverage industry, cellulase is used to produce fruit juices. Cellulase is also used to produce detergents, cleaning agents, and cleaning products [4, 5].

In nature, many microbial strains that efficiently synthesize cellulase enzymes have been identified. These include bacteria, fungi, and actinomycetes. Some highly valued strains, including *Trichoderma*, *Aspergillus*, *Bacillus*, *Clostridium*, and *Cellulomonas*, have been isolated from various sources, such as soil, decaying plant matter, and insect digestive systems [6]. Cellulase obtained from bacteria has a more outstanding potential than that from fungi. The advantages of producing cellulase from bacteria are ease of cultivation, ability to withstand harsh conditions (high temperature, low pH, high salt concentration), rapid growth rate, formation of extracellular enzyme complexes which facilitate easy extraction, purification, and increased substrate processing efficiency, and easy genetic modification to improve enzyme biosynthesis efficiency [6, 7]. Typically, thermophilic bacterial strains of the genus *Bacillus* including *B. velezensis*, *B. subtilis*, *B. mojavensis* and *B. cereus* have been reported to be capable of synthesizing cellulase with high temperature stability up to 70°C, salt concentrations up to 2 M and a wide pH range (4–11) [6]. Nevertheless,

enzyme yield is affected by many factors, especially bacterial strains and culture conditions [5, 7]. Hence, research on the application of native bacterial strains with high cellulase production capacity and the establishment of specific culture conditions is of great importance in developing biotechnological solutions for treating cellulose-containing agricultural by-products. The goal of this study was to evaluate the stress tolerance to several abiotic factors and screen the culture conditions that have the most significant impact on CMCase activity in the crude enzyme solution of the native *B. subtilis* DSC.03 strain.

Materials and Methods

Materials and chemical. *Bacillus subtilis* DSC.03 strain, capable of degrading cellulose and heat-resistant, was isolated from durian peel compost samples. This strain has been identified and its 16S rRNA gene sequence has been registered in the gene bank (GenBank) with accession number PP851408.1 [8].

Chemicals: Carboxymethyl Cellulose-Na (CMC), 3,5-dinitrosalicylic acid (DNS) reagent, tryptone, NaCl, HCl, NaOH (Xilong, China). All chemicals meet analytical grade standards.

Evaluation of the growth potential of the *B. subtilis* DSC.03 strain. Bacterial suspension preparation of: A bacterial colony was inoculated into 50 ml of liquid Luria-Bertani (LB) medium and shaken at 150 rpm for 12 h at 37°C [8]. Bacterial growth was measured by turbidity at OD₆₀₀ and adjusted to OD₆₀₀ = 1.0 with liquid LB medium. The bacterial suspension was stored at 4°C and used within 30 min.

Construction of the growth curve: the suspension (1 ml) was transferred of into an Erlenmeyer flask containing 50 ml of liquid medium containing 1% CMC-Na (CB) [8]. The mixture was incubated at 37°C by shaking at 150 rpm. The OD₆₀₀ bacterial suspension was measured for every 2 h until stable.

To determine tolerance to abiotic stress conditions: Transfer 1 ml of the suspension to an Erlenmeyer flask containing 50 ml of CB medium adjusted to pH values of 3–10, supplemented with NaCl at a concentration of 0–10% (w/v), and incubated at 4–60°C, shaking at 150 rpm. After 48 h of incubation and shaking, the absorbance of the suspension was measured at a wavelength of 600 nm. The results of this experiment serve as the basis for selecting appropriate parameters for the experimen-

tal design to determine the optimal culture conditions for obtaining highly active cellulase.

Effect of culture conditions on the cellulase activity of *B. subtilis* DSC.03 strain. The experiments were designed as a completely randomized design with one factor: 1 ml of the prepared bacterial suspension was aspirated into 50 ml of pH-adjusted CB medium (4, 5, 6, 7, and 8). The mixture was then shaken at 150 rpm for 48 h at 37°C. After incubation, 5 ml of the suspension was centrifuged at 12,000 xg at 4°C for 15 min, and the crude enzyme solution was collected to determine the endoglucanase (carboxymethyl cellulase - CMCCase) activity. Additional experiments were conducted to determine the effect of different culture conditions on CMCCase activity. The experiments included varying culture temperatures (20, 25, 30, 35, 40, 45, 50, and 55°C), NaCl concentrations (0, 1, 2, and 3%) (w/v), CMC concentrations (0.5; 1; 1.5; 2, and 2.5%), fermentation times (24, 36, 48, 60, 72, 84, and 96 h) (w/v), and inoculum ratios (1, 2, 3, 4, and 5%) (v/v).

The Plackett-Burman design was applied to screen for culture condition factors that had a major impact on the CMCCase activity of *B. subtilis* DSC.03 strain. The experiment included 12 treatments, and each factor was investigated at two levels: low (-1) and high (+1) (Table 1). The effect of each factor on the response value, CMCCase activity, was estimated by the difference between the average enzyme activity value of the measurement placed at the low (-1) and high (+1) levels of the corresponding factor.

Determination of the cellulase activity. The CMCCase enzyme activity of the crude enzyme solution (U/ml) was determined by reaction with 3,5-dinitrosalicylic acid (DNS) reagent. The crude enzyme solution (0.5 ml) was mixed with 0.5 ml of 50 mM citrate buffer (pH 4.8) containing 2% CMC (w/v) and incubated for 30 min at 50°C. After incubation, 3 ml of DNS reagent was added to 1 ml of the reaction mixture, and the mixture was then heated in a boiling water bath for 5 min. Next, the mixture was then cooled to room temperature by placing the test tubes containing the samples in ice water. The absorbance of the reaction mixture was measured at 540 nm. The amount of reducing sugars was determined using the glucose standard curve ($y = 3.2995x + 0.03$, $R^2 = 0.9957$). A CMCCase unit is defined as the amount of enzyme that can release 1 μ mol of reducing sugar (expressed as glucose) per minute under standard conditions [9].

Table 1. Plackett-Burman design with actual values for six variables representing culture conditions

Treatment	Encoding variable						Real variable						Y-CMC _{Case} , U/ml
	A	B	C	D	E	F	A	B	C	D	E	F	
1	-1	+1	+1	+1	-1	+1	5	40	1.5	1.5	48	2	1.43 ± 0.02
2	-1	-1	-1	+1	+1	+1	5	35	1.0	1.5	60	2	1.31 ± 0.03
3	+1	+1	-1	+1	+1	-1	7	40	1.0	1.5	60	1	1.48 ± 0.04
4	-1	+1	+1	-1	+1	-1	5	40	1.5	1.0	60	1	1.32 ± 0.03
5	-1	-1	+1	+1	+1	-1	5	35	1.5	1.5	60	1	1.37 ± 0.04
6	+1	+1	-1	+1	-1	-1	7	40	1.0	1.5	48	1	1.39 ± 0.04
7	+1	-1	+1	-1	-1	-1	7	35	1.5	1.0	48	1	0.75 ± 0.04
8	-1	-1	-1	-1	-1	-1	5	35	1.0	1.0	48	1	0.64 ± 0.05
9	+1	-1	+1	+1	-1	+1	7	35	1.5	1.5	48	2	1.33 ± 0.02
10	-1	+1	-1	-1	-1	+1	5	40	1.0	1.0	48	2	1.11 ± 0.08
11	+1	+1	+1	-1	+1	+1	7	40	1.5	1.0	60	2	1.72 ± 0.03
12	+1	-1	-1	-1	+1	+1	7	35	1.0	1.0	60	2	0.91 ± 0.02

Note. A – pH of the medium; B – culture temperature (°C); C – NaCl concentration (%) (w/v); D – CMC concentration (%) (w/v); E – culture time (h); F – percentage of added bacteria (%)

Data processing. All treatments in each experiment were replicated three times. Data were entered, calculated, and plotted using Microsoft Excel 2019. Data are presented as mean ± standard deviation (SD). The standard deviation is presented as error bars in the graphs, the length of which corresponds to the standard deviation. One-way ANOVA was performed using the Minitab 16 statistical software to determine statistical differences at a 95% confidence level using the Tukey's test.

Results and Discussion

Growth curve of *B. subtilis* DSC.03. The division rate and metabolic activity of microorganisms vary throughout the growth stages. To determine the appropriate age of the DSC.03 strain for experiments investigating medium composition and culture conditions, the growth curve of the bacterial strain was monitored in CB medium supplemented with 1% CMC (w/v), shaken at 150 rpm at 37°C for 48 h. Cell density was measured at 600 nm every 2 h and processed using Minitab 16 software with a high correlation coefficient of $R^2 = 0.9650$ (Fig. 1).

The results in Fig. 1 show that in the first 4 h, the DSC.03 bacterial strain grew slowly due to adaptation to the environment (lag phase) (suspension turbidity increased from 0.212 to 0.367). Next, the bacterial strain grew vigorously in the logarithmic phase for approximately 16 h (4–20 h after inocu-

lation) with suspension turbidity values increasing from 0.367 to 1.116. After that, the bacterial suspension reached its highest turbidity and remained relatively stable after 20–48 h of cultivation with OD_{600} values ranging from 1.116 to 1.089. Therefore, in CB medium, the suspension or biomass of the *B. subtilis* DSC.03 strain can be harvested for inoculation within 18–19 h of cultivation. According to [10], the extracellular enzyme activity of bacteria is also proportional to the number of cells through suspension turbidity. Bacteria are reported to produce maximum enzymes in the logarithmic phase of growth, whereas others in the stationary phase [11]

Abiotic stress tolerance in strain *B. subtilis* DSC.03. Table 2 shows the growth of the DSC.03 bacterial strain after 48 h under stressful environmental conditions of temperature, pH, and high NaCl salt concentration.

The tested bacterial strain exhibited good environmental stress tolerance. This strain had a wide temperature tolerance range, growing well at temperatures of 15–45°C with suspension turbidity values of 0.531 ± 0.02 and 0.941 ± 0.01 , respectively. The most suitable conditions for DSC.03 were slightly acidic to neutral (1.338 ± 0.04 at pH 6 and 1.047 ± 0.03 at pH 7), although it could also grow in strongly acidic environments at pH 4 with a suspension turbidity of 0.398 ± 0.05 . Furthermore, DSC.03 could survive under high salt stress conditions of up

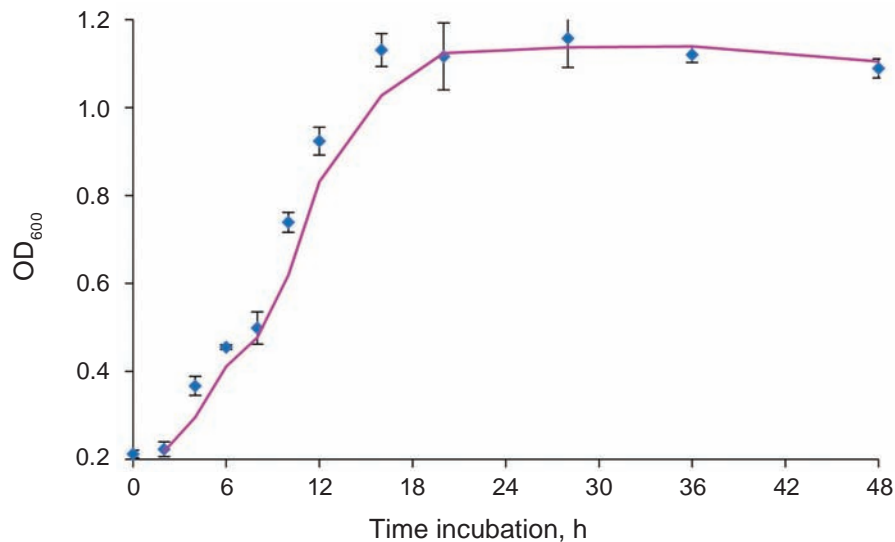


Fig. 1. Growth curve of the *B. subtilis* DSC.03 strain in CB medium

Table 2. Growth performance testing of DSC.03 under stressful environmental conditions

Temperature, °C					pH								NaCl, % w/v			
4	15	37	45	60	3	4	5	6	7	8	9	10	0	6	8	10
-	+	+	+	-	-	+	+	+	+	+	-	-	+	+	+	-

Note. “+” positive, increased turbidity, “-” negative, no increase in turbidity

to 8% with a suspension turbidity of 0.701 ± 0.06 . Similar studies have reported that *Bacillus* sp., including *B. subtilis*, possess high tolerance to abiotic stress factors [12, 13]. This result demonstrates the broad application potential of the *B. subtilis* DSC.03 strain.

Effect of culture conditions on CMCase activity.

Impact of the independent factors. The effect of environmental pH on the CMCase activity of the *B. subtilis* DSC.03 was highest at pH 7 (1.39 ± 0.02 U/ml) (Fig. 2, a). *Bacillus* sp. strains have a wide adaptability to environmental pH ranging from 3.0 to 9.0. However, the optimal pH for their growth and development was determined to be a slightly acidic to slightly alkaline environment [14, 15]. The study by Fouda et al. [16] and Malik et al. [17] showed that cellulase enzyme produced by the bacterium *B. subtilis* F3 and *B. subtilis* TD11 has the highest stability and activity at pH 7. Therefore, pH 7 was selected for further experimentation.

Similar to the effect of environmental pH, temperature is one of the most important parameters for fermentation process. In this study, after 48 h of fermentation, the highest CMCase activity

of the *B. subtilis* DSC.03 strain was observed in the incubation treatment at 40°C (reaching a value of 1.18 ± 0.03 U/ml) and decreased sharply when the temperature was increased to 55°C or 60°C. Similar to this result, Cai et al. [18] determined that the optimal temperature for cellulase production from *B. velezensis* to be 40°C at neutral pH. However, the cellulase enzyme from *B. velezensis* has a temperature limit of up to 60–65°C [19]. Microorganisms grow slowly at temperatures lower or higher than their optimal temperature due to reduced cell division rate. Therefore, a temperature of 40°C was chosen to continue the experiment.

To study the effect of salt concentration, NaCl salt was added to the medium at the highest concentration tested, which was 3% (w/v). The results showed that the *B. subtilis* DSC.03 strain had good adaptability, with the highest CMCase activity recorded at the 1.5% NaCl treatment (1.54 ± 0.03 U/ml) and decreasing at higher NaCl concentrations (Fig. 2, b). Notably, the CMCase activity under the 1.5% NaCl condition was 4.16 times higher than the treatment without added NaCl. This result is similar to that of previous reports confirming that culture

temperature is a positive factor that significantly increases the cellulase production efficiency of *Bacillus* sp. [18, 20]. According to Djelid et al. [19], *B. velezensis* is a bacterial strain that can synthesize salt-tolerant endoglucanase (an enzyme that can function well in both conditions with and without NaCl). Salt tolerance can significantly improve the industrial usefulness of cellulase under harsh conditions [21]. Therefore, a NaCl concentration of 1.5% (w/v) was chosen to continue the experiment.

Each bacterial strain has different substrate requirements. The results in Fig. 2,d show that CMC concentration significantly affects CMCase activity ($P < 0.05$). After 72 days of culture, the enzyme activity increased sharply when the CMC concentration was increased from 0% to 1.5%, reaching 1.27 ± 0.02 U/ml. However, enzyme activity decreased when the CMC concentration was increased to 2, 2.5, and 3% (w/v). This result is similar to those of previous reports. Islam et al. [22] reported that *Paenibacillus* sp. strains produced the highest amount of cellulase media with CMC as a carbon nutrient source. The appropriate CMC concentration for cellulase synthesis mainly depends on the bacterial strain used. The appropriate CMC concentration of some typical *Bacillus* strains, such as *Paenibacillus* sp. CMC, was 1% [22] and *Brevibacillus* sp. was 2% [23]. CMC has been reported to induce cellulase production. Concentrations of CMC, yeast extract, and peptone have a significant positive effect on cellulase production in *Bacillus* [24]. Therefore, a 1% (w/v) CMC concentration was selected for further experimentation.

Regarding incubation time, CMCase activity increased rapidly from 24 h to 60–72 h and reached its highest value of 1.38 ± 0.03 U/ml at 72 h after inoculation, with no statistically significant difference at 60 h after inoculation (1.33 ± 0.03 U/ml) (Fig. 2, e). Subsequently, CMCase activity decreased significantly, maintaining approximately 80.44% and 59.42% at 84 and 96 h after inoculation, respectively, compared to the peak. Djelid et al. [19] also determined that the cellulase biosynthesis efficiency from *B. velezensis* H1 reached its highest value after 72 h of fermentation. This shows that fermentation time significantly affects the cellulase activity. Therefore, proper fermentation time control is necessary to optimize the benefits in cellulase production. Thus, a culture time of 60 h was chosen to continue the experiment.

The final experiment focused on the rate of bacterial inoculum addition. As shown in Fig. 2, f, the highest cellulase activity of *B. subtilis* DSC.03 was achieved at a bacterial inoculum volume of 2% (v/v). At the next three higher inoculum rates of 3, 4, and 5% (v/v), cellulase activity was lower and statistically significantly different from the 2% inoculum concentration. This inoculum rate is consistent with previous reports for strain *B. velezensis* M2 isolated from piglet feces [15] and strain *B. velezensis* Z2.6 isolated from compost [18]. Increasing the initial inoculum concentration in the fermentation system leads to a higher bacterial population density. This promotes cellulase synthesis within a certain range. However, when the inoculum size exceeds the optimal level, excessively high bacterial density increases the rate of dissolved oxygen and nutrient consumption in the medium, creating unfavorable, unstable conditions and ultimately degrading enzyme production efficiency. Therefore, an inoculum supplementation rate of 2% was selected to continue the experiment.

Screening for key influencing factors using Plackett-Burman design analysis. The Plackett-Burman design was applied to screen for culture conditions that had the most significant influence on cellulase production of strain DSC.03. The results in Table 1 show that the Y-response value (CMCase activity (U/ml)) of the crude enzyme extract of bacterial strain DSC.03 under different combined culture conditions significantly differed. The highest CMCase activity value of 1.72 U/ml was achieved in the treatment with a pH-adjusted medium of 7, supplemented with 1.5% NaCl (w/v), 1% CMC (w/v), and 2% inoculum (v/v), cultured for 60 h at 40°C. Under these conditions, the CMCase activity of the bacterial suspension was higher than that determined in the single-factor experiment (1.57 U/ml) at pH 7, supplemented with 1.5% NaCl (w/v), 1.5% CMC (w/v), and 2% inoculum (v/v), and cultured for 60 h at 40°C.

The ANOVA results in Table 3 show that three factors, including culture temperature, CMC substrate concentration, and culture time, have a statistically significant positive impact on the CMCase activity of strain DSC.03 ($P < 0.05$).

The impact levels of the six variables on the objective function are presented in the Pareto plot in Fig. 3.

The results show that the order of impact levels of culture environment factors on the CMCase

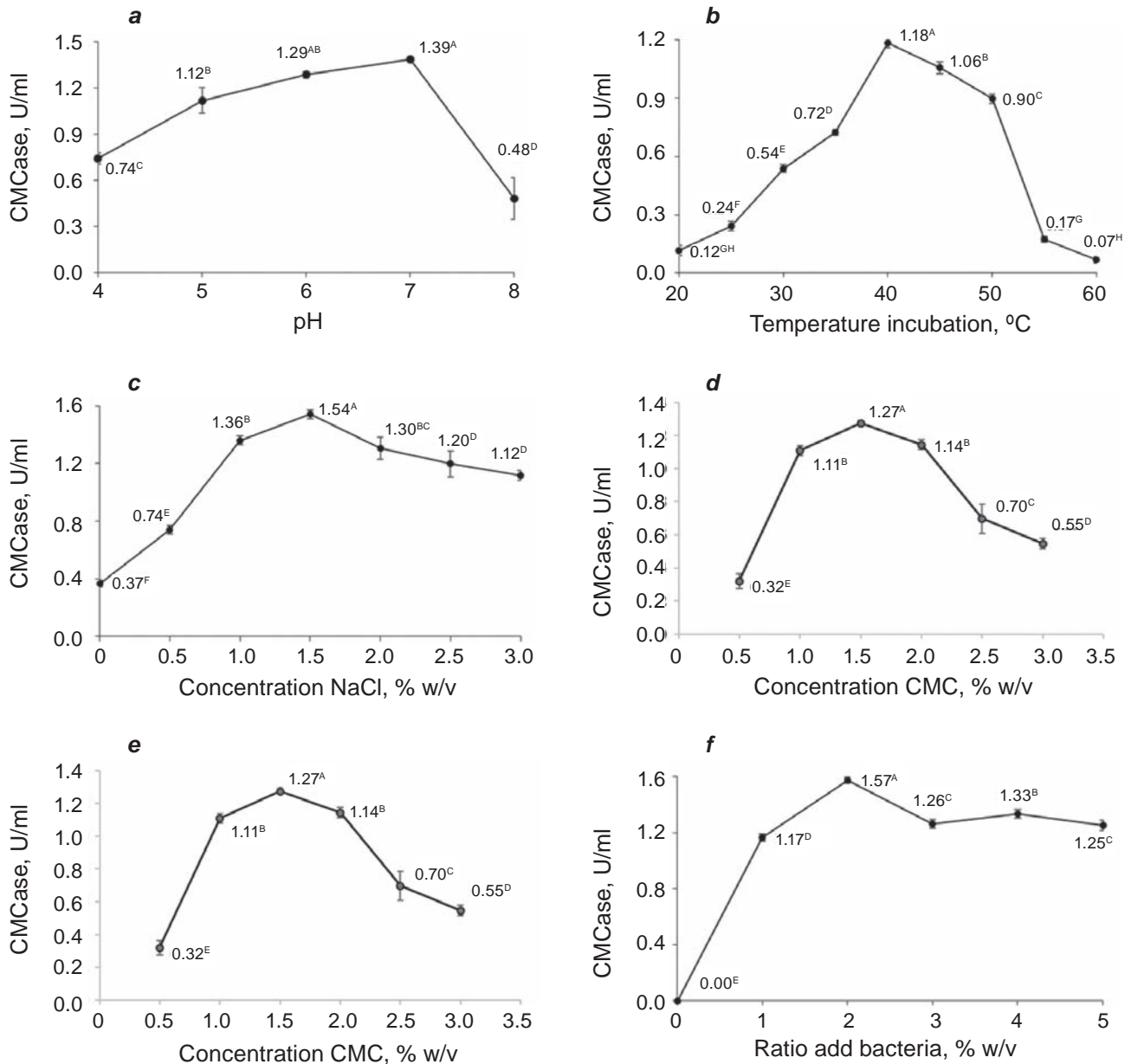


Fig. 2. CMCase activity of crude enzyme extract of *B. subtilis* DSC.03 strain under different culture conditions with CB culture medium containing 1% (w/v) CMC-Na as carbon source and 0.2% (w/v) tryptone as nitrogen source. (a) Initial pH; (b) temperature (°C); (c) Salinity (%); (d) CMC substrate concentration (%); (e) culture time (h); (f) bacterial inoculum ratio (%) (v/v). Values are the mean of three independent experiments. Different uppercase letters following the values indicate statistically significant differences at the 95% confidence

activity of strain DSC.03 is culture temperature > CMC substrate concentration > culture time. Our results are consistent with some research findings of several groups of authors such as [25-27], which all indicate that temperature significantly affects the activity of cellulase produced by bacterial strains of the genus *Bacillus*. As temperature increases, en-

zyme activity increases, reaching its maximum at the optimal temperature, and decreasing with further increases in temperature.

The three variables, salt concentration, inoculum ratio, and pH, are showed negligible effects on the objective function value ($P > 0.05$). Therefore, the coefficients of these three variables will be re-

Table 3. Statistical analysis of the Plackett-Burman design for CMCase activity of *B. subtilis* DSC.03

Term	Effect	Coef	SE Coef	t-value	P-value
Constant		1.230	0.038	32.14	0.000
A	0.067	0.033	0.038	0.87	0.424
B	0.357	0.178	0.038	4.66	0.006
C	0.180	0.090	0.038	2.35	0.065
D	0.310	0.155	0.038	4.05	0.010
E	0.243	0.122	0.038	3.18	0.025
F	0.143	0.072	0.038	1.87	0.120

Note. A – pH of the medium; B – culture temperature (°C); C – NaCl concentration (%) (w/v); D – CMC concentration (%) (w/v); E – culture time (h); F – inoculum size (%)

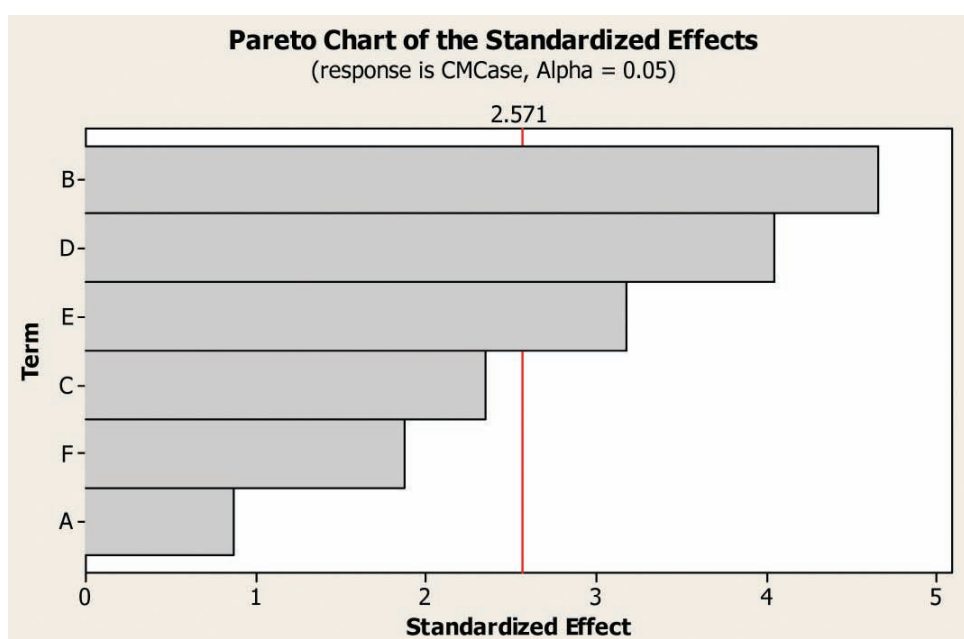


Fig. 3. Pareto plot showing the effect of different culture conditions on the CMCase activity of *B. subtilis* DSC.03. Note: A – pH of the medium; B – culture temperature (°C); C – NaCl concentration (%) (w/v); D – CMC concentration (%) (w/v); E – cultivation time (h); F – inoculum size (%)

moved from the model's regression equation. The regression equation of the model is as follows:

$$\text{CMCase (U/ml)} = -4.18 + 0.35667B + 0.31000D + 0.24333E \quad (R^2 = 92.07\%, \text{Adj. } R^2 = 82.55\%)$$

The fit of the model was tested through the correlation coefficient R^2 . A higher R^2 value indicates that the model is more suitable for explaining the variation of the dependent variables. According to Guan and Yao [28], the R^2 value of the model should be at least 80%. The obtained R^2 value of the model reached 92.07%, indicating that the model can explain 92.07% of the variation in the data (i.e., only

7.93% of the variation was not taken into account by the model), and the adj. R^2 value was 82.55%.

Conclusion. This study shows that the native cellulolytic bacterial strain *B. subtilis* DSC.03 isolated from durian peel compost samples exhibits relatively rapid growth and the ability to survive and thrive under stressful pH, temperature, and salinity conditions. The CMCase activity of the DSC.03 strain is profoundly affected by these culture conditions.

Through model analysis using the Plackett-Burman design, the three main factors affecting

cellulase production by *B. subtilis* DSC.03 was identified as culture temperature, CMC substrate concentration, and culture time. The optimal culture conditions for cellulase synthesis with the highest CMCase activity of *B. subtilis* DSC.03 were as follows: pH 7, 1.5% NaCl (w/v), 1% CMC (w/v), 2% inoculum (v/v), and 60 h at 40°C.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЦЕЛЮЛАЗНА АКТИВНІСТЬ *BACILLUS SUBTILIS* DSC.03 ЗАЛЕЖИТЬ ВІД ПАРАМЕТРІВ КУЛЬТИВУВАННЯ ШТАМУ

T. Q. Truong¹✉, K. D. Nguyen²

¹Faculty of Agriculture – Fisheries, University of Cuu Long, Vinh Long, Vietnam;

²Faculty of Biotechnology, Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam;

✉e-mail: truongquocdat@mku.edu.vn

Целюлаза, отримана з целюлозолітичних бактерій, відіграє дедалі важливішу роль у біотехнології та характеризується високим попитом на світовому ринку ензимів. Відомо, що бактеріальні штами роду *Bacillus* здатні синтезувати целюлазу, яка розщеплює β-1,4-глікозидні зв'язки з утворенням глюкози як кінцевого продукту. Проте активність і вихід ензиму залежать від багатьох чинників, зокрема від штаму бактерій і умов культивування. У дослідженні оцінювали активність целюлази *Bacillus subtilis* DSC.03 залежно від параметрів культивування штаму. *Bacillus subtilis* DSC.03 було ізольовано зі зразків компосту зі шкірки дуріану. Активність карбоксиметилцелюлази (КМЦ-ази) визначали за реакцією з 3,5-динітросаліциловою кислотою у сирому ензимному екстракті бактеріального штаму. Показано, що тривалість культивування, температура та концентрація субстрату суттєво впливали на активність КМЦ-ази штаму DSC.03. Оптимальні параметри становили: 60 год при 40°C, 1% карбоксиметилцелюлози та 2% інокуляту. За цих умов штам проявляв максимальну активність КМЦ-ази – 1,72 од/мл.

Ключові слова: *Bacillus subtilis* DSC.03, бактеріальна целюлаза, активність карбоксиметилцелюлази, умови культивування.

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