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Selection of the Constitutive Mutant of *Bacillus firmus* var. *alkalophilus* and its Characteristics of Cyclodextrin Glucanotransferase Production

KIM, CHAN, HYUN-DONG SHIN, AND YONG-HYUN LEE*

Department of Genetic Engineering, College of Natural Sciences
Kyungpook National University, Taegu 702-701, Korea

To investigate the role of induction on CGTase production for alkalophilic *Bacillus firmus* var. *alkalophilus* H609, the constitutive mutants that form a halo around its colonies at non-inducible AG agar media containing amylose and glucose were selected. The selected constitutive mutants could produce CGTase in the range of 18.9 to 28.8 units/ml · A_{600} in the alkaline basal medium, and finally a constitutive mutant *Bacillus firmus* var. *alkalophilus* CM46 was selected. The constitutive nature of CM46 was also confirmed in protein level using SDS-PAGE. The effects of induction and catabolite repression for both parent strain *Bacillus firmus* var. *alkalophilus* H609 and constitutive mutant CM46 were also compared by adding soluble starch and glucose during cultivation. The selected mutant CM46 was a non-inducible but a catabolite regulated type mutant. Even though inductive regulation was released, the specific CGTase activity defined as CGTase activity per cell concentration was not increased compared with that of parent strain. The cell growth and CGTase production patterns of constitutive mutant *Bacillus firmus* var. *alkalophilus* CM46 were compared with the parent strain to identify CGTase production characteristics.

Cyclodextrin glucanotransferase (EC 2.4.1.19: 1,4-α-glucan 4-α-D-(1,4-glucano) transferase, cyclizing; CGTase) is a multi-functional enzyme which catalyzes intramolecular transglycosylation (cyclization and coupling), intermolecular trans-glycosylation (disproportionation), and ring opening of cyclodextrins (CDs) (1). The major role of CGTase is to catalyze the formation of α-, β-, and γ-CD from starch or starch related materials (9).

CGTase is exclusively produced by several bacteria, mostly by genus *Bacilli,* and most of the CGTase from *Bacillus* sp. is an extracellular enzyme except for CGTase which is intracellularly accumulated in *Bacillus macerans* ATCC8514 (4). The CGTase biosynthesis in bacteria is usually triggered by inducers, and on the other hand, repressed by easily metabolizable carbon sources, such as glucose. The major inducible compounds for CGTase production include various glucose polymers, such as starch, dextrin, amylopectin, cyclodextrin, and glycogen (11), however, the precise induction mechanism have not yet been clarified.

CGTase production is also regulated by catabolite repression, however the detailed mechanisms of catabolite repression on CGTase biosynthesis have not yet been studied intensively. However, the catabolite repression for amylases production have been exculsively studied in their molecular, biological, and biochemical aspects. For *Bacillus* sp., Saier (13) suggests that the catabolite repression appears to be the result of inducer exclusion mechanism caused by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) that represses the metabolism of other sugars.

On the other hand, Stewart (15) insists that the catabolite repression in the gram-positive bacteria appears to be transcriptional regulation by negatively acting catabolite repressors. In *Bacillus* sp. and other gram-positive bacteria, *cis* elements within the promoter region have been identified as the target sequence for these repressors, and catabolite control protein A (Ccp A) from *Bacillus subtilis* has been identified by Henkin *et al.* (7).

In our previous work (6), an alkalophilic strain that produced β-type CGTase was isolated from hot-water spring soil, and identified as a *Bacillus firmus* var. *alkalophilus* H609. In the follow-up work (5), several catabolite repression resistant mutants were selected, and the catabolite repression effect on CGTase excretion was investigated in order to clarify catabolite repression

*Corresponding author
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mechanism and to achieve the overproduction of CGTase. The selected catabolite repression resistant mutant CR41 seemed to be damaged in the regulatory region of the CGTase gene, and could produce CGTase amounts of 250 units/ml three times higher than that of the parent strain. However, the detail of the induction mechanism or the role of the induction in CGTase biosynthesis cannot be clarified because the induction phenomenon of catabolite repression resistant mutant did not differ from that of the parent strain *Bacillus firmus* var. *alkalophilus* H609.

In order to understand the role of induction mechanism for CGTase biosynthesis, a constitutive mutant that released from induction regulation need to be selected and the induction effects of both parent and mutant strains have to be compared. In this work, several constitutive mutants of *Bacillus firmus* var. *alkalophilus* were selected by mutagenesis with NTG. The amount of CGTase produced and the production pattern of constitutive mutants were compared with those of the parent strain after cultivation under various cultivation conditions, such as presence of various inducible and catabolite repressive compounds. The role of induction effect in CGTase biosynthesis can be inferred, and will help in understanding the regulation mechanism of CGTase biosynthesis and strain development for overproduction of CGTase.

MATERIALS AND METHODS

Strain and Cultivation

The strain used in this work was alkalophilic *Bacillus firmus* var. *alkalophilus* H609 screened from hot-water spring soil previously in our laboratory (6). The cultivation media were alkaline basal medium I containing glucose (G medium) and alkaline basal medium II containing soluble starch (S medium) as suggested by Horikoshi *et al.* (11). For selection of the constitutive mutant, an alkaline basal medium containing 0.5% amylose and 0.5% glucose was formulated in this work designated as amylose and glucose medium (AG medium). A alkaline basal medium without any carbon sources (N medium) was also used in our work for confirmation of constitutive mutants. The cultivation was carried out at 37℃, pH 11.2, and 200 rpm in 250 ml shake flask.

Selection of Constitutive Mutants

The medium used for selection of the constitutive mutants was AG medium containing non-inducible compounds, amylose and glucose. The cells grown to exponential phase in G medium were harvested, and washed twice in saline solution (0.85% NaCl), and then 50 μg/ml of NTG disolved in 50mM Na-phosphate buffer (pH 7.0) was added for mutagenesis. The NTG treated cells were incubated for 60 min at 37℃ without shaking, and then washed again with saline solution. The treated cells were plated onto non-inducible medium (AG agar medium) supplemented with CGTase indicator of 0.02% phenolphtalein and 0.01% methyl orange (12). Yellow halo-forming colonies were selected first, and then selected colonies were recultivated in G, N, and S liquid media seperately for 72 hours. The cells that can produce CGTase in both G, N, and S media were seleceted as the constitutive mutants.

Measurement of Cell Growth and CGTase Activity

Cell concentration was determined indirectly by measuring the absorbance at 600 nm. For measuring the CGTase activity in the culture broth, 0.1 ml of culture filterate was added to the mixture solution of 0.4 ml of 40 mM tris-maleic NaOH buffer and 0.5 ml of 10% soluble starch desolved in the same buffer. The reaction mixture was incubated at 50℃ for 30 min, and then β-CD formed was measured by phenolphtalein method (8). One unit of CGTase activity was defined as the amount of enzyme that produce 1 mg of β-CD per hour and specific CGTase activity defined in this work as the enzyme activity per unit cell growth.

Cultivation by Addition of Inducible and Catabolite Repressible Compounds

To investigate the induction effect, the parent strain *Bacillus firmus* var. *alkalophilus* H609 and selected mutant strains were cultivated in alkaline basal media (G media) containing 1% glucose to its exponential phase, and then soluble starch was added to the final concentration of 0.1 and 1.0% (w/v), respectively, and then further cultivated for 48 hours. For the catabolite repression effect, the glucose was added instead of soluble starch to reach the final concentration of 0.1 and 1% (w/v), respctively. The changes of cell growth and CGTase activity of culture broth were measured during cultivation.

Electrophoresis of Culture Filtrate for Identification of CGTase

To identify the CGTase in the culture filtrate, SDS-PAGE was performed on 10% polyacrylamide gel after dialysis and concentration (3). Proteins were stained with coomassie brillant blue R-250, and CGTase activities were also detected by staining the gel with iodine reagent (0.02% I_2 + 0.2% KI solution) after incubation for 30min in 10% soluble starch that dissolved in 40 mM tris-maleic NaOH buffer (pH 6.0).

Analytical Methods

Glucose concentration was determined by DNS method (10), and soluble protein was measured by Bradford method (2) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Induction Phenomenon of *Bacillus firmus* var. *alkalophilus* H609 for CGTase Production

Bacillus firmus var. *alkalophilus* H609 was cultivated in various alkaline basal medium containing 1.0% of carbohydrates as carbon sources to investigate the induction effects on CGTase biosynthesis. Table 1 compares the cell growth, activity of CGTase, and specific CGTase activity defined in this work as the enzyme activity per unit cell growth after cultivation for 48 hours. As can be seen at Table 1 the CGTase was not excreted in the media containing simple carbohydrates, such as, glucose, fructose, lactose, and sucrose. On the other hand, *Bacillus firmus* var. *alkalophilus* H609 can synthesize CGTase from the range of 5.7 to 21.4 units/ml · A_{600} in the media containing various glucose polymers, such as, soluble starch, amylopectin, and β-cyclodextrin. However, among the glucose polymers, exceptionally amylose could not induce CGTase excretion of *Bacillus firmus* var. *alkalophilus* H609. It is well known that amylose can be used as a substrate for cyclodextrin production reaction of CGTase. Therfore it is a noticeble phenomenon that amylose can not induce CGTase production, and this unexpected result needs to be investigated.

Selection of Constitutive Mutants from *Bacillus firmus* var. *alkalophilus* H609

The role of induction effect on CGTase biosynthesis of *B. firmus* var. *alkalophilus* H609 can be clarified if the constitutive mutant that can produce CGTase without any inducible compounds is selected and then compared to the induction effects both of the parent and constitutive mutant strains. In order to select the constitutive mutants, a selection medium need to be formulated, in which CGTase production can not induce but can confirm the excreted CGTase using the halo around the colony on the agar plate. As can be seen in Table 1, amylose did not induce CGTase production but converted starch to CDs by CGTase. Based on the above observation, an AG medium containing amylose of 0.5% and glucose of 0.5% was formulated as a selection medium for constitutive mutant.

To confirm the susceptibility of AG medium for selection of constitutive mutant, one plate was incubated *B. firmus* var. *alkalophilus* H609 for 48 hours and the other was incubated by dropping the CGTase excreted by the same microorganism for 12 hours. As shown in Fig. 1 (A, B), for *B. firmus* var. *alkalophilus* H609 a halo was not formed around the colonies in AG medium (A), however a clear halo was formed around the dropped CGTase excreted by the same strain (B). This observation indicates that AG medium can be suitably utilized as a selection medium for constitutive mutants that can excrete CGTase without the presence of any inducible compounds.

Mutagenesis was performed with NTG to their survival rate of 5~10%, and then the treated cells were spread on the AG agar medium. After incubation for 72 hours, the yellow halo forming colonies in the AG medium were preliminarily isolated, and then the isolates were recultivated in S and AG agar medium for confirmation. Finally five halo-forming mutants were selected on the basis of halo clearness and then named as CM11, CM13, CM17, CM23, and CM46.

Fig. 2 compares the sizes of the halos formed during cultivation by the parent strain *B. firmus* var. *alkalophilus* H609 and the selected five mutants grown in S and AG media. In S medium, the sizes of the halo of mutant strains were larger than that of the parent strain during the early growth phase, however the size of the halo

Table 1. Comparison of cell growth and CGTase production of *Bacillus firmus* var. *alkalophilus* H609 grown on various carbon sources

Carbon sources	Growth (A_{600})	CGTase activity (units/ml)	Specific activity (units/ml · A_{600})
None	2.0	N.D.*	N.D.
Glucose	2.2	N.D.	N.D.
Fructose	2.4	N.D.	N.D.
Lactose	3.4	N.D.	N.D.
Sucrose	1.9	N.D.	N.D.
β-Cyclodextrin	2.8	15.9	5.7
Amylose	2.2	N.D.	N.D.
Amylopectin	2.0	42.7	21.4
Soluble strach	2.8	47.8	17.1

N.D.*: not detected

Bacillus firmus var. *alkalophilus* H609 was cultivated at 37℃ for 72 hours in 60 ml cap tube filled with 10 ml of alkaline basal media containing 1%(w/v) different carbon sources.

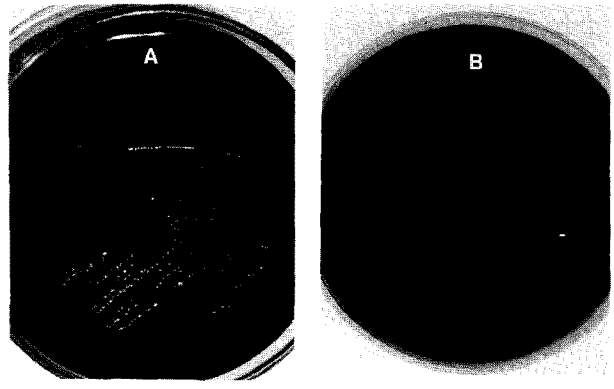

Fig. 1. Photograph of *Bacillus firmus* var. *alkalophilus* H609 grown in AG agar media (A) and the halo formed by CGTase produced by same strain grown in AG agar media (B).

of selected mutant strains became similar to that of parent strain after 48 hours. In AG medium, the selected mutants formed the halo, on the other hand, the parent strain could not formed. From the above results it can be confirmed that all selected mutants are constitutive type mutants, however, the amounts of excreted CGTase was not increased compared to the parent strain *B. firmus* var. *alkalophilus* H609.

Comparison of Selected Constitutive Mutants for CGTase Production

Table 2 compares cell growth, CGTase production, and specific CGTase activity of the parent and constitutive mutants that were cultivated in S, N, and G media, respectively. The cell growth of the selected mutants did not differ with that of the parent strain in the case of the same media. However, cell growth of all strains including *B. firmus* var. *alkalophilus* H609 showed differences among media, in N medium not containing carbon sources cell growth was as small as 60~80% compared with that grown in S and G media, due to the limitation of carbon sources for growth.

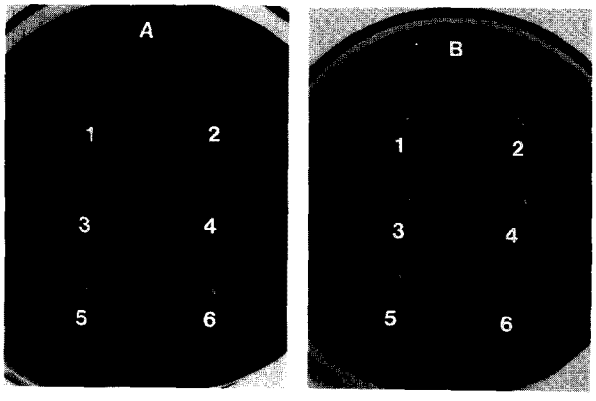

Fig. 2. Comparison of halo formed by *Bacillus firmus* var. *alkalophilus* H609 and selected constitutive mutants grown in S (A) and AG agar plates (B).
1: H609, 2: CM11, 3: CM13, 4: CM17, 5: CM23, 6: CM46

For the specific CGTase activity, the selected mutants showed similar activity to that of the parent strain in S medium. The specific activity of the parent strain in N medium was negligible, however the mutants could produce in the ranges from 18.9 to 28.8 units/ml · A_{600}, simillarly both in N and S media. This results also supported previous observation that the selected mutants could produce CGTase whether inducible compounds were present or not, but the capability of producing CGTase was not changed.

In G medium, mutant strains could produce CGTase in the ranges from 6.8 to 13.4 units/ml · A_{600}, however, parent strain could not produce CGTase. The amount of produced CGTase by mutants in G medium was around 40~60% of those of N and S media. This decrease in CGTase production in G medium may be due to the catabolite repression caused by glucose contained in the medium.

Among the five selected mutants, strain CM46 showed not only the highest amount of CGTase both in N and G media as 28.8 and 13.4 units/ml · A_{600}, respectively, but also similar cell growth to the parent strain *B. firmus* var. *alkalophilus* H609, therefore, CM46 was selected as a constitutive mutant used for the follow-up work.

SDS-PAGE Patterns of Excreted CGTase of Constitutive Mutant CM46 Grown in Different Carbon Sources

Fig. 3 compares the SDS-PAGE patterns of protein excreted in cultrue broth by the parent strain and the selected constitutive mutant strain CM46 that grown in G, N, and S media, respectively. The purified CGTase from *B. firmus* var. *alkalophilus* H609 whose molecular weight was 77 kDa (14) was used as the standard marker. The CGTase band could be easily identified among proteins contained in the culture broth at shown in Fig. 3 (A), and the CGTase activity could be also confirmed in the gel stained by iodine reagent after reaction with soluble starch for 30 min as could be seen in Fig. 3

Table 2. Comparison of cell growth and CGTase production of *Bacillus firmus* var. *alkalophilus* H609 and selected constitutive mutants grown in S, N, and G media

Strain	S media			N media			G media		
	G.[1]	C.A.[2]	S.A.[3]	G.	C.A.	S.A.	G.	C.A.	S.A.
Paraent strain									
H609	2.2	52.9	18.2	1.5	N.D.*	N.D.	2.9	N.D.	N.D.
Mutant strains									
CM11	1.9	55.5	19.8	1.3	25.0	19.2	2.7	18.5	6.8
CM13	2.2	40.4	19.2	1.5	31.1	20.7	2.6	24.3	9.3
CM17	2.5	51.0	22.8	1.3	25.0	19.2	2.3	13.1	5.7
CM23	2.4	40.1	17.5	1.8	34.0	18.9	2.0	26.4	13.2
CM46	2.4	53.1	23.1	1.4	40.3	28.8	2.2	29.5	13.4

G.[1]:Growth(A_{600}), C.A.[2]:CGTase activity(units/ml), S.A.[3]:Specific activuty(units/ml · A_{600}), N.D.*:Not Detected, *Bacillus firmus* var. *alkalophilus* H609 and its constitutive mutants were cultivated at 37℃ for 72 hours in 60 ml cap tube containing 10 ml of S, N, and G media.

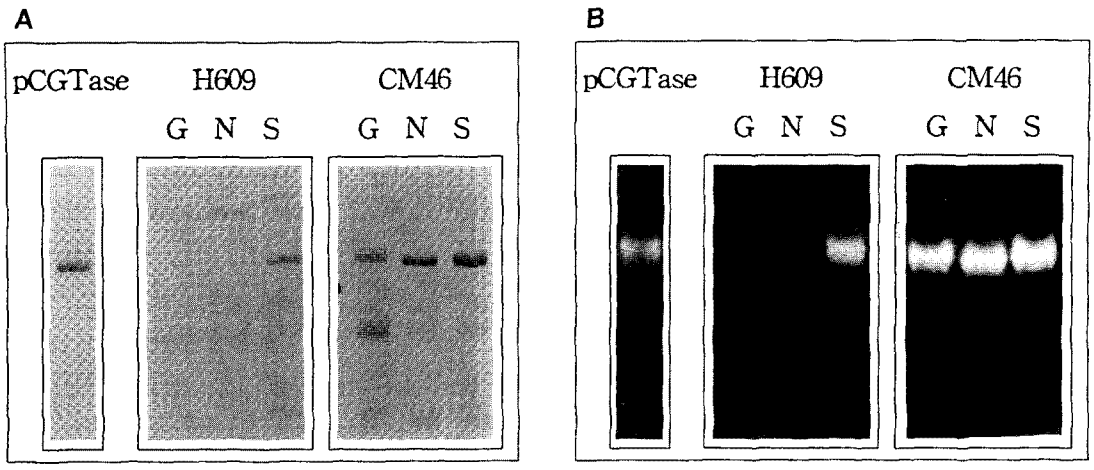

Fig. 3. Comparison of SDS-PAGE patterns of excreted CGTase from *B. firmus* var. *alkalophilus* H609 and its constitutive mutant CM46.
pCGTase: purified CGTase from *Bacillus firmus* var. *alkalophilus* H609, N, G, S: proteins in culture broth grown in G, N, and S media, respectively, A: PAGE gel was stained by coomassie brilliant blue R-250, B: PAGE gel was stained by 0.02% I_2-0.2% KI solution after 10min reaction in 10% soluble starch solution.

(B).

For parent strain *B. firmus* var. *alkalophilus* H609, the CGTase band cultivated in S medium was seen clearly in the same position with that of purified CGTase, however, the band did not appear in G and N media. By contrast, constitutive mutant CM46 showed the CGTase bands in all G, N, and S media, however, the intensity of the CGTase band in G medium was less strong than those of N and S media. CGTase activity staining by iodine reagent also showed similar results to the above result as shown in pannel (B). Above observations also support the fact that the selected mutant CM46 can constitutively produce the CGTase regardless the presence of inducible compounds, however CGTase production is still regulated by catabolite repression.

Regulatory Mechanism for CGTase Production of Constitutive Mutant CM46

Induction: The selected constitutive mutant *B. firmus* var. *alkalophilus* CM46 was cultivated in an alkaline basal medium containing 1.0% of various carbohydrates. Table 3 shows cell growth, CGTase production, and the specific CGTase activity after cultivation for 48 hours. The constitutive mutant CM46 produced the CGTase in the range of 23.1~30.8 units/ml · A_{600} in the medium containing inducible compounds, such as β-cyclodextrin, amylopectin and soluble starch. Especially mutant strain CM46 could produce noticeable amounts of CGTase corresponding to 26.5 units/ml · A_{600} even in the medium not containing carbon sources and 21.0 units/ml · A_{600} in the medium containing amylose that could not induce the CGTase production for the parent strain *B. firmus* var. *alkalophilus* H609. This result also indicates that constitutive mutant CM46 can constitutively produce the CGTase due to the release of induction mechanism for CGTase expression.

Also, the constitutive mutant CM46 could produce a small quantity of CGTase of 5.0~10.1 units/ml · A_{600} in the medium containing various mono- or di-saccharides that did not induce the CGTase production for the parent strain, which were around 19~38% of that of parent strain grown in S medium. This observation indicates that the constitutive mutant CM46 is still subjected to catabolite repression even though the strain is released from induction.

To compare the induction effect caused by soluble starch for the parent and constitutive mutant strains, soluble starch was added to the culture broth at an early stationary phase(O.D. ≥ 2.0), and the changes of specific CGTase activities during the cultivation was measured as shown in Fig. 4. The parent strain *B. firmus* var. *alkalophilus* H609 could not produce CGTase without soluble starch, however, adding to the final concentration of 0.1 and 1.0%, the CGTase production initiated from after 3 hours and reached 4.0 and 16.0 units/ml · A_{600} after 72 hours. The specific CGTase activity was changed significantly by the addition of soluble starch, which was proportionally increased as starch concentration increased.

Otherwise constitutive mutant CM46 produecd CGTase to 12.5 units/ml · A_{600} without the addition of soluble starch, and adding to the final concentration of 0.1 and 1.0% the CGTase production reached 13.0 and 14.0 units/ml · A_{600} after 72 hours, respectively. The pattern of specific CGTase activity was scarcely changed regardless of the addition of soluble starch, indicating that the constitutive mutant CM46 is not regulated by in-

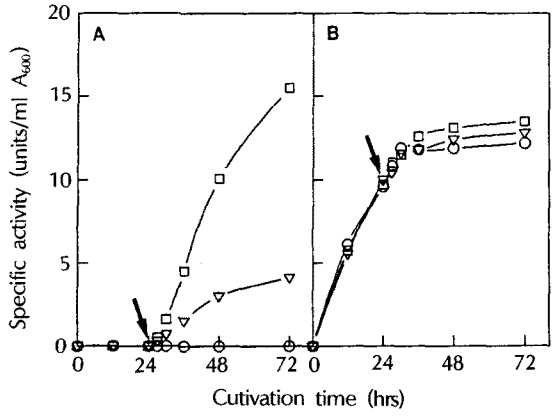

Fig. 4. Effect of soluble starch addition on CGTase pro duction during the cultivation of *B. firmus* var. *alkalophilus* H609 (A) and constitutive mutant CM46 (B) using glucose as a carbon source. Soluble strach were added to be a final concentration of 0.1, and 1.0% (w/v), respectively, at the point indicated by the arrow.
○–○: no addition, ▽–▽: soluble starch of 0.1% □–□: soluble starch of 1.0%

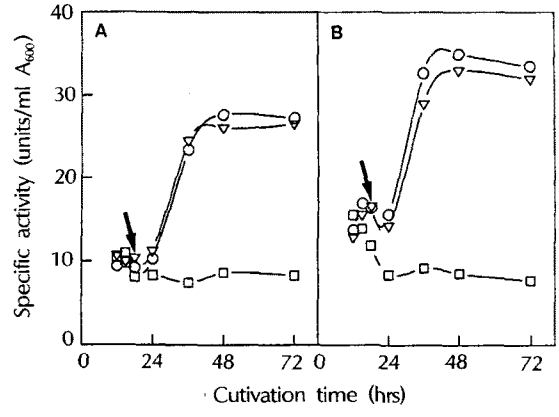

Fig. 5. Effect of glucose addition on CGTase production during the cultivation of *B. firmus* var. *alkalophilus* H609 (A) and constitutivemutant CM46 (B) using soluble starch as a carbon source. Glucose were added to be a final concentration of 0.1, and 1.0% (w/v), respectively, at the point indicated by the arrow.
○–○: no addition, ▽–▽: glucose of 0.1%, □–□: glucose of 1.0%

Table 3. Comparison of cell growth and CGTase production of constitutive mutant CM46 grown on various carbon sources

Carbon sources	Growth (A_{600})	CGTase activity (units/ml)	Specific activity (units/ml · A_{600})
None	1.7	45.1	26.5
Glucose	4.6	23.0	5.0
Fructose	3.7	19.0	5.1
Maltose	3.3	24.0	7.2
Lactose	2.1	21.3	10.1
Sucrose	3.1	25.4	8.2
β-Cyclodextrin	1.9	43.8	23.1
Amylose	2.6	54.5	20.9
Amylopectin	1.9	58.5	30.8
Soluble strach	2.3	61.4	26.7

Constitutive mutant CM46 was cultivated at 37℃ for 72 hoursin 60 ml cap tube containing 10 ml of alkaline medium containing 1%(w/v) of various carbon sources.

duction. It can be postulated that the enhancement of CGTase productivity may be scarcely achieved through the release from induction.

Catabolite Repression: As shown in Table 3, the constitutive mutant CM46 produced only a small quantity of CGTase in the media containing simple carbohydrates, so it might be still subjected to catabolite repression even though the induction regulation is released. To confirm the above postulation, glucose was added to the culture broth both in the parent and mutant strains when the CGTase production was initiated(10~15 units/ml · A_{600}). Fig. 5 shows the changes of the specific activities of both parent and mutant strains during cultivation.

When glucose was not added, CGTase production of the parent strain *B. firmus* var. *alkalophilus* H609 reached up to 26.0 units/ml · A_{600} after 72 hours, however when glucose was added to the final concentration of 0.1 and 1.0% CGTase production decreased to 25.0 and 8.0 units/ml · A_{600}, respectively. The production of the constitutive mutant CM46 also decreased by the addition of glucose similar with parent strain. This fact indicates that the constitutive mutant CM46 is still regulated by catabolite repression regardless of the released induction.

Cultivation of Constitutive Mutant *B. firmus* var. *alkalophilus* CM46 for CGTase Production

Fig. 6 compares the changes in cell concentration, pH and the CGTase activity of parent strain (A) with those of constitutive mutant *B. firmus* var. *alkalophilus* CM46 (B). The parent strain showed relatively long lag time, on the other hand, the constitutive mutant CM46 grew more rapidly without lag time, and the stationary phase was reached after 24 hours in contrast to 36 hours for the parent strain. The CGTase production of constitutive mutant CM46 was also more rapid compare to the parent strain, and CGTase produced was in proportional relationship with cell growth.

However the amount of CGTase produced was increased up to 110.0 units/ml after 72 hours for mutant CM46, which was higher than 80.0 units/ml for the parent strain, this may be due to the accumulated CGTase at an early cultivation stage.

Conclusively, the constitutive mutant *Bacillus firmus* var. *alkalophilus* CM46 can produce CGTase without induction, however it is still strongly regulated by catabolite repression. The specific CGTase activity defined the amount of CGTase per unit cell growth is not sub-

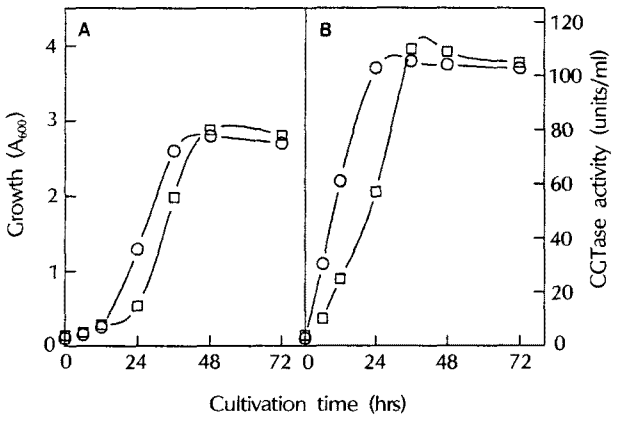

Fig. 6. The cell growth and CGTase production patterns of constitutive mutant CM46(A) and *B. firmus* var. *alkalophilus* H609(B) grown in S medium
○—○ : cell growth, □—□ : CGTase activity

stantially increased compared to that of the parent strain *Bacillus firmus* var. *alkalophilus* H609, even though the total amount increased slightly. It can be postulated that the role of induction seems to contribute to the initiation of CGTase production, however, it is not solely engaged in the determination of the amount of CGTase. The interrelationship between the induction and catabolite repression in CGTase production needs to be studied to understand precisely, not only, the regulation mechanism of CGTase production of *Bacillus firmus* var. *alkalophilus* H609, but also, the strain development to achieve overproduction of CGTase.

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