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Selection and Characterization of Catabolite Repression Resistant Mutant of *Bacillus firmus* var. *alkalophilus* Producing Cyclodextrin Glucanotransferase

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In order to elucidate the mechanism which regulates the production of cyclodextrin glucanotransferase (CGTase) and to achieve overproduction of CGTase by releasing catabolite (glucose) repression, several catabolite repression resistant mutants were selected from newly screened *Bacillus firmus* var. *alkalophilus* H609, after NTG (N-methyl-N'-nitro-N-nitrosoguanidine) treatment, using 2-deoxyglucose as a nonmetabolizable analog of catabolite glucose and as a selection marker. Five catabolite repression resistant mutants were selected from about 30,000 2-deoxyglucose resistant colonies. Relative catabolite repression indices of the selected mutants were in the range of 8~80% assuming 100% for parent strain. The amount of CGTase produced by the mutant strain CR41, which was 250 units/ml, was three times larger than that produced by its parent strain. The mutation seems to have occurred in the regulatory region of CGTase gene and not in the structural region or the glucose transporting system in cell membrane. The enzymatic properties of CGTase excreted from parent and mutant strains were also compared.

Cyclodextrin glucanotransferase (EC 2.4.1.19 : 1,4-α-glucan 4-α-D-(1,4-glucano) transferase, cyclizing; CGTase) is an enzyme which can convert starch or other α-1,4-glucans to cyclodextrins(CDs) by intramolecular transglycosylation. CGTase is produced by two groups of *Bacillus* sp.; one is neutrophilic *Bacillus*, such as *Bacillus macerans, B. circulans, B. megaterium, B. licheniformis,* and *B. stearothermophilus* (11), and the other is alkalophilic *Bacillus*, such as *B. circulans* var. *alkalophilus* and *B. ohbensis* sp. nov. C-1400. The enzymatic properties and the gene structure of *B. circulans* var. *alkalophilus* (ATCC 21783) was intensively investigated by Nakamura and Horikoshi (7, 13), and those of *B. ohbensis* sp. nov. C-1400 was mainly investigated by Yagi et al. (17, 18).

In our previous work (5), an alkalophilic microorganism appropriate for overproducing CGTase was newly screened from hot-water spring soil, and identified as *Bacillus firmus* var. *alkalophilus* H609. The newly screened strain excreted the considerable amount of CGTase producing mainly β-cyclodextrin, corresponding to 75 units/ml at 37℃, initial pH of 11.2, and after 40 hours. Because of its high stability during prolonged preservation and the efficient enzyme production, the selected strain could be used for a large scale production of CGTase.

It is well known that the production of various carbohydrate-hydrolyzing enzymes by microorganisms is subjected to the catabolite repression by rapidly utilizable carbon sources such as glucose, and to the induction caused by an inducer such as starch. Utilization of catabolite repression resistant mutants is one of the most common methods used to improve industrial strain for the overproduction of various carbohydrate-hydrolyzing enzymes.

The regulation mechanism of CGTase synthesis by various alkalophilic *Bacillus* has not been thoroughly investigated yet. However, judging from the fact that the production of CGTase was enhanced in media containing starch when compared with other media containing glucose or maltose (13), it can be postulated that CGTase production from alkalophilic *Bacillus* sp. was also subjected to catabolite repression and induction.

The objectives of this work were; to screen catabolite repression resistant mutants of *Bacillus firmus* var. *alkalophilus* H609 to achieve overproduction of CGTase;

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and to characterize enzyme synthesis properties of the selected mutants. Several catabolite repression resistant mutants were selected after NTG (N-methyl-N'-nitro-N-nitrosoguanidine) treatment, using 2-deoxyglucose, a nonmetabolizable analog of catabolite glucose(antimetabolite) as a selection marker. The growth and the CGTase synthetic characteristics of the selected mutant were investigated, along with the physiological and microbiological characteristics. These studies will help not only to achieve the overproduction of CGTase but also to elucidate the producing mechanism of CGTsae from alkalophilic *Bacillus* sp..

MATERIALS AND METHODS

Strain and Growth Media

The strain used in this study was newly screened *Bacillus firmus* var. *alkalophilus* H609 in our laboratory (5). The growth media used were Horikoshi's alkaline basal medium I containing glucose as carbon source (in this work : G medium) and Horikoshi's alkaline basal medium II containing soluble starch (in this work : S medium) (13). An alkaline basal medium containing 0.5% glucose and 0.5% soluble starch were used as repressive media for CGTase production which was designated as GS medium in this work. The cell was cultivated at 37℃, pH 11.2 and 200 rpm, and cell concentration was determined by measuring the absorbance at 600 nm.

Mutagenesis and Screening of Catabolite Repression Resistant Mutant

Bacillus firmus var. *alkalophilus* H609 cell grown in G medium to exponential phase was treated with 200 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG) dissolved in 50 mM Na-phosphate buffer(pH 7.0) for 1 hour at 37℃. The NTG treated cell was plated onto a 0.1% 2-deoxyglucose (2-DG) supplemented S medium containing 0.02% phenolphthalein and 0.01% methyl orange as indicators of CGTase production (15). After 24 hours, the yellow halo-forming colonies were selected, and then the selected colonies were reinoculated in both glucose supplemented (GS media) and non-supplemented agar media (S media). The yellow halo-forming colonies in both media were selected as the catabolite repression resistant mutants.

Cultivation of Catabolite Repression Resistant Mutant in Repressive Media

The selected mutants were cultured in non-repressive S medium until the exponential phase, and then harvested and washed with saline solution. The washed mutant cells were suspended to equal volume of repressive GS media containing 0.5% glucose, and successively cultivated for 24 hours during which the cell growth and the CGTase production were monitored.

Measurement of CGTase Activity

0.1 ml of enzyme solution was mixed with 0.9 ml of 5% soluble starch in 40 mM tris-maleic-NaOH buffer (pH 6.0) and incubated at 50℃ for 1 hour. The formed β-CD which was formed in result, was determined by colorimetric method (9) which monitored the reduction of color intensity of phenolphthalein, resulting from complex formation with produced β-CD. One unit of the produced CGTase was defined as the amount of enzyme that can form 1 mg of β-CD per hour.

Cell Cultivation at the Presence of Glucose Analog

Parent and mutant cells were cultivated in alkaline media containing non-phosphotransferase system (PTS) sugar, maltose as carbon source. After cell growth reached the exponential phase, various glucose analogs were added to reach the final concentration of 5 mM, and then further cultivated for 24 hours.

Polyacrylamide Gel Electrophoresis of CGTase Concentrate and Activity Staining of Starch Dextrinizing Activity

The enzyme concentrates of culture broth were electrophoresed by Davis method (4) with 3 mA per well. Half of the electrophoresed gel was stained with coomassie brilliant blue R250 and the other half was stained with Iodine reagent (0.02% I2/ 0.2% KI solution) after 15 min of reaction in 5% soluble starch dissolved in 10 mM tris-maleic NaOH buffer (pH 6.0).

Analytical Methods

Glucose concentration was determined by DNS method (12). Cyclodextrin concentration was determined by colorimetric method (9) or high performance liquid chromatography (HPLC) using the following; Cosmosil 5NH_2 (Nacalai Tesque Co.), acetonitrile/water (65 : 35), 1 ml/min, and RI detector. Soluble protein was determined by Bradford method (3) using bovine serum albumin as standard.

Determination of Repression Index

Repression index, which shows the degree of the reduction of catabolite repression to glucose, was determined by measuring the CGTase activity and the residual glucose concentration after 24 hours of cultivation in S and GS medium, respectively. The repression index was defined as follows;

$$\text{Repression index(RI)} = \frac{[\text{CGTase}]_S/[\text{CGTase}]_{GS}}{[\text{Glucose}]_S/[\text{Glucose}]_{GS}}$$

Where $[\text{CGTase}]_S$ = CGTase activity in S medium after 24 hours

$[\text{CGTase}]_{GS}$ = CGTase activity in GS medium after 24 hours

$[\text{Glucose}]_S$ = Residual glucose concentration in S medium after 24 hours

$[Glucose]_{GS}$=Residual glucose concentration in S medium after 24 hours

RESULTS AND DISCUSSION

Catabolite Repression of *Bacillus firmus* var. *alkalophilus* H609 for CGTase Production

In order to investigate whether the CGTase production of *Bacillus firmus* var. *alkalophilus* H609 was also subjected to the catabolite regulation by rapidly utilizable carbon sources, its parent strain was cultivated in an alkaline basal media containing 0.5% soluble starch and different amounts of glucose. As shown in Table 1, glucose concentration did not affect cell growth greatly, however, the CGTase production was affected substantially by the presence of glucose and decreased proportionally to glucose concentraion. The amount of CGTase produced per unit cell was the greatest value at the non-glucose supplemented media, and gradually decreased as glucose concentration increased, and especially, at a glucose concentration higher than 0.6%, the amount of CGTase produced was the smallest. This observation shows that CGTase production of *Bacillus firmus* var. *alkalophilus* H609 is also closely controlled by the presence of rapidly utilizable catabolite glucose. The observed catabolite repression phenomenon for CGTase production in other *Bacillus* sp. has not been previously documented.

Mutagenesis and Screening of Catabolite Repression Resistant Mutants

The observation from above shows that CGTase production in *Bacillus firmus* var. *alkalophilus* H609 is initiated only when glucose concentration in culture media remained low level. Therefore the CGTase production can be enhanced by developing catabolite repression resistant mutant, in which the CGTase production will not be affected by the presence of high level of glucose. Catabolite derepressed mutants have been widely used in other bacteria (1, 8), yeasts (2), and fungi (6) for the overproduction of different kinds of inducible enzyme.

Nonmetabolizable glucose analog, 2-DG, was used as a selection marker in this work, its optimal concentration was determined to be 0.1%, because about 5~10% of cells, which is a appropriate amount, survived in S medium after 24 hours. Halo-forming colonies in S medium containing nonmetabolizable glucose analog, 2-DG were selected as the potential glucose catabolite repression resistant mutant for CGTase production.

After NTG treatment, about 30,000 halo-forming colonies, with sizes similar or larger than that of the parent strain, in 0.1% 2-DG supplemented S medium were preliminarily selected. In order to confirm the CGTase production capability, the selected colonies were recultivated both in GS and S agar media, and five halo-forming colonies were finally selected, and named as CR41, CR 45, CR102, CR103, and CR106.

Fig. 1 compares the characteristics of the halos formulated by the parent and by the selected 5 mutant strains both in S and GS media. The sizes of halos formulated by the mutant in S media were similar or larger than that formulated by the parent strain (Fig. 1(A)), and much clearer halos were also formulated by the mutant in S media compared with that formulated by the parent strain in GS media containing glucose (Fig. 1(B)). The ratios of the halo size to the colony diameter were quite different among strains, showing that the CGTase production of parent strain was more severely suppressed than selected mutant strains.

Table 1. Effect of the mixing ratios of glucose and starch on the growth and CGTase production of *Bacillus firmus* var. *alkalophilus* H609.

Carbon source (% w/v)		Cell growth (A_{600})*	CGTase activity (Unit/ml)	CGTase activity/ Cell growth (Unit/ml·A_{600})
Glucose	Starch			
1.0	0.5	4.4	13.72	3.12
0.8	0.5	3.8	18.10	4.76
0.6	0.5	4.0	23.80	5.95
0.4	0.5	4.3	43.62	10.14
0.2	0.5	3.4	44.48	13.08
0.0	0.5	2.3	37.56	16.50

Bacillus firmus var. *alkalophilus* H609 was cultivated in the alkaline basal medium containing 0.5% of soluble starch (S medium) and different glucose concentration at 37℃ for 48 hours.

*Absorbance at 600 nm

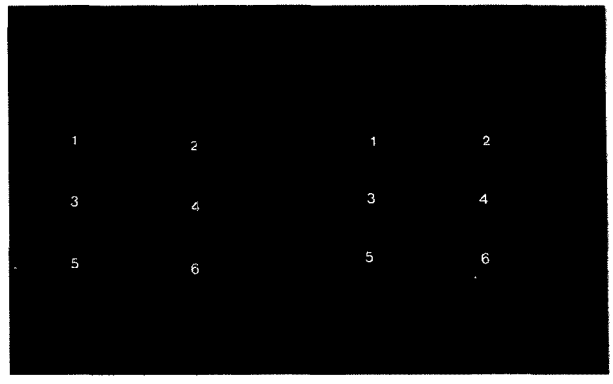

Fig. 1. The clear zone appeared around the colonies of parent and isolated mutant strains in non-repressive S media (A) and repressive GS media (B).

1; parent starin *Bacillus firmus* var. *alkalophilus* H609, 2; catabolite repression resistant mutant strain CR41, 3; CR45, 4; CR 102, 5; CR103, 6; CR106

Utilization of catabolite repression resistant mutant for strain improvement was also has been carried out for cellulase production by the hypercellulolytic mutant of *Cellulomonas* sp. (1), and α-amylase production by the mutants of *B. cereus* (19).

Comparison of Selected Catabolite Repression Resistant Mutant Strains for Growth and CGTase Production

Table 2 compares relative growth of the parent and the selected mutant strains on S medium containing 2-DG, their CGTase activities and repressive indices. The growth of the selected mutants was in the range of 54.8 % to 95.6% whereas that of parent strain was in 7.6% in 0.1% 2-DG supplemented S media, considering 2-DG unsupplemented as 100%. The amounts of CGTase produced by the selected strains were also higher than that by parent strain 1.57 to 2.66 times.

The relative catabolite repression indices to glucose of selected mutants varied from the lowest 7% for mutant strain CR103 to the highest 55% for CR102, considering that of parent strain as 100%. Most of the selected mutants were not subjected to absolute repression but were partially repressed. This may be due to the partial desensitization of the regulatory region of the catabolite repression CGTase operon. A phenomenon simillar to catabolite repression has been observed in α-amylase production of *B. subtilis* 168 harvoring *gra*10 mutation, which bring to decrease the binding affinity of the catabolite repression mediating protein factor (14).

Among the five isolated mutants, CR103 was the least susceptible to catabolite repression, however, the amount of CGTase production was relatively low compared to other mutant strains. This may be due to the occurence of other type of mutation that affects the CGTase production, and not its catabolite repression resistance. Meanwhile, for the mutant strain CR41, the repression index was relatively high, being around 50%, however, its CGTase production was the highest, 2.66 times higher than that of parent strain. Therefore, among the five isolated strains, CR41 was selected for further studies.

Table 2. The comparison of growth, CGTase activity, and repression index of parent and selected catabolite repression resistant mutant strains

Strains	Relative growth on 2-DG (%)[a]	CGTase activity[b] (Unit/ml)	Repression index[c]
Parent strain			
H609	7.62	90.3	100.0
Mutant strains			
CR41	69.00	239.7	54.2
CR45	93.18	158.8	17.2
CR102	95.65	150.0	54.9
CR103	54.76	142.1	7.1
CR106	94.70	168.5	20.9

[a]Cultivated both in S medium and S medium supplemented with 0.1% 2-DG for 24 hours, and then calculated comparing them.
[b]Cultivated in S media for 72 hours.
[c]Grown in S and GS media for 24 hours. The repression indices were determined as described in Materials and Methods.

The Differential Rate of CGTase Synthesis of Parent and Catabolite Repression Resistant Mutant Strain CR41

In order to compare the effect of catabolite glucose on CGTase production for both parent and mutant strain CR41, both cells were cultivated in S media till the exponential growth phase, and then transferred to repressive GS media. As shown in Fig. 2, in contrast to the parent strain, CGTase production of mutant strain was not affected seriously after being transferred to GS media from S media. On the other hand, the glucose utilization rates of both parent and mutant strain CR41 were similar during the growing period, which indirectly indicated that the catabolite repression resistance was not resulted from the decreased capability of glucose utilization.

CGTase Production Characteristics of Catabolite Repression Resistant Mutant Strain CR41

Glucose Repression: As shown in Table 3, the degree of decrease in CGTase production by catabolite glucose was not severe compared with that by parent strain previously described in Table 1. At the glucose concentration of 0.6%, the parent strain only produced around 36% of CGTase, whereas the mutant strain produced about 75% in terms of CGTase activity per cell growth considering to 100% for non-glucose supplemented media.

Induction: The tendency of growing cells at various carbon sources was not much different between the mutant and parent strain as shown in Table 4. However

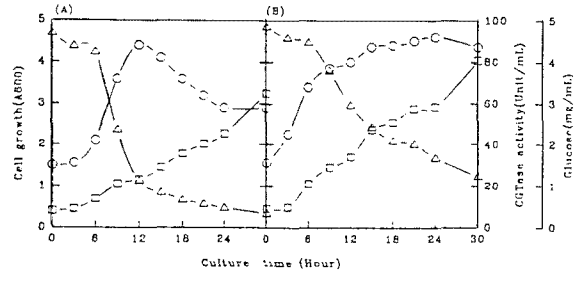

Fig. 2. Effect of glucose on the differential rate of CGTase production in parent strain (A) and catabolite repression resistant mutant CR41 (B).
Cells grown in S media to exponential phase were collected and then the same amount of cell was recultivated in GS media. ○−○; Cell density, □−□; CGTase activity, △−△; Residual glucose concentration.

Table 3. Effect of mixing ratio of glucose and starch on the growth and CGTase production of catabolite repression resistant mutant strain CR41.

Carbon source (% w/v)		Cell growth (A_{600})*	CGTase activity (Unit/ml)	CGTase activity/ Cell growth (Unit/ml·A_{600})
Glucose	Starch			
1.0	0.5	3.0	33.34	11.30
0.8	0.5	2.8	44.74	15.98
0.6	0.5	2.4	46.16	19.23
0.4	0.5	2.3	47.58	21.15
0.2	0.5	2.2	62.04	28.20
0.0	0.5	2.1	74.24	25.50

Mutant strain CR41 was cultivated in the alkaline basal medium containing 0.5% of soluble strach (S medium) and different glucose concentration for 48 hours.

the amount of CGTase produced increased substantially in mutant strain CR41. It increased from about 3.0 to 5.0 times relative to parent strain. This increase in CGTase production by mutant strain was the consequence of the catabolite repression being released, which lead enzyme production in early growth phase and thereafter sustained production.

In both parent and mutant strains, it is noticeable that the CGTase production increased more in media containing various glucose-polymers such as, β-cyclodextrin, amylopectin, corn starch and soluble starch, compared with the media containing various simple mono- or disaccharides as carbon sources. This indicates that induction has an important role in CGTase production for both the parent and the mutant strains. A similar unchanged induction pattern was also observed in the α-amylase hyperproducing catabolite derepressed mutant of *Bacillus* sp. 2B(10).

It is also noticeable that CGTase production was not induced at all by amylose that consisted of unbranched α-1,4 glycosidic bond. It means that the induction compound of CGTase sythesis probably was caused effectively by branched chain structures containing α-1,6 glycosidic bonds, not by unbranched chain structure. This fact could be utilized for developing inducers containing branched α-1,6 type glycosidic bonds as an effective inducer compound.

Repression of Other Simple Carbohydrates: Table 5 shows the influence of simple carbohydrates on the CGTase production by the parent and the mutant strains. For parent strain CGTase production was greatly repressed by the added simple carbohydrate, whereas repression was not so great in mutant strain CR41. Arabinose and galactose scarcely affected the CGTase production in both parent and mutant strains showing similar relative productivity in medium containing soluble

Table 4. The effects of various carbon sources on cell growth and CGTase production of parent strain and catabolite repression resistant mutant strain CR41

Carbohydrate	*B. firmus* var. *alkalophilus* H609		Mutant CR41	
	Growth (A_{600})	CGTase (Unit/ml)	Growth (A_{600})	CGTase (Unit/ml)
Xylose	1.9	6.9	2.1	21.0
Glucose	2.1	6.1	2.8	19.5
Fructose	1.9	7.2	2.8	13.7
Maltose	3.0	5.7	3.2	19.5
Lactose	2.2	5.3	2.4	13.7
Sucrose	1.5	4.1	2.8	13.7
Inositol	1.6	4.1	2.3	12.2
Sorbitol	1.0	7.2	0.8	15.2
β-cyclodextrin	1.5	24.4	2.8	170.0
Amylose	2.2	5.3	2.5	11.4
Amylopectin	2.1	28.8	2.1	129.7
Corn starch	2.4	31.0	2.9	140.5
Soluble starch	2.2	45.4	1.9	226.7

Cells were cultivated in alkaline basal medium containing 1.0% of different carbohydrates as carbon sources for 48 hours.

starch. Whilst xylose and glycerol severely repressed the parent strain, on the other hand, mutant strain was not so repressed.

Glucose Transport System of *Bacillus firmus* var. *alkalophilus* and Mutant Strain

It is well known that the transport of monosaccharides was mediated by phosphotransferase system (PTS) and, that the transport of disaccharides was mediated by a non-PTS system, both in Gram negative and positive bacteria (16). If the selected catabolite repression resistant mutant is caused by the defection in the glucose transport system, the cell growth can not be surpressed by nonmetabolizable glucose analogs, such as, 2-DG and α-methylglucoside (α-MG). To investigate the possibility of such defection in glucose transport system, both cells were cultivated in alkaline basal medium containing 1.0% maltose instead of glucose, and then 2-DG or α-methylglucoside was added to the culture broth at the exponential growth phase.

As shown in Fig. 3, both strains grew equally well on non-PTS sugar, maltose, as the sole carbon source. When 2-DG or α-methylglucoside was added to the medium, the grown of both strains were immediately surpressed. This shows that mutant CR41 does not have any defects in glucose transport system, indicating that the catabolite repression resistance is not resulted from the decreased capability of glucose utilization caused by defection in glucose transport system, but by other mutation mechanism.

Table 5. The influences of various simple carbohydrates on catabolite repression resistance of parent and mutant strain CR41.

Carbohydrate (0.5+0.5)%	*B. firmus* var. *alkalophilus* H609				Mutant CR41			
	Growth (A_{600})	CGTase (Unit/ml)	CGTase/Growth (Unit/ml·A_{600})	RP* (%)	Growth (A_{600})	CGTase (Unit/ml)	CGTase/Growth (Unit/ml·A_{600})	RP* (%)
Arabinose	2.1	41.6	19.8	96.6	2.9	121.1	41.8	94.1
Maltose	2.7	15.2	5.6	27.3	2.3	54.9	23.9	53.8
Glucose	2.9	15.5	5.3	25.9	3.8	83.8	22.1	49.8
Xylose	5.6	34.0	6.1	29.8	3.3	118.2	35.8	49.8
Galactose	3.0	56.0	18.7	91.2	3.3	124.1	37.6	84.7
Ribose	4.5	59.5	13.2	64.6	2.4	124.1	51.7	116.4
Lactose	2.2	32.5	14.8	72.2	2.6	95.2	36.6	82.4
Glycerol	2.0	10.1	5.1	24.9	2.5	95.2	38.1	85.8
Sorbose	2.7	25.2	9.3	45.4	3.4	83.8	24.6	55.4
Fructose	3.1	21.8	7.0	34.1	2.8	89.5	32.0	72.1
Sucorse	3.0	6.3	2.1	10.2	2.8	66.7	23.8	53.6
Sluble starch (Control)	2.3	47.1	20.5	100.0	3.2	142.2	44.4	100.0

Cell was cultivated in alkaline basal media containing 0.5% soluble starch (S medium) and 0.5% of various kinds of simple carbohydrates.

*RP: Relative productivity;

$$\frac{\text{CGTase activity/Growth in different carbohydrates}}{\text{CGTase activity/Growth in soluble starch alone}} \times 100$$

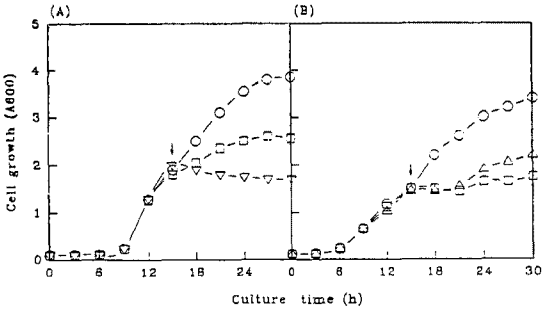

Fig. 3. The Inhibitory cell growth of parent strain (A) and catabolite repression resistant mutant strain CR41 (B) on non-PTS sugar, maltose, by non-metabolizable glucose analog.

α-MG and 2-DG were added to the final concentration of 5 mM at the point indicated by the arrow.

○−○; Control, □−□; α-Methylglucoside (α-MG), △−△; 2-Deoxyglucose (2-DG)

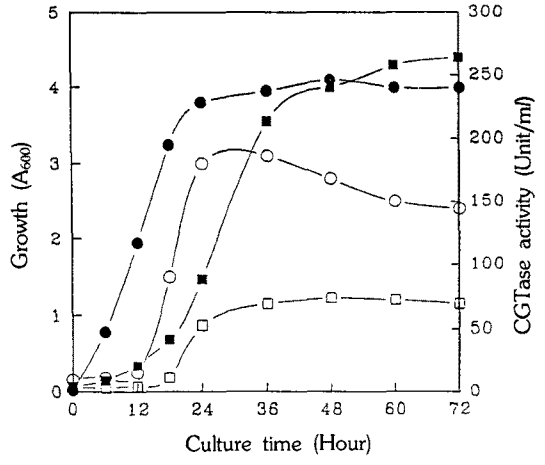

Fig. 4. Time course of cell growth and CGTase production patterns of parent and catabolite repression resistant mutant strain CR41 in S medium.

Cells were cultivated in 5.0*l* jar fermentor at 37℃, 200 rpm, and 1.5 vvm for 72 hours.

Cell density; ○−○ parent strain, ●−● mutant strain CR41

CGTase activity; □−□ parent strain, ■−■ mutant strain CR41

Cultivation of Mutant Strain CR41 for CGTase Production

Fig. 4 illustrates the cell growth and CGTase production patterns of mutant strain CR41 cultivated in S media. The CGTase production increased consistently in proportion to the cell growth, similarly with parent strain, except that the parent strain had a long lag time of CGTase production in early growth phase. The amount of CGTase in culture broth increased consistently upto 250 units/ml after 72 hours cultivation, which was about 3.0 times higher than by parent strain.

Comparison of CGTase Properties of *Bacillus firmus* var. *alkalophilus* and Mutant Strain CR 41

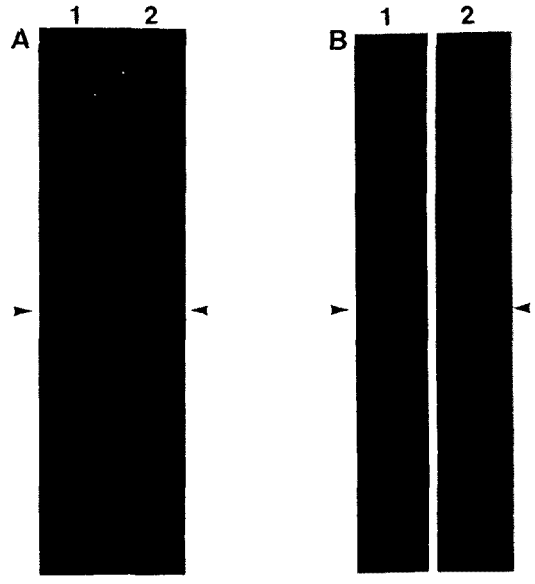

Fig. 5. Protein bands and activity staining of CGTase concentrates of culture filtrates of parent (Lane 1) and that of mutant strain CR41 (Lane 2).
A: Polyacylamide gel was stained by coomassie brilliant blue R-250.
B: Polyacylamide gel was stained by 0.02% I_2-0.2% KI solution after 15 min reaction in 5% soluble starch solution.

The V_{max} and K_m values of CGTase from parent and mutant strains were determined after their reaction with soluble starch used as a substrate. The V_{max} and K_m values of CGTase of mutant strain CR41 was 2.453 mg/ml·min and 0.015 2mg/ml, respectively, and similar values were also obtained for parent strain *B. firmus* var. *alkalophilus* H609. The mixing ratio of produced cyclodextrin was 0 : 6.88 : 1 (α-: β-: **d**-CD) after 24 hours of reaction with 5% soluble starch similar values for both parent and mutant strains, indicating indirectly that the genetic modification did not occur in structural region of CGTase gene.

Proteins in culture filtrate of the both strains were electrophoresed, and their activity was stained as described in Materials and Methods. One strong starch-hydrolyzing band was observed in the same position in both strains as shown in Fig. 5. These observation also showed that the mutant CR41 had a defect in the regulatory region which resulted in catabolite repression resistance, but was not mutated in structural region of CGTase gene.

Further studies for optimization of CGTase production conditions of selected mutant strain need to be conducted along with further efforts to illucidate control mechanisms of enzyme production, which will help to clarify regulating mechanism more precisely and achieve industrial scale production of CGTase.

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