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Regulatory mutations affecting the synthesis of cellulase in *Pseudomonas fluorescens*

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Pseudomonas fluorescens, was cultured in basal medium containing carboxymethyl-cellulose (CMC) as inducer and glucose or glycerol as carbon and energy sources. Ethylmethanesulphonate (EMS) was used to mutagenize the wild-type organism to produce mutants. The isolated mutants were screened for the isolation of catabolite repression resistant mutants in the presence of 1% (w/v) glucose as carbon source. A total of fifty mutants were isolated. All the mutants produced cellulase in the presence of CMC as an inducer with specific activity of 0.057, 0.088 and 0.074 units/mg protein for the wild-type, catabolite repression resistant mutant4 (CRRmt$_4$) and catabolite repression resistant mutant24 (CRRmt$_{24}$), respectively. It was observed that addition of glucose or glycerol as carbon and energy sources to the culture medium resulted into considerable reduction in the cellulolytic activity. However, glycerol appeared to be a better carbon and energy source than glucose which inhibited enzyme expression in most of the strains used in this study. It was also observed that potent cellulase production occurred at the exponential growth phase of the organism. The isolated mutants were grouped into three classes based on their induction ratios namely; unimproved mutants, catabolite repression resistant mutants and mutants with highest induction ratio but sensitive to catabolite repression in the presence of high glucose concentration. The overall results obtained showed that cellulolytic activity in *P. fluorescens* was regulated by catabolite repression.

Key words: *Pseudomonas fluorescens*, ethylmethanesulphonate, mutants, cellulose, catabolite repression, induction ratio.

INTRODUCTION

Pseudomonas fluorescens is a bacterium which belongs to the taxonomic family IV of Pseudomonadaceae, genus Pseudomonas. *Pseudomonas* is an aerobic, mesophilic, rod-shaped, motile with polar flagella and gram-negative bacterium (Buchanan and Gibbons, 1974). The organism can be isolated from soil, water, sewage, and faeces. The pseudomonads are ecologically important organisms in the soil and water for the aerobic degradations of many soluble compounds derived from the breakdown of plant and animal materials (Brock and Madigan, 1991). Members of the genus have been reported to have cellulolytic activity towards cellulosic materials (Hazlewood et al., 1992; Yamane et al., 1970). Apart from the cellulolytic enzymes, *Pseudomonas* species also produce -amylases, proteases, rennin, lipases and penicillin acylases (Crueger and Crueger, 1990; Merieau et al., 1993; Hellio et al., 1993). The potential of cellulose, majority of which occurs as waste, as an alternative energy source has stimulated research into bioconversion processes which hydrolyse cellulose to soluble sugars for feedstock in alcoholic fermentations and other industrial processes (Coughlam, 1990). Large quantities of cellulose are locked up in manufactured products such as textiles, paper and building materials. Disposal of solid wastes by biological method using cellulolytic microorganisms can reduce environmental pollution, compared to other methods of waste disposal such as incineration which increases the atmospheric carbon dioxide concentrations (Wyman et al., 1993).

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The specific objectives of the present research are therefore to produce mutants of *P. fluorescens* capable of producing large quantity of cellulase, screen the mutants for the isolation of catabolite repression resistant mutants, study the regulation of cellulase synthesis in the mutants and the wild-type.

MATERIALS AND METHODS

Materials

The organism, *Pseudomonas fluorescens* NCIB3756 for this study was supplied from the culture collection of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria. The culture was originally obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. The stock culture was maintained on nutrient agar slants and stored at 4°C in the refrigerator. Subculturing of the organism into new agar slants was carried out every month in order to have fresh culture of the organism throughout the duration of the study. Several preliminary tests including Gram, motility, oxidase, catalase, and indole tests, were carried out on the organism in order to confirm the authenticity of the culture.

Media

The organism was cultured on liquid basal medium (BM) containing tryptone 4 g, K_2HPO_4 0.6 g, $MgSO_4.7H_2O$ 0.2 g and NaCl 0.15 g per 200 ml of 0.1 M phosphate buffer pH 7.0 (Kotchoni and Shonukan, 2002). The basal medium was supplemented with carbon source; high viscosity carboxymethylcellulose (CMC). The CMC was autoclaved separately and added to the BM to give a final concentration of 0.2% (w/v). All other carbon sources used in this work were also autoclaved separately before they were added to the BM to give a final concentration of 0.2% (w/v) substrate. The medium was aseptically adjusted to pH 7.0 after autoclaving.

Isolation of *P. fluorescens* mutants

The modified procedure of Shonukan and Nwafor (1989) was employed to mutagenize the cells of *P. fluorescens*. Ethylmethanesulphonate (EMS) was used as the mutagenic agent. Broth culture of the organism was incubated for 18 to 24 h at 37°C. The culture was centrifuged at 6,000 g for 15 min and the sediment obtained was washed twice with normal saline. Then 0.08 ml of EMS was added to 2.0 ml of the washed cells. The mixture of EMS and the cell was incubated for 1 h at 37°C. At the end of the incubation period, the cell was washed twice with normal saline and diluted 1:10 in a minimal medium (MM) containing 1.0 mM $MgSO_4$, 1.25 mM $Fe_2(SO_4)_3$, 50 mM $CaCl_2$, 15 mM $(NH_4)_2SO_4$ and 0.2% (w/v) glucose. The culture was incubated at 37°C for 18 to 24 h to allow segregation of the mutants. Then, 0.2 ml of the growing cells was plated out on nutrient agar plate using sterile glass spreader. The plates were incubated at 37°C for 18 to 24 h to give rise to individual mutant colonies.

Fifty isolated mutant colonies were identified properly and transferred to a fresh nutrient agar plate by replica plating technique. These mutants and the wild-type strains of *P. fluorescens* were used throughout in this work. The mutants were grouped into classes according to their enzyme synthesis pattern, enzyme characteristics and induction ratio of the mutants with respect to cellulase production using CMC as inducer.

Growth and enzyme production

The basal medium containing 0.2% (w/v) CMC was inoculated with an aqueous broth suspension of the organism from a 24 h old culture. The mixture was incubated at 37°C for 18 to 24 h on a rotary shaker at 100 rev/min. This procedure was applied for the wild-type and all the isolated mutants.

For the study of the enzyme synthesis and its regulation, the time course of the enzyme production was determined and compared with growth. Each of the mutants and the wild-type of *P. fluorescens* was inoculated into separate 500 ml BM containing 0.2% (w/v) CMC as inducer. During the growth, samples were collected aseptically at 2-h interval. The bacterial growth was monitored by measuring the optical density (OD) of the culture at 670 nm using Cecil spectrophotometer. The cellulase activity of the sample filtrates towards carboxymethyl cellulose was measured by the appearance of reducing end groups in solution of CMC using Nelson (1944) method. The protein content of the enzyme was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Screening for catabolite repression resistant mutants

All the mutants of *P. fluorescens* and the wild-type strain were cultured on the basal medium containing high concentration of glucose as carbon source (1% w/v glucose). The cultures were incubated for 24 h as described above under growth and enzyme production. Cellulase synthesis by each mutant was induced by using 0.2% (w/v) CMC. After incubation, mutants which exhibited appreciable enzyme activity were referred to as catabolite repression resistant mutants (CRRmt). These mutants were used throughout in this work with respect to cellulase synthesis and regulation.

RESULTS

Isolation of mutants of *P. fluorescens*

A total of fifty isolates were identified as mutants of the wild-type. Cellulase was synthesized by all the strains at varying degrees. Specific activity of the wild-type and all the mutants in the culture medium containing CMC only ranged from 0.029 to 0.088 units/mg of protein. For culture medium containing CMC and glucose, the specific activity ranged from 0.007 to 0.044 units/mg of protein while 0.018 to 0.053 units/mg of protein was the range obtained from the culture medium containing CMC and glycerol. However, in the culture medium with only glucose or glycerol, the specific activity was low, ranging from 0.004 to 0.034 and 0.008 to 0.025 units/mg of protein for glucose and glycerol media, respectively.

The induction ratio of all the mutants and the wild-type is shown in Table 1. Based on the induction ratio, the mutants were grouped into three classes as shown in Table 2. The induction of cellulase production in Class I mutants was low ranging from 1.0 to 2.49. About 48% of the mutants were in this class. The second class known as Class II mutants showed higher cellulase induction than the class I mutants and the wild-type. The induction of cellulase in class II mutants ranged from 2.5 to 3.49. The class III mutants showed the highest induction ratio

Table 1. Induction ratios of mutants and wild-type of *P. fluorescens*.

Strain	IRa	IRb
Mt1	1.64	2.40
Mt2	1.50	2.27
Mt3	1.67	2.33
Mt4	1.29	3.20
Mt5	1.41	2.12
Mt6	2.29	2.10
Mt7	1.25	2.13
Mt8	1.38	3.00
Mt9	1.00	2.25
Mt10	2.67	2.57
Mt11	1.73	2.47
Mt12	0.71	2.82
Mt13	2.29	2.60
Mt14	1.31	2.53
Mt15	1.93	2.65
Mt16	1.91	2.11
Mt17	1.67	3.17
Mt18	1.18	2.00
Mt19	1.39	2.56
Mt20	0.77	2.75
Mt21	2.00	2.82
Mt22	1.46	1.63
Mt23	0.81	2.25
Mt24	1.52	2.91
Mt25	1.86	2.58
Mt26	1.38	2.42
Mt27	1.82	1.69
Mt28	2.14	2.64
Mt29	1.70	2.82
Mt30	1.30	2.68
Mt31	0.58	2.25
Mt32	1.36	2.25
Mt33	1.90	2.35
Mt34	1.33	2.75
Mt35	0.78	4.38
Mt36	1.40	1.90
Mt37	2.25	3.38
Mt38	1.68	3.82
Mt39	1.68	2.87
Mt40	1.61	2.22
Mt41	1.63	2.58
Mt42	0.87	2.73
Mt43	1.75	2.09
Mt44	1.35	3.47
Mt45	1.38	2.07
Mt46	2.50	3.63
Mt47	1.57	2.17
Mt48	0.73	2.25
Mt49	1.44	2.47
Mt50	1.58	2.63
WT	1.67	1.75

IRa = Induction ratio (CMC+Glucose/Glucose only).
IRb = Induction ratio (CMC+Glycerol/Glycerol only).
CMC = Carboxymethyl cellulose.
Mt = mutant.

**Table 2.** Classification of the mutants based on their induction ratio (IRb).

Classes	Similar strains	Range of induction ratio
I	Mt1, Mt2, Mt3, Mt5, Mt6, Mt7, Mt9, Mt11, Mt16, Mt18, Mt22, Mt23, Mt26, Mt27, Mt31, Mt32, Mt33, Mt36, Mt40, Mt43, Mt45, Mt47, Mt48, Mt49.	1.0 – 2.49
II	Mt4, Mt8, Mt10, Mt12, Mt13, Mt14, Mt15, Mt17, Mt19, Mt20, Mt21, Mt24, Mt25, Mt28, Mt29, Mt30, Mt34, Mt37, Mt39, Mt41, Mt42, , Mt44, Mt50.	2.5 – 3.49
III	Mt35, Mt38, Mt46	3.5 – 4.49

Mt = mutants of *P. fluorescens*.

Table 3. Screening for catabolite repression resistant mutants of *P. fluorescens* using 1% (w/v) glucose.

Strain	Spec. Act. (Unit/mg Prot.)	Strain	Spec. Act. (Unit/mg Prot.)
Mt1	.000	Mt26	.000
Mt2	.003	Mt27	.000
Mt3	.009	Mt28	.005
Mt4	.027	Mt29	.007
Mt5	.003	Mt30	.006
Mt6	.003	Mt31	.000
Mt7	.005	Mt32	.000
Mt8	.008	Mt33	.000
Mt9	.000	Mt34	.003
Mt10	.006	Mt35	.000
Mt11	.003	Mt36	.003
Mt12	.003	Mt37	.003
Mt13	.005	Mt38	.000
Mt14	.005	Mt39	.007
Mt15	.007	Mt40	.000
Mt16	.000	Mt41	.006
Mt17	.007	Mt42	.003
Mt18	.000	Mt43	.000
Mt19	.007	Mt44	.009
Mt20	.005	Mt45	.000
Mt21	.003	Mt46	.003
Mt22	.000	Mt47	.003
Mt23	.000	Mt48	.000
Mt24	.017	Mt49	.000
Mt25	.007	Mt50	.009
		WT	.003

when compared with the first two classes. About 6% of the mutants were in this class.
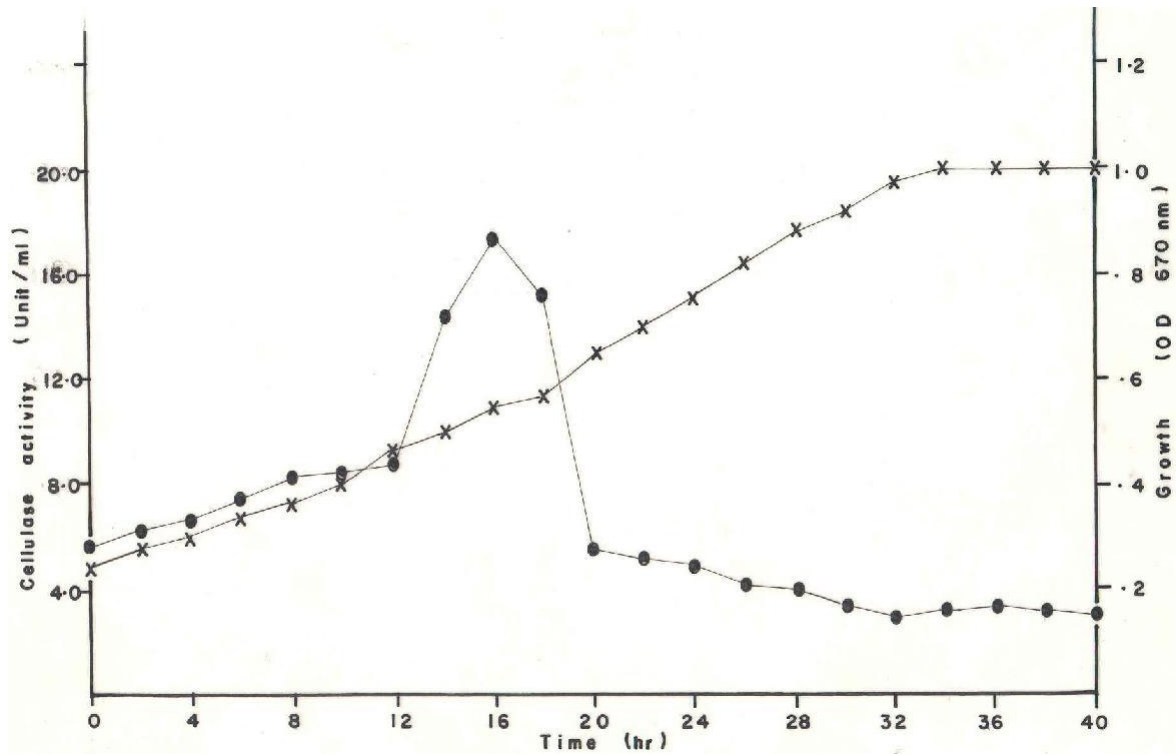
Figure 1. Growth (X), and cellulase production ( ), by wild-type strain of *Pseudomonas fluorescens* using CMC as inducer. The growth was monitored by measuring optical density (OD) at 670nm. The activity of cellulase was determined following the procedure as described in the text.

Isolation of catabolite repression resistant mutants

The isolated mutants and the wild-type of *P. fluorescens* were subjected to high concentration of glucose (1%, w/v) as shown in Table 3. It could be observed that cellulase synthesis was repressed in all the mutants and the wild-type to varying degrees. Only 2% of the mutants could still synthesise cellulase in appreciable amounts. About 40% of the mutants appeared to have lost the ability to produce the enzyme in the presence of high concentration of glucose. From Table 3, mutants 4 and 24 produced cellulase under high glucose concentration with specific activity of 0.027 units/mg protein and 0.017 units/mg protein, respectively. The two mutants were regarded as catabolite repression resistant (CRR) mutants i.e. $CRRmt_4$ and $CRRmt_{24}$.

Growth and regulation of cellulase production

The cellulase activity of the improved mutants ($CRRmt_4$ and $CRRmt_{24}$) and the wild-type increased with increase in incubation time during the growth of the mutants and the wild-type. The improved mutants and the wild-type exhibited more or less similar growth phases and

cellulase synthesis pattern Figures 1, 2 and 3. It could be observed that the wild-type and the two improved mutants had a lag phase of about 0 to 4 h. The exponential phase of growth was observed to vary with the mutants and the wild-type. The three strains produced cellulase during the exponential growth phase. It could also be observed that during the exponential growth phase, highest cellulase activity was obtained at 12, 14 and 16 h for $CRRmt_4$, $CRRmt_{24}$ and the wild-type, respectively. After 26, 30 and 34 h of growth, $CRRmt_4$, $CRRmt_{24}$ and the wild-type reached the stationary phase with little or no metabolic activity occurring in the culture medium.

DISCUSSION

In this study, the culture filtrates of the wild-type of *P. fluorescens* and all the isolated mutants cultured on either glucose or glycerol as the sole carbon and energy source exhibited cellulase activity. Cellulase synthesis was found to be higher in the CMC medium incorporated with glycerol than in the CMC medium containing glucose. Consequently, glucose is regarded as an

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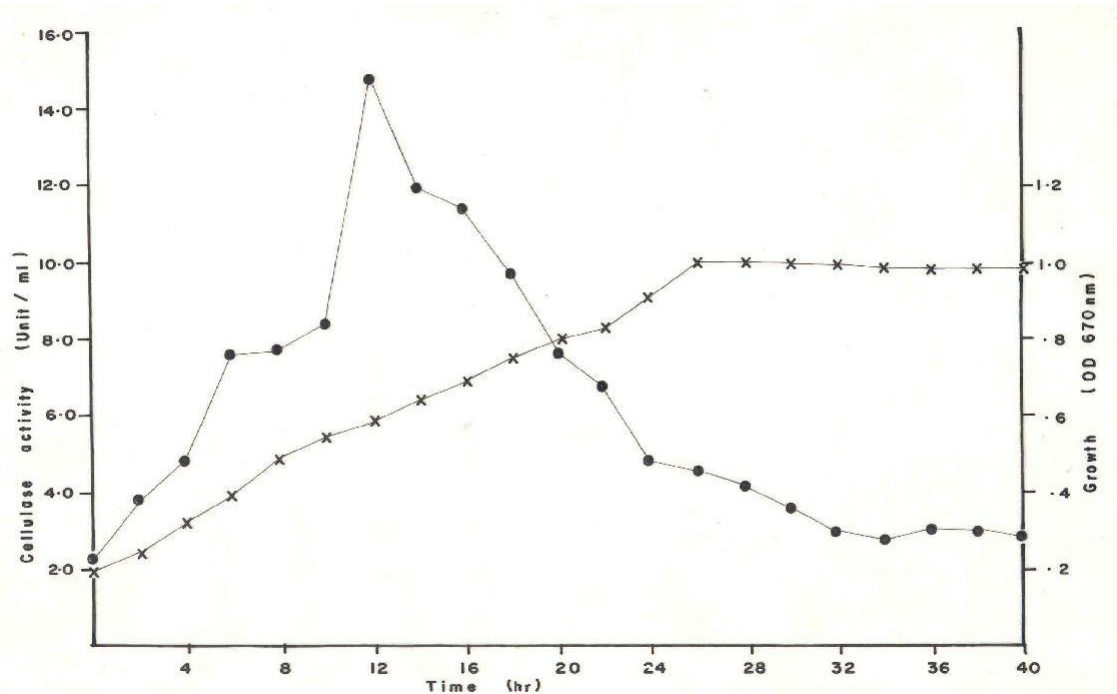
Figure 2. Growth (X), and cellulase production ( ), by catabolite repression resistant mutant 4 (CRRmt4) of *Pseudomonas fluorescens* using CMC as inducer. Growth monitoring and cellulase activity determination were as in Figure 1 legend.
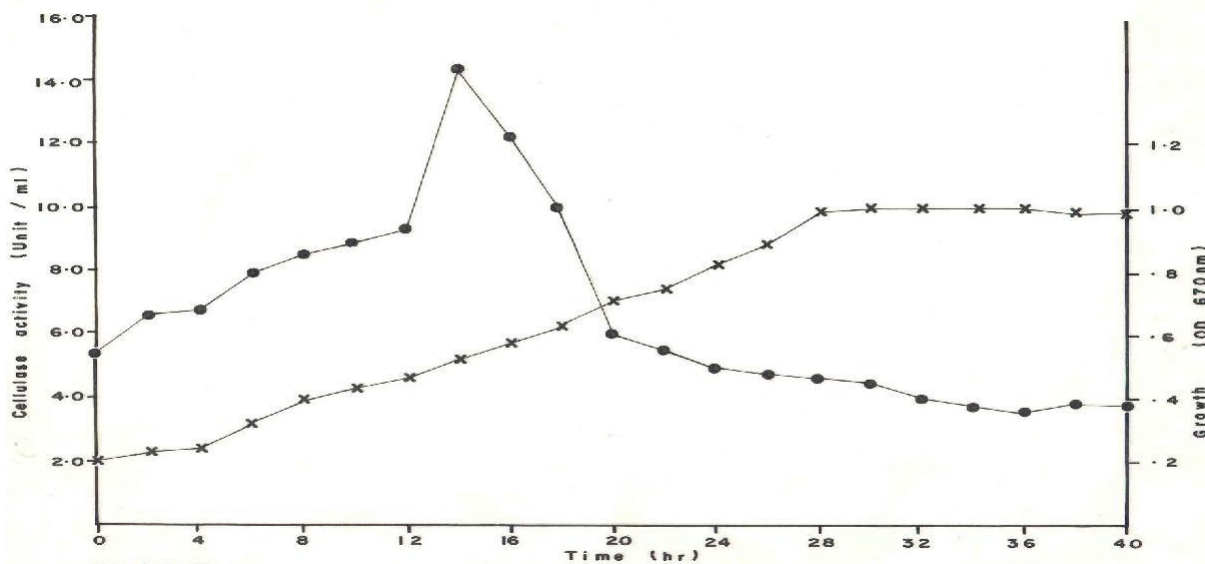
Figure 3. Growth (X) and cellulase production ( ) by catabolite repression resistant mutant 24 (CRRmt 24) of *Pseudomonas fluorescens* using CMC as inducer. Growth monitoring and cellulase activity determination were as in Figure 1 legend.

unsuitable carbon source for cellulase production in this organism.

When the organism was grown on glucose and glycerol as carbon sources, in the absence of inducer, all the strains investigated released cellulase into the culture medium. This showed that *P. fluorescens* can produce cellulase constitutively. However, there was increase in cellulase activity when a cellulosic material was added to the culture medium. This indicates that cellulase production in this organism is inducible. It has been

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reported that inducible enzymes are normally produced in small amounts in the absence of inducers because the repressor system will not function as an absolute block, thus allowing constitutive production of cellulase which could easily yield soluble hydrolysis products of natural cellulose (Eriksson, 1993). The soluble products of hydrolysis could then enter the cells and function as inducers because cellulose cannot enter the cell and act as an inducer as a result of its large size and complexity (Eriksson, 1993; Beguin et al., 1977).

The wild-type of *P. fluorescens* and all the mutants were sensitive to catabolite repression. The mutagenic agent may not have affected the regulatory gene. Cellulase production in the wild-type and its mutants is regulated by induction and catabolite repression mechanisms. It has been reported by Brock and Madigan (1991) that cyclic AMP plays a crucial role in catabolite repression mechanism of exoenzyme production. This molecule exerts its influence through an allosteric protein known as catabolite gene activation protein (CAP) or cyclic AMP receptor protein (CRP). The cyclic AMP-CRP complex facilitates binding of RNA polymerase to the promoter site of catabolite sensitive operon which alleviates the repression state. As long as glucose is available as an energy source, the cyclic AMP concentration is low, thereby preventing the synthesis of enzymes capable of utilizing other energy sources through CRP (Brock and Madigan, 1991). This observation may also be applicable to cellulase production in *Pseudomonas fluorescens*, since cellulase activity is considerably reduced in almost all the strains studied in the presence of glucose.

Two mutants from class II mutant were regarded as improved mutants because of their ability to produce cellulase in the presence of high glucose concentration. These two mutants were referred to as catabolite repression resistant mutants. The regulatory gene in these mutants has probably lost its regulatory role thereby allowing the improved mutants to produce cellulase without inhibition by glucose.

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