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CMCase production under conditions of catabolite repression by a mutant strain of *Pseudomonas* sp.

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Abstract

Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to glucose units. A wild strain of *Pseudomonas* sp. (PSC-11) isolated from soil sample was found to be a cellulase producer. A stable mutant, $PsCNT_9$ that no longer exhibits catabolite repression was produced through treatment of the wild type organism with UV and N-methyl-N'-nitro-N-nitrosoguanidine treatment. $PsCNT_9$ strain exhibited even at 35 mM of glucose concentration and hence designated as catabolite repression resistant NT mutant strain ($PsCCRRNT_9$). $PsCCRRNT_9$ exhibited maimum activity under conditions of catabolite repression at 72 hours of incubation period. A higher cellulolytic activity was observed in combine wild type and mutant strain.

Introduction

Cellulases are being studied increasingly due to their application in the hydrolysis of cellulose, the most abundant biopolymer and potential source of utilizable sugars, which serve as raw materials in the microbial production for a wide variety of chemicals and fuel (Ekperigin, 2007). The regulation of cellulolytic activity, although not understood, is a very effective control on both the rate and extent of cellulose degradation. Cellulolytic microbial cultures do not accumulate soluble sugars during their active growth phase due to their regulatory mechanisms. However, the effective utilization of the products of cellulose hydrolysis will depend on the ability to increase the rate and amount of cellulase activity produced by the microorganism (stewart and Leatherwood, 1976). Cellulose research has been concentrated mostly in fungi but there is increasing interest in cellulase production by bacteria (crowford, 1986; Li and Gao, 1996; Ekperigin, 2007). *Pseudomonas* sp is a bacterium which belongs to the taxonomic family IV of Pseudomonodaceae. *Pseudomonas* is an aerobic, mesophilic, rod shaped, motile with polar flagella and gram-negative (Holt *et al*. 1994). Members of the genus have been reported to have cellulolytic activity towards cellulosic materials (Hazlewood *et al*., 1992; Yamane *et al*., 1970).

The regulation of cellulolytic enzyme production in various organism has been investigated and it has been found that induction and repression functions go together (coughlan, 1985 C; Fusee and Leather wood, 1972; Mandels *et al*., 1962; Mandel and Reese, 1960; Nisizawa *et al*., 1971). One of the key factors that affects cellulose hydrolysis includes end product inhibition of cellulolytic enzymes (Coughland, 1985C). Cellulolytic microorganisms in their typical habitat in nature, probably depend on the phenomenon of catabolite repression and act as a control on cellulase synthesis. Repression of cellulase biosynthesis severely limits the ability of wild type cultures to produce cellulase on a commercial scale and hampers its application in biomass conversion processes. Therefore, methods have been developed to obtain catabolite repression – resistant mutants (Hofsten and Berg, 1972; Montenecourt and Eveleigh, 1979). The potential utilization of cellulose will require organisms that have high cellulase activity and are no longer under repressive control.

In light of the above discussion, an attempt was made to screen the NTG mutants of *Pseudomonas* sp for CMCase production under catabolite repression conditions.

Materials and Methods

Microorganisms

The strain of *Pseudomonas* used in these experiments was isolated from the soil by standard serial-dilution techniques on CMC agar media. This strain was grown on Luria-Bertani (LB) agar slants at 28 ± 2°C for 24-48 hours and stored for further use.

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Growth and cellulase production:

The growth of the bacteria grown on LB agar slants was transferred into 250ml Erlenmeyer flask containing 45ml of production medium (Reese and Mandels, 1963) with following composition (g/l): KH_2PO_4, 2; $(NH_4)_2SO_4$, 1.4; $CaCl_2$, $2H_2O$, 0.3; $MgSO_4$. $7H_2O$, 0.3; Urea, 0.3; Proteose peptone , 0.25; Yeast extract , 0.2; CMC, 10.0; Trace metal solution, 1ml [$FeSO_4$. $7H_2O$, 5mg/l; $MnSO_4$. $7H_2O$, 5.6 mg/l; $ZnSO_4.7H_2O$, 3.34 mg/l; $CoCl_2.2H_2O$, 2mg/l) and Tween-80, 1ml. pH of the medium was adjusted to 7.0. The flasks were incubated at 28 ± 2°C on a rotary shaker at 150 rpm. Cultures were centrifuged at 8000 rpm for 15min and the clear supernatant was used as the source of crude enzyme.

Assay:

CMCase activity was measured as described previously (Ghosh, 1987) with slight modification. An aliquot of appropriately diluted enzyme sample was mixed with 0.5 ml of 1% CMC prepared in 0.05 M sodium phosphate buffer (pH7.0). The reaction mixture was incubated for 30 min at 50°C. The release of reducing sugars was determined by dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of CMCase or endoglucanase activity was defined as the amount of enzyme releasing 1µ(mole) of reducing sugar per ml per min.

Mutagenesis

The procedure of was followed as described by Chand *et al*. (2005) with slight modification. Single mutant clones were further grown in new plates till the third generation. The third generation (M_3) of the mutants were replicated in new plates containing CMC as sole carbon source. Selection of mutants was based on the diameter of the clear some (Kotchoni and Shukan, 20020. The selected colonies were transferred on to LB agar slants, incubated at 28 ± 2°C for 24-48 hours and were tested for production of CMCase.

Catabolite repression studies

NTG mutants and the wild type strain was cultured on the basal medium containing high concentration of glucose i.e. 15mM, 20mM, 25mM 30mM and 35mM concentration and a control was maintained. After 48 hours of incubation, CMCase activity was determined. The mutants which exhibited appreciable CMCase activity were used for further investigations.

CMCase production by catabolite repression mutant strains in presence of glucose at concentrations; 15mM; 20mM; 25mM; 30mM and 35mM at different incubation periods, 24 hours; 48 hours and 72 hours were studied.

CMCase production in combined wild type and catabolite repression resistant mutant strain in presence of glucose at 35mM concentration was studied. A control was also maintained i.e without addition of glucose.

Statistical analysis

The pooled data obtained from these investigations were subjected ANOVA analysis.

Results

Isolation and characterization

The isolation procedure consisted of making serial dilutions of the soil and subsequently plating on CMC agar medium. Cellulolytic organisms were detectable through the formation of distinct clear zones in the medium (Teather and Wood, 1982). Colony formation generally appears after 24 to 48 hours of incubation and clear zone around the colony indicate that the organism is cellulase positive. The isolated cellulolytic bacteria was identified as *Pseudomonas* sp by referring Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). This isolate was selected for further studies and some of the characteristics of the isolate, *Pseudomonas* sp (PsC-11) is shown in table 1.

Screening of NTG mutants for CMCase under conditions of catabolite repression

The wild type strain of *Pseudomonas* sp (PsC-11) is subjected to random mutagenesis (UV/NTG). All the NTG mutants of *Pseudomonas* sp obtained were screened for the production of CMCase under catabolite repression

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conditions. A concentration of 15, 20, 25, 30 and 35 mM of glucose and also a control was employed in the medium and the CMCase activity was measured at 48 hours of incubation period. The results are presented in table 2. a critical perusal of table 2 reveals that the mutant strains PSCNT., $Pscnt_2$ and $PsCNT_5$ exhibited CMCase activity upto 30mM of glucose concentration. But the isolate $PsCNT_9$ exhibited CMCase activity only up to 20mM of glucose concentration. At 20mM concentration of glucose $PsCNT_9$ exhibited approximately 4.36 times higher CMC activity than wild strain. The strain $PsCNT_9$ which exhibited CMCase at higher concentrations of glucose found to be catabolite repression resistant and hence designated as ($PsCCRRNT_9$). A similar screening of NTG mutants of *Pseudomonas* sp for CMCase activity under catabolite repression conditions was reported by Bakare *et al*., (2005). Kotchoni *et al*. (2003) reported that at 20mM of glucose concentration, no CMCase activity was detected by the wild type but the mutant strain of *Bacillus* sp (BpCRI 6) exhibited significant amounts of CMCase. The mutant strains of *Trichoderma reesei* exhibited glucose derepression while the wild type strain showed glucose repression (Choi *et al*. 1998). A mutant of *Cellulomonas* sp that no longer exhibits catabolite repression was produced through treatment of the wild type organism with NTG. Interestingly, this mutant produced cellulase even in presence of high levels of glucose (Stewart and Leatherwood, 1976).

CMCase production by $PsCCRRNT_9$ in presence of glucose at different incubation periods.

Figure 1 ($P < 0.001$) depicts the CMCase activity of $PsCCRRNT_9$ strain in presence of glucose at different concentrations at different incubation periods. Glucose at a concentration of 15, 20, 25, 30 and 35 mM were added to the cultivation medium. A control was also maintained without addition of glucose. It was noticed that although the addition of glucose did not stop the growth of the organism but enzyme secretion was partially arrested. However, CMCase secretion stopped until the glucose was depleted in the medium and the rate of cellulase resumed at a lower level. At 72 hours, it was found that the glucose in the medium might be depleted and hence the activity was detected more when compared to 24 and 48 hours. A 40.18%, 45.11% and 52.82% of CMCase activity was observed by $PsCCRRNT_9$ strain at 24, 48 and 72 hours of incubation period respectively 35 mM glucose concentration ($p < 0.01$).

Enhanced CMCase production in combined wild type and mutant strain

Interestingly, a higher CMCase activity was detected under conditions of catabolite repression in a combined wild type and mutant strain of *Pseudomonas* sp (Table 3) ($p < 0.05$). It is evident from the results that the wild type strain PsC-11 did not exhibit CMCase activity at a concentration of 35 mM glucose. A higher cellulolytic activity was exhibited in combined wild type and mutant strain. Similar to the present observations, Kotchoni *et al*. (2003) reported a high cellulase yield in a combined wild type and a mutant strain of catabolite repression insensitive strain of *Bacillus* sp BPCRI6. A mixture of culture containing a cellulolytic and non-cellulolytic strain has been reported to be the best condition for degrading cellulose (Lewis *et al*., 1988). As demonstrated in table 3 an improvement in cellulase synthesis in a combined wild type and mutant strain was observed.

Discussion

The organism isolated and characterized in these studies belongs to the genus *Pseudomonas* sp. It, like previously reported cellulolytic organisms was shown to be under the control of catbolite repression (Nisizawa *et al*., 1972). Low level of cellulolytic enzymes in the presence of glucose could be attributed to repression of synthesis of cellulolytic enzymes involved in the utilization of cellulose by easily metabolizable carbon, glucose and demonstrated by many organisms (Ruijter and Visser, 1977; Suto and Tomita, 2001). However, insensitization of repression by mutations resulted higher production of cellulase even in the presence of glucose (Kotchani *et al*., 2003). The mutant strain $PsCCRRNT_9$ in our study exhibited cellulase activity even under conditions of catabolite repression. 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 exo-enzyme 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, there by preventing the synthesis of

enzymes capable of utilizing other energy sources though CRP (Brock and Madigan, 1991). This observation may also be applicable to cellulase production in *Pseudomonas* sp, since cellulase activity is considerably reduced in presence of glucose. A similar report by *Pseudomonas* sp was observed by Bakare *et al*. (2005).

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Table 1 : Morphological and biochemical tests of the isolate PsC-11

Characteristic	PsC-11
Morphology	
Gram strain	--
Shape	Rods
Motility	+
Biochemical reaction	
Starch hydrolysis	+
Gelatin hydrolysis	+
Catalase	+
Oxidase	+
H_2S	--
Nitrate reduction	+
Carbohydrate fermentation test	
Glucose	A
Lactose	A
Sorbitol	A
Mannitol	A
Fructose	A
IMV:C test	
Indole	--
Methyl red	--
Voges proskauer	--
Citrate	+

A – Acid production

Based on above characteristics the isolate PsC-11 is tentatively identified as *Pseudomonas* sp.

Table – 2 : Screening for catabolite repression resistant NTG mutant of *Pseudomonas* sp for the production of CMCase[#]

NTG mutant of *Pseudomonas* sp	Conc. of glucose (in mM)				
	15	20	25	30	35
$PsCNT_1$	2.86	2.51	1.89	1.11	0.00
$PsCNT_2$	2.62	2.04	1.38	0.65	0.00
$PsCNT_3$	0.00	0.00	0.00	0.00	0.00
$PsCNT_4$	1.48	0.98	0.51	0.00	0.00
$PsCNT_5$	3.04	2.86	2.16	1.84	0.00
$PsCNT_6$	1.68	1.22	0.81	0.00	0.00
$PsCNT_7$	2.42	1.98	1.09	0.00	0.00
$PsCNT_8$	1.04	0.85	0.00	0.00	0.00
$PsCNT_9$	4.85	4.26	3.59	1.86	1.29
$PsCNT_{10}$	2.95	2.61	1.09	0.00	0.00
$PsCNT_{11}$	0.00	0.00	0.00	0.00	0.00
$PsCNT_{12}$	0.00	0.00	0.00	0.00	0.00
$PsCNT_{13}$	0.00	0.00	0.00	0.00	0.00
Wild strain PsC-11	1.11	0.62	0.00	0.00	0.00

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[#] Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxy methyl cellulose per min.
Values are mean of three replicates significant at $p < 0.001$ according to Fisher's one way ANOVA analysis

Table 3 : CMCase production by combined wild type (PsC-11) and catabolite repression resistant mutant strain ($PsCCRRNT_9$)

Glucose conc. (in mM)	Substreates	CMCase activity[#] (U/ml)				
		PsC-11	$PsCCRRNT_9$	PSc-11 + $PsCCRRNT_9$	LSD 0.05	LSD 0.01
Control	Cellulose	2.24	4.82	5.62	0.18	0.25
	Filter paper	2.01	2.86	2.94	0.07	0.09
Control + 35 mM glucose	Cellulose	--	1.65	1.98	0.03	0.07
	Filter paper	--	1.26	1.39	0.02	0.05

[#] Carboxymethyl cellulase (CMCase) is expressed in terms of units. One unit is the amount of enzyme releasing 1 µmole of reducing sugar from carboxy methyl cellulose per min.
Values are mean of three replicates significant at $p < 0.001$ according to Fisher's one way ANOVA analysis

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Fig. 1 : Production of CMCase by catabolite repression resistant NTG mutant of Pseudomonas sp (PsCCRRNT9) at different incubation periods.