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IMPROVEMENT OF CARBOXYMETHYL CELLULASE PRODUCTION FROM *CHAETOMIUM CELLULOLYTICUM* NRRL 18756 BY MUTATION AND OPTIMIZATION OF SOLID STATE FERMENTATION

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Abstract

Cellulase producing fungus *Chaetomium cellulolyticum* NRRL 18756 was subjected to various doses of gamma irradiation to enhance the production of the industrially important enzyme carboxymethyl cellulase (CMCase). Among all the mutants tested, M-7 obtained through 0.5 KGy irradiation showed highest extracellular CMCase production which is 1.6-fold higher than that of the wild type. Optimal conditions for the production of CMCase by the mutant fungal strain using solid-state fermentation were examined. The optimized medium consisted of sugarcane bagasse supplemented with 1% (w/w) peptone, 2.5mM $MgSO_4$, and 0.05% (v/w) Tween 80. Optimal moisture content and initial pH was 40% (v/w) and 5.0-6.5, respectively. The medium was fermented at 40° C for 4 days. The resulting CMCase yield was 4.0-fold more than that of the wild type strain grown on the basal wheat bran medium. Some characteristics of partially purified CMCase from the mutant and wild type of *C. cellulolyticum* were investigated. The partially purified mutant CMCase was more stable than the wild type CMCase. Thus, the higher thermostability of mutant CMCase makes it a potential candidate for commercial and industrial process.

Introduction

Cellulase(s) are industrially important enzymes that are sold in large volumes for use in industrial applications, for example in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp, paper and textile industries (Ögel *et al*. 2001, Abo-State *et al*. 2010). Moreover, there are growing markets for produced cellulases in the field of detergent industry and saccharification of agriculture wastes for bioethanol technology (Camassola and Dillon 2009, Vu *et al*. 2011).

The cellulase complex secreted by filamentous fungi consists of three major enzyme components, an endo-1,4-β-glucanase [Carboxymethyl cellulase (EC 3.2.1.4)], a 1,4-β-cellobiohydrolase [Exoglucanase (EC 3.2.1.91) and a 1,4-β-glucosidase [Cellobiase (EC 3.2.1.21)], which act synergistically during the conversion of cellulose to glucose (Almin *et al*. 1975, Bucht and Ericksson 1969).

The cost of production and low yield of cellulases are the major problems for industrial applications. It has been reported that solid state fermentation (SSF) as an attractive process to produce cellulases economically is mainly due to its lower capital investment and lower operating expenses (Singhania *et al*. 2009). Production of cellulases by fungi in SSF using agricultural wastes has been reported (Fawzi 2009, Abo-State *et al*. 2010). Therefore, investigation on the ability of fungal strains to utilize inexpensive substrates and improvement of enzyme productivity are important.

In the last few decades, the exponential increase in the application of cellulases in various fields demands extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the

overproduction of the enzyme as the quantities produced by wild strains are usually too low (Li *et al*. 2009, Pradeep and Narasimha 2011). The spectacular successful examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Vu *et al*. 2011). Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics (Karanam *et al.* 2008). Irradiation by gamma ray may cause some mutations to the genes of cells through the DNA repair mechanisms within cells. Gamma radiations are short wave high energy electromagnetic radiations emitted from certain radioactive isotopes such as $Cobalt^{60}$. The effect of gamma radiation doses on fungal enzyme production was studied by many workers (Macris 1983, Shimokawa *et al*. 2007, Yousef *et al*. 2010).

Thus, the aim of this study was to investigate high level production of extracellular carboxymethyl cellulase (CMCase) through mutating *C. cellulolyticum* by gamma radiation method, and optimizing some parameters in solid-state fermentation medium.

Materials and Methods

Plant products like soya stalks (*Glycine max*), barley straw (*Hordeum vulgare*), corn cobs and stalks (*Zea mays*), rice straw (*Oryza sativa*), sugarcane bagasse (*Saccharium officinalis*), wheat straw (*Triticum aestivum*) and Compositae; sunflower stalks (*Helianthus annuus*) were evaluated as carbon sources to produce CMCase.

The materials were soaked in 1N HCl in the ratio 1 : 10 (substrate: solution) for 60 min at room temperature and then washed with double distilled water and autoclaved at 121° C for one hour. The treated substrate was filtered and neutralized by washing with dilute aqueous sodium hydroxide and double distilled water until the filtrate became neutral. The material was then dried at 60°C for 12 hours and finely milled into small pieces (3 - 5 mm).

Chaetomium cellulolyticum Chahal & Hawksw. NRRL 18756 was obtained from NRRL (Agricultural Research Service Culture Collection) through United States Department of Agriculture (USDA), New Orleans, Louisiana 70179. The strain was kept on malt extract agar at 4° C and routinely cultured. The medium used for fermentation contained 20 g of wheat bran and 8 ml of mineral salt solution [0.05% $MgSO_4$ $7H_20$, 0.2% $NH_4H_2PO_4$ and 0.1% KH_2PO_4 and nature pH].

Spore suspension (containing about 2 × 106 spores/ml^{-1}) was freshly prepared from 6-day-old cultures of *C. cellulolyticum* on malt extract agar slants at 35° C using deionised double distilled water.

The spore suspension was exposed to 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 KGy by the Indian gamma cell of Co^{60} located at the National Center for Radiation Research and Technology (NCRRT), Nasr City, Cairo, Egypt. The irradiated spore suspensions were plated on to the MEA medium and cultured at 35° C, D-sorbitol (1M) was used as the osmotic stabilizer. Six days later, the growing colonies were transferred before sporulating on to MEA slants for further studies.

The spore suspension from each mutant (10^6 spores/ml) was cultured on to sterilized 250 ml Erlenmeyer flask containing fermentation medium: 20 g of wheat bran with 8 ml of mineral salt solution [0.05% $MgSO_4$ $7H_2O$, 0.2% $NH_4H_2PO_4$ and 0.1% KH_2PO_4) and nature pH] at 35° C. Six day later, the potentiality of CMCase production was investigated. The mutants which showed superior cellulase production were selected for hereditary stability studies. The mutants that produced high CMCase activity were studied for their stability for enzyme production for nine generations.

Acid (HCl 5%) hydrolyzed lignocellulosic wastes (listed in Table 3) were analyzed in terms of cellulose, hemicelulose and lignin contents as described by Jermyn (1955).

The solid substrate culture broth was prepared by adding 10-fold distilled water by keeping the flasks on a rotary shaker for 1 h at 200 rpm. The mixture was filtered through muslin cloth and the filtrate was centrifuged at 10000 rpm for 20 min at 4º C and served as crude CMCase preparation.

The activity of CMCase was determined following Li (2009). The 1 ml enzyme reaction mixture was composed of 0.5 ml of enzyme and 0.5 ml of 1% (w/v) carboxymethyl cellulose (CMC; Sigma, St. Louis, MO, USA) in citrate buffer (0.1M, pH 5). The reaction mixture was incubated at 50° C for 30 min and the released reducing sugar was determined by the 3,5-dinitrosalicylic acid method (Miller 1959). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μM of glucose from CMC per min under the assay conditions. Cellulase activity was expressed as unit per 1 g of fermented solid substrate (U/g).

Protein content was determined using bovine serum albumin dissolved in 0.17 M NaCl as a standard (Bradford 1976).

Optimising CMCase biosynthesis was performed by optimizing various physico-chemical process parameters. The parameters that were optimized were carbon sources: (barley straw, corn cobs & stalks, rice straw, soya stalks, sugarcane bagasse, sunflower stalks and wheat straw), moisture (20~70% v/w, where v and w represent water and water + dried agricultural substrate, respectively), incubation temperature (25~50°C), pH of solid culture (3~8.5) and incubation time (2~8 days). The additives were; nitrogen sources (Beef extract, Casein, Peptone, Urea, yeast extract, Ammonium chloride, Ammonium nitrate, Ammonium sulphate, Sodium nitrate, Sodium sulphate at 1% w/w); metal salts ($CaSO_4$, $CoSO_4$, $CuSO_4$, $FeSO_4$, KCl, $MgSO_4$, $MgCl_2$, $MnSO_4$, $MnCl_2$, Na_2CO_3, $ZnSO_4$ at 2.5mM), surfactant (Tween 20, Tween 80, Triton X-100 at 0.05% v/w, sodium dodecyl sulfate [SDS] and ethylenediaminetetraacetic acid [EDTA] at 0.4 mM).

Partially purified CMCase was prepared from the wild and mutant strain which was superior in CMCase production grown in optimized solid state fermentation (SSF) medium. Protein content of 200 ml of crude CMCase of *C. cellulolyticum* was precipitated overnight with 60% ammonium sulphate, collected by centrifugation at 12,000 ×g for 15 min, dissolved in 5 ml acetate buffer (0.2 M, pH 6.0), dialyzed overnight against the same buffer and fractionated on Sephadex G-100 column (2.5 x 82 cm) of Fraction Collector (Fra100, Pharmacia-Fin Chemicals) preequilibrated with acetate buffer (Plummer 1978). The column was eluted with the same buffer at 20 ml h^{-1}. Active fractions (5 ml each) were pooled, lyophilized and subjected for investigation the enzyme characteristics.

The optimum pH of the enzyme was evaluated by measuring the CMCase activity with CMC as the substrate at 50° C and different pHs for 30 min. The buffers were 0.1M citrate buffer (pH 3.5-6.0), 0.2 M phosphate buffer (pH 7.0-8.0) and 0.2 M glycine/NaOH buffer (pH 9.0). To determine pH stability, enzyme was incubated in the presence of pH values within the above cited range (3.5 - 9.0) for 60 min. The residual activity for enzyme was assayed. The optimum temperature of the enzyme was evaluated by measuring the CMCase activity at the optimum pH, at different temperatures (40 - 90° C) with CMC as the substrate. For determination of thermal stability, the enzyme was incubated for variable durations (30 to 90 min) at fixed temperatures (50 - 60° C).

The mean, standard deviation, T-score and probability "P" values of 3 replicates of the investigated factors and the control were computed according to the mathematical principles described by Glantz (1992).

Results and Discussions

The wild type of *C. cellulolyticum* when exposed to different gamma radiation doses ranging from 0.25 to 1.75 KGy gave 15 mutants with different abilities to produce CMCase. The lethality rate of *C. cellulolyticum* spores crossed 100% when exposed to 2.0 KGy (Table 1). The results revealed that the highest CMCase production and protein were obtained by M7 when exposed to 0.5 KGy dose which represents a 1.45-fold improved enzyme activity than that of wild type. Abo-State *et al*. (2010) found enhanced productivity in CMCase by gamma-irradiation at dose 0.5 KGy with 21% increase as compared with un-irradiated control. CMCase activity of a mutant of *Aspergillus terrus* showed two-fold improved activity than that of the wild type (Vu *et al*. 2011).

Table.1. Effect of gamma radiation on CMCase production by *C. cellulolyticum* on SSF. n = 3, ± standard deviation.

Mutant strains	Radiation dose (KGy)	CMCase activity (U/g)	Protein mg/ml
Cont. (wild type)	0	20.1 ± 0.2●	0.10●
M1	0.25	21.3 ± 0.5 (+HS)	0.11 ± 0.01(+NS)
M2	0.25	22.2 ± 0.2 (+HS)	0.11 ± 0.02 (+NS)
M3	0.25	22.6 ± 0.3 (+HS)	0.20 ± 0.06 (+HS)
M4	0.5	23.1 ± 0.08 (+HS)	0.21 ± 0.001 (+HS)
M5	0.5	23.4 ± 0.04 (+HS)	0.21 ± 0.004 (+HS)
M6	0.5	25.6 ± 0.3 (+HS)	0.23 ± 0.002 (+HS)
M7	0.5	29.2 ± 0.04 (+HS)	0.26 ± 0.01 (+HS)
M8	0.5	26.5 ± 0.2 (+HS)	0.23 ± 0.04 (+HS)
M9	0.5	27.1 ± 0.3 (+HS)	0.23 ± 0.02 (+HS)
M10	0.5	26.0 ± 0.01 (+HS)	0.22 ± 0.01 (+HS)
M11	0.5	25.3 ± 0.07 (+HS)	0.22 ± 0.03 (+HS)
M12	0.75	21.8 ± 0.1 (+NS)	0.14 ± 0.02 (+S)
M13	1.0	5.1 ± 0.08 (–HS)	0.05 ± 0.005 (–HS)
M14	1.5	0.4 ± 0.03 (–HS)	0.03 ± 0.004 (–HS)
M15	1.75	0.06 ± 0.001(–HS)	0.01 ± 0.001 (–HS)
(No growth)	2.0	-	-
L.S.D 1%		2.7	0.05
L.S.D 5%		1.9	0.03

HS = Highly significant, $p \leq 0.01$; S = Significant, $p \leq 0.05$; NS = Non significant, $p > 0.05$; ● = Value to which other data was statistically compared using t-test.

Six mutant stains (M6-M11) were selected as hypercellulase-producing mutants. The mutants M7-M10 maintained the same production yield after being subcultured nine times, indicating that the mutation is stably heritable (Table 2). Li *et al*. (2009) also found that the mutant strains produced high levels of CMCase (obtained by compound mutation of microwave and ultraviolet) were stable for a long period of nine generations to produce cellulase.

Chemical composition of lignocellulosic components of the investigated substrates after acid treatment are given in Table 3. The analytical data represents the percentage of celluloses, hemicelluloses and lignin as referred to the original weight. The results showed that the high amount of celluloses was found in sugarcane bagasse (*Saccharium officinalis*) (39.2%). In previous studies, Javed *et al*. (2007) found that the treatment of sugarcane bagasse enhanced the production of high yield cellulases by *Humicola insolens*.

Sugarcane bagasse resulted in a favorably high production of CMCase (37.4 U/g) than the other substrates (Fig. 1A) and higher amounts of cellulose (Table 3). Mekala *et al*. (2008) using treated sugarcane bagasse as solid substrate obtained maximum production of CMCase by *Trichoderma reesei* (25.6 U/g). Therefore, sugarcane bagasse was chosen for further experiments.

Table 2. CMCase activities of mutant strains for nine generations.

Strains	CMCase activity (U/g) in 5 selected generations				
	1st	3rd	5th	7th	9th
M6	25.6	24.9	25.2	24.5	24.8
M7	29.2	29.2	29.3	29.2	29.4
M8	26.5	26.1	26.5	26.2	26.4
M9	27.1	26.5	26.9	27.1	27.0
M10	26.0	26.1	26.2	26.1	26.0
M11	25.3	24.4	25.0	24.8	25.1

Table 3. Main components of lignocellulosic wastes after acid treatment as % of the original weight.

Substrate	Cellulose	Hemicellulose	Lignin
Barley straw (*Hordeum vulgare)*	31.3 ± 2.1(HS)	32.2 ± 0.7 (HS)	7.4 ± 0.4•
Corn cobs (*Zea mays)*	34.1 ± 2.4 (HS)	39.5 ± 2.9 (HS)	12.1 ± 0. 9 (HS)
Corn stalks (*Z. mays)*	25.4 ± 1.3•	35.7 ± 1.5 (HS)	9.8 ± 0.3 (S)
Rice straw (*Oryza sativa)*	32.3 ± 0.6 (HS)	33.0 ± 1.2 (HS)	7.7 ± 0.4 (NS)
Soya stalks (*Glycine max*)	31.7 ± 0.4 (HS)	23.2 ± 0.6 (NS)	10.4 ± 0.5 (HS)
Sugarcane bagasse (*Saccharium officinalis*)	39.2 ± 0.9 (HS)	31.4 ± 0.8 (HS)	12.0 ± 0. 7 (HS)
Sunflower stalks (*Helianthus annuus)*	38.3 ± 1.6 (HS)	37.5 ± 1.2 (HS)	13.7 ± 0.4 (HS)
Wheat straw (*Triticum aestivum)*	35.7 ± 0.9 (HS)	23.1 ± 0.4•	10.6 ± 0.6 (HS)
L.S.D 1%	2.97	1.33	2.67
L.S.D 5%	2.07	0.93	1.85

Abbreviations same as in Table 1.

Forty per cent moisture content resulted in CMCase production (40.5 U/g) that was higher than obtained at other moisture levels (Fig. 1B). Vu *et al*. (2010) showed that in SSF, moisture level plays an important role in biosynthesis and secretion of many kinds of enzymes, especially cellulases. Very high moisture content in solid medium resulted in decreased substrate porosity as well as reducing oxygen penetration among the substrate particles, but excessively low moisture levels in solid medium lead to poor microbial growth, poor development and poor accessibility to nutrients (Singhania *et al*. 2009).

The optimal temperature for the highest production of CMCase was 40° C, with decreasing of enzyme production at higher temperatures (Fig.1C). Chahal and Wing (1978) found as 37º C the optimum temperature of thermotolerent *C. cellulolyticum* to produce cellulases.

The highest production of CMCase was observed at a wide range of pH 5.0-6.5 (Fig.1D). Pamment *et al*. (1978) found 6.0 as the optimum pH for CMCase production by *C.cellulolyticum.*

The highest production of the enzyme was observed after 4 days of fermentation (Fig. 2A). This finding was in accordance with previous studies (Vu *et al*. 2010, Yousef *et al*. 2010) who

showed that thermophiles or thermotolerent fungi in general, possess a more faster rate of enzyme production than mesophiles.

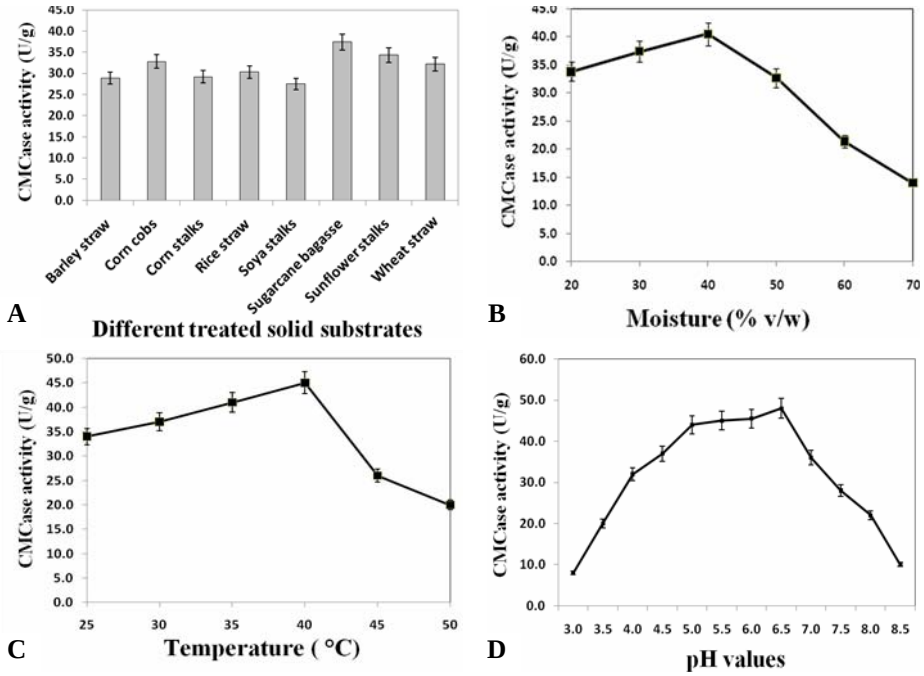

Fig. 1. Effects of solid substrates (A), moisture levels (B), temperature (C) and pH on CMCase production (D).

Peptone was the best nitrogen additive which enhanced the CMCase production followed by casein, yeast and beef extract (Fig. 2B). Organic nitrogen showed superiority over inorganic nitrogen sources for the production of enzymes (Fawzi 2011).

The result in Fig. (2C) revealed that when the solid medium supplemented with $MgSO_4$ highest yield of CMCase was obtained. Fraústo da Silva and Williams (1993) stated that $MgSO_4$ and CuSO4 were essential salts for some organisms. However, all other tested metal salts slightly reduced CMCase production except $ZnSO_4$ which showed inhibitory effect (Fig. 2C).

Surfactants like Tween 80 and EDTA were most effective in CMCase production which others do not appear to have any effect (Fig. 2D). The use of Tween 80 is beneficial because it does not denature the enzymes (Shahriarinour *et al*. 2011).

The optimum pH for activities of partially purified CMCase from the mutant and wild type was found to be 6.0 and 5.5 respectively (Fig. 3A). Acidic pH optima were reported for CMCase excreted from other fungi (McHale and Coughlan 1981). The pH stability exhibited by the mutant CMCase was a wide range between 5.5-7.0, but at 5.5 pH for wild type CMCase (Fig. 3B). As pH value diverged from the optimum level, the efficient functioning of the enzyme was affected, most probably, due to the change in active site conformation which is determined, in part, by ionic and hydrogen bonding that can be affected by pH. It is also clear that mutant CMCase was more stable than wild type CMCase. This result was confirmed by the previous results repoted by Macris (1984) and Gao *et al*. (2008).

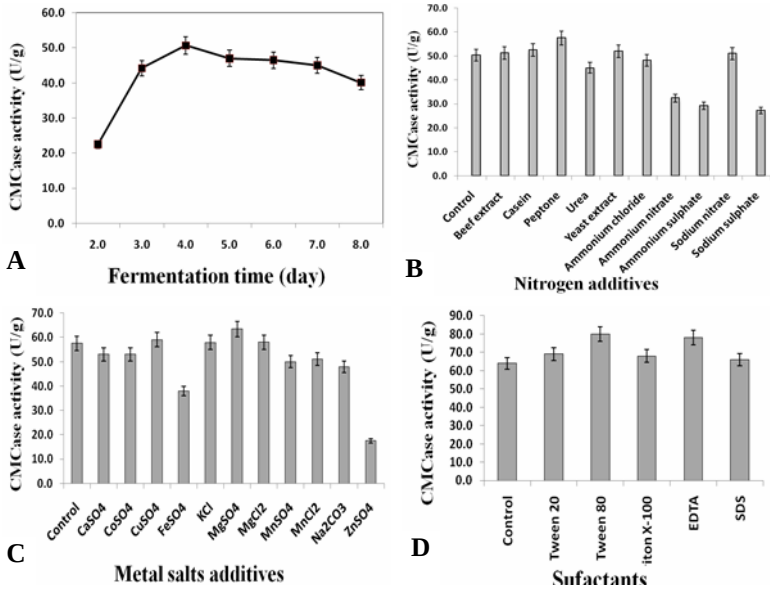

Fig. 2. Effects of fermentation time (A), nitrogen additives (B), metal salt additives (C) and surfactant (D) on CMCase production.

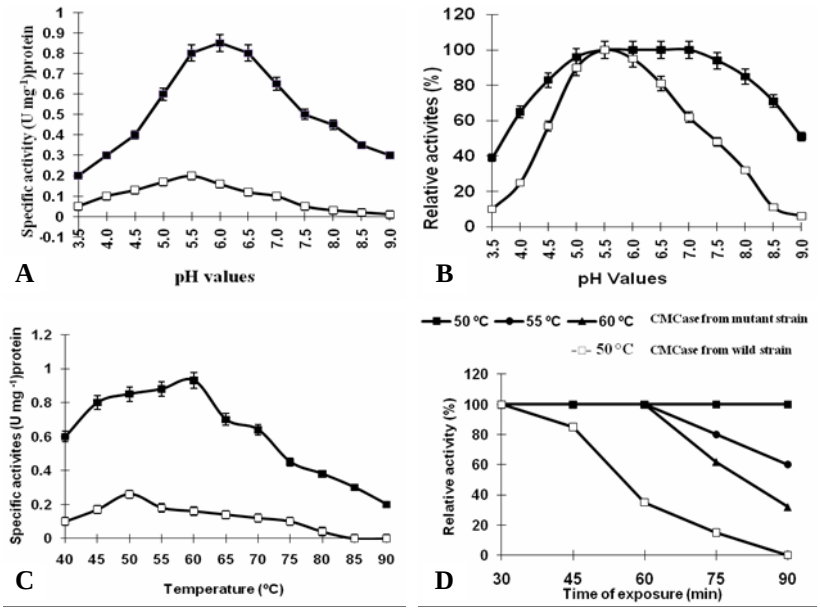

Fig. 3. Characterization of partially purified CMCase of mutant and wild type of *Chaetomium cellulolyticum* cultured in optimized solid fermentation medium. (A) Effect of different pH values on the specific activity of partially purified CMCases from mutant (-■-) and wild type (-□-). (B) Effect of different pH values on the stability of the partially purified CMCase from mutant (-■-) and wild type (-□-). (C) Effect of different temperature on the specific activity of CMCases from mutant (-■-) and wild type (-□-). (D) Thermal stability of the partially purified CMCase from mutant and wild type.

CMCase retained its original activity after heating to 60ºC for 1 hr. and to 50ºC for 1.5 h (Fig. 3D). However, wild type CMCase retained its original activity at 50ºC only for 30 min. At the same degree, it lost 65% for 1 hour and no activity was recorded after 1.5 hr (Fig. 3D). Mutant CMCase showing high stability than the wild type CMCase. This result is in agreement to a certain extent with the results obtained from Vu *et al*. (2011) who reported that the thermostability was increased by mutation and is a very important property. Cellulolytic enzymes that are stable at high temperatures can be used in cellulose saccharification processes at elevated temperatures to protect both substrate and products of the enzymic reaction from microbial contamination and deterioration (Mekala *et al*. 2008).

A mutant fungus strain, *Chaetomium cellulolyticum* M7 was developed by irradiating with Co^{60} γ-rays radiation. Under the deduced optimized medium and SSF conditions, the CMCase production of the mutant fungus was 80.0 U/g, representing a 4.0-fold increase in CMCase production than that produced in wheat bran basal medium by the wild type strain. The partially purified mutant CMCase was more stable than the wild type CMCase.

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