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Full Length Research Paper

Improvement of carboxymethyl cellulase production from *Chaetomium cellulolyticum* NRRL 18756 by mutation and optimization of solid state fermentation

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The purpose of the present investigation was to enhance the production of the industrially important enzyme carboxymethyl cellulase (CMCase) by subjecting the wild cellulase producing fungal strain *Chaetomium cellulolyticum* NRRL 18756 to various doses of gamma irradiation (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0 KGy). Among all the mutants tested, M-7 obtained at 0.5 KGy of *C. cellulolyticum* strain showed highest extracellular CMCase production in a yield 1.6-fold exceeding 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, 2.5 mM $MgSO_4$, and 0.05% (v/w) Tween 80. Optimal moisture content and initial pH was 40% (v/w) and 5.0 to 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.

Key word: *Chaetomium cellulolyticum,* carboxymethyl cellulase, mutation, optimization, solid state fermentation, characterization.

INTRODUCTION

Cellulase(s) are industrially important enzymes that are sold in large volumes for use in different 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 industry (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; Dogaris et al., 2009; Sukumaran et al., 2009; Xu et al., 2011; 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; Beldman et al., 1985; 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

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Abbreviations: CMCase, Carboxymethyl cellulose; **SSF,** solid state fermentation; **USDA,** united states department of agriculture; **NCRRT,** national center for radiation research and technology; **SDS,** sodium dodecyl sulfate; **EDTA,** ethylenediaminetetraacetic acid; **MEA ,** malt extract agar.

attractive process to produce cellulases economically is mainly due to its lower capital investment and lower operating expenses (Yang et al., 2004, Singhania et al., 2009). Production of cellulases by fungi in SSF using agricultural wastes has been reported (Gao et al., 2008; Dogaris et al., 2009; Fawzi, 2009; Abo-State et al., 2010). Therefore, investigation on the ability of fungal strains to utilize inexpensive substrates and improvement of enzyme productivity remains an important object for research.

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 (Szengyel et al., 2000; Chand et al., 2005; Li et al., 2009; Pradeep and Narasimha, 2011). The spectacular success examples of strain improvement in industry are mostly attributed to the extensive application of mutation and selection (Xu et al., 2011; 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 and Medicherla, 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. Also, the comparison between some characteristics of both partially purified CMCases from mutant and wild type was investigated. *C. cellulolyticum*, a cellulolytic fungus, showed 50 to 100% faster growth rates and over 80% more final biomass-protein formation than *Trichoderma viride*, a well-known high cellulase-producing cellulolytic organism. With this knowledge, the present study sought to modify a fungal strain and optimize solid medium and culture conditions to enhance CMCase production.

MATERIALS AND METHODS

Cellulosic materials

Several natural plant products belonging to three angiosperm families were evaluated as carbon sources to produce CMCase. These included: Leguminosae; soya stalks (*Glycine max*); Graminae; 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*).

Acid treatment (HCl)

These agricultural materials were firstly soaked in 1 N HCl in the ratio 1: 10 (substrate : solution) for 60 min at room temperature and then washed with double distilled water for removal of chemicals and autoclaved at 121°C for 1 h. The treated substrate was filtered for freeing it from fibers and neutralized by washing with dilute aqueous sodium hydroxide. The treated substrate was then washed with double distilled water until the filtrate became neutral. The material was then dried at 60°C for 12 h and finely milled into small pieces (3 to 5 mm). These milled was stored in dry flasks under dark conditions at room temperature.

Microorganism

The strain *C. cellulolyticum* Chahal and Hawksw NRRL 18756 used throughout this work was obtained from NRRL (Agricultural Research Service Culture Collection). It was provided by United States Department of Agriculture (USDA), New Orleans, Louisiana 70179. The strain was kept on malt extract agar (MEA) at 4°C and routinely cultured.

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.

Fermentation media

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).

Mutagenesis with gamma ray treatment

The spore suspension which was previously prepared was exposed to different doses, 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 dose rate was 1.0 KGy/18 min at the time of experiment at room temperature.

After treatment with different doses of γ-ray, the mutant spore suspensions were plated onto the MEA medium and cultured at 35°C, D-sorbitol (1 M) was used as the osmotic stabilizer. Six days later, the growing colonies were transferred before sporulating onto MEA slants for further studies.

Screening of *Chaetomium cellulolyticum* mutant

After mutagenesis, spore suspension from each mutant (10^6 spores ml^{-1}) was cultured onto sterilized 250 ml Erlenmeyer flask containing fermentation medium: 20 g of wheat bran with 8 ml of mineral salt solution [(0.05% $MgSO_4$ $7H_20$, 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 that were superior in cellulase production were selected and used for hereditary stability studies.

Hereditary stability studies of mutants

The mutant strains obtained by the mutation of the aforementioned method that produced high CMCase activity were studied for their stability for enzyme production for 9 generations. The mutants after fermentation were inoculated on the wheat bran fermentation medium and used for inoculating next fermentation.

Determination of main components of lignocellulosic wastes

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).

Enzyme extraction

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 4°C and served as crude CMCase preparation. Each trial was made in triplicates.

Enzyme activity assay

The activity of CMCase was determined as previously detailed by 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) (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 liberate 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 process parameters for carboxymethyl cellulase (CMCase) production

Optimising CMCase biosynthesis was performed by optimizing various physico-chemical process parameters where the optimum condition found for each parameter was settled for the subsequent experiments. The parameters that were optimized were carbon sources: (barley straw, corn cobs and 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), incubation time (2~8 days). Studies were also conducted to examine the effect on cellulase production of various additives supplemented into solid culture. The examined 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 2.5 mM), 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]. Each experiment was replicated three times.

Partial purification of carboxymethyl cellulase (CMCase)

Partially purified CMCase was prepared from the wild and mutant strain which was superior in CMCase production grown in optimized 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.

Characteristics of partially purified carboxymethyl cellulase (CMCase) from wild type and selected mutant

The optimum pH of enzyme was evaluated by measuring the CMCase activity with CMC as the substrate at 50°C and at different pH for 30 min. The following buffers were used: 0.1 M citrate buffer (pH 3.5 to 6.0), 0.2 M phosphate buffer (pH 7.0 to 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 previously mentioned range (3.5 to 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 to 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 to 60 °C).

Statistical validation of treatment effects

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 were considered highly significant, significant or non-significant where $P < 0.01$, > 0.01 and < 0.05, > 0.05, respectively.

RESULTS AND DISCUSSION

Mutagenesis of *Chaetomium cellulolyticum* and screening of mutant

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 (29.2 U/g) and highly protein content (0.26 mg/ml) were obtained by mutant No (7) when exposed to 0.5 KGy dose which represent a 1.45 fold improved enzyme activity than that of wild type (20.1 U/g). This result was confirmed by a previous study of Abo-State et al. (2010), who found enhanced productivity in CMCase by gamma-irradiation at dose 0.5 KGy with present increase 21% as compared with un-irradiated control. Also, the CMCase activity of a mutant of *Aspergillus terrus* was represented 2-fold improved activity than that of the wild type (Vu et al., 2011).

Carboxymethyl cellulase (CMCase) activity and hereditary stability of mutant

After the mutagenesis of gamma radiation, six mutant stains (M6-M11) were selected as hypercellulase-producing mutants. The stability of these mutants for

Table 1. Effect of gamma radiation on CMCase production by *C. cellulolyticum* on SSF.

Mutant strains	Radiation dose (kgy)	CMCase activity (U/g)	Protein (Mg/ml)
Cont. (the wild type)	0	20.1±0.2●	0.10●
M1	0.25	21.3±0.5 (+H.S)	0.11±0.01(+N.S)
M2	0.25	22.2±0.2 (+H.S)	0.11±0.02 (+N.S)
M3	0.25	22.6±0.3 (+H.S)	0.20±0.06 (+H.S)
M4	0.5	23.1±0.08 (+H.S)	0.21±0.001 (+H.S)
M5	0.5	23.4±0.04 (+H.S)	0.21±0.004 (+H.S)
M6	0.5	25.6±0.3 (+H.S)	0.23±0.002 (+H.S)
M7	0.5	29.2±0.04 (+H.S)	0.2**6**±0.01 (+H.S)
M8	0.5	26.5±0.2 (+H.S)	0.23±0.04 (+H.S)
M9	0.5	27.1±0.3 (+H.S)	0.23±0.02 (+H.S)
M10	0.5	26.0±0.01 (+H.S)	0.22±0.01 (+H.S)
M11	0.5	25.3±0.07 (+H.S)	0.22±0.03 (+H.S)
M12	0.75	21.8±0.1 (+N.S)	0.14 ±0.02 (+S)
M13	1.0	5.1 ±0.08 (-H.S)	0.05±0.005 (-H.S)
M14	1.5	0.4 ±0.03(-H.S)	0.03±0.004 (-H.S)
M15	1.75	0.06±0.001(-H.S)	0.01±0.001 (-H.S)
(No growth)	2.0	-	-
L.S.D 1%		2.7	0.05
L.S.D 5%		1.9	0.03

Value in the table represents the mean of 3 reading expressed as U g^{-1} ± standard deviation. (H.S) Highly significant, p ≤0.01; (S) significant, p≤ 0.05; (N.S) non significant, p >0.05; (●) = value to which other data was statistically compared using T-Test.

Table 2. CMCase activities of mutant strains for 9 generations.

Strains	CMCase activity (U/g)				
	1st generation	3rd generation	5th generation	7th generation	9th generation
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

CMCase production was determined by successive subculturing for 9 generations. After each subculture, mutants were tested for their ability to stably produce CMCase by SSF. The mutants (M7-M10) maintained the same production yield after being subcultured 9 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 9 generation to produce cellulase.

Chemical composition of lignocellulosic components

Chemical composition of lignocellulosic components of the investigated substrates after acid treatment was demonstrated 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 (*S. 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*.

Optimising process parameters for carboxymethyl cellulase (CMCase) production

Treated substrate

In the present work, treated sugarcane bagasse resulted

Table 3. Main components of lignocellulosic wastes after acid treatment.

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

Value in the table represents the mean of 3 reading expressed as % ± standard deviation. (H.S) Highly significant, p ≤0.01; (S) significant, p≤ 0.05; (N.S) non significant, p >0.05; (●) = value to which other data was statistically compared using T-Test.

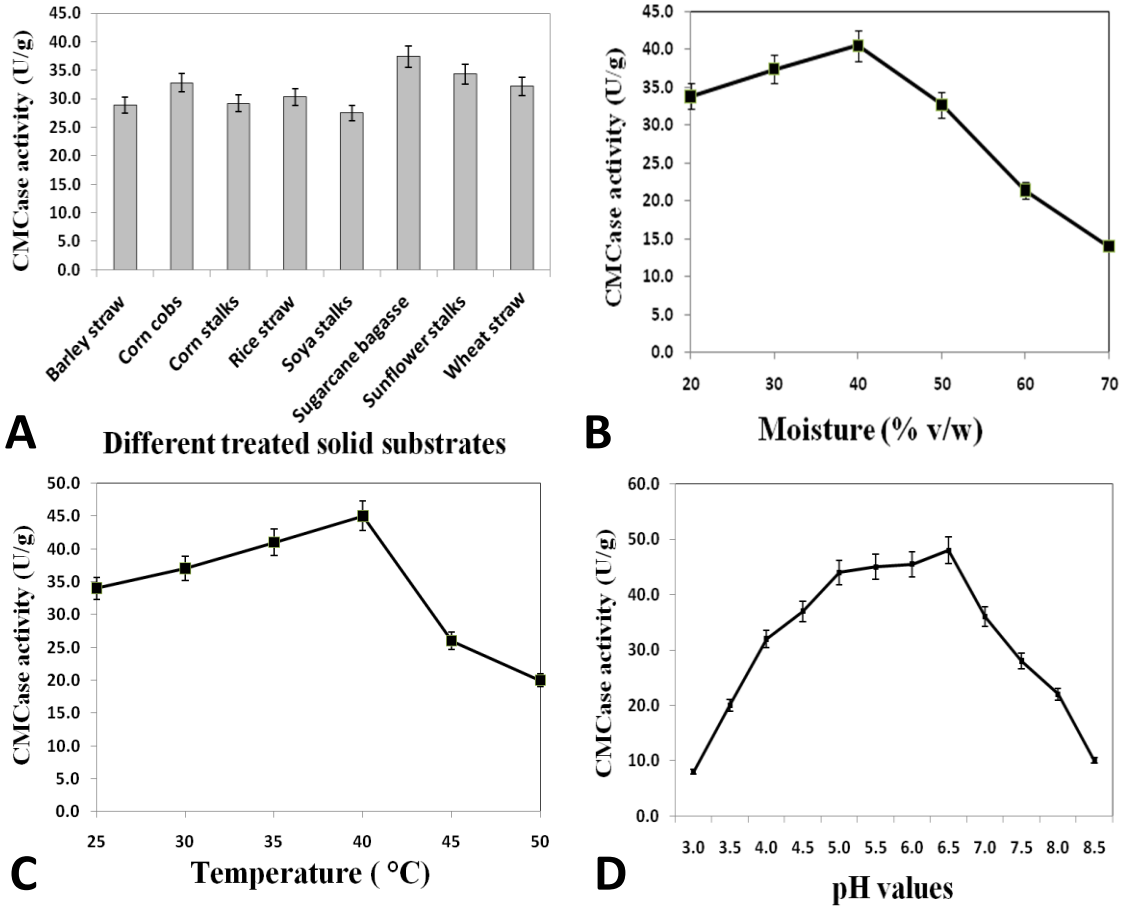

Figure 1. Effect of various culture conditions on CMCase production from *C. cellulolyticum*. A, Enzyme production in different treated solid substrates; B, effect of initial moisture levels on CMCase production; C, effect of incubation temperature on CMCase production; D, effect of pH of solid medium on CMCase production.

in a favorably high production of CMCase (37.4 U/g) than the other treated substrates used in solid media (Figure 1A). This result was confirmed by the obtained data in Table 3 which revealed that the sugarcane bagasse contained higher amounts of cellulose than that in other substrates. Moreover, Mekala et al. (2008) used treated

sugarcane bagasse as solid substrate for maximum production of CMCase by *Trichoderma reesei* (25.6 U/g). Therefore, sugarcane bagasse was chosen for further experiments.

Initial moisture content in the solid substrate

Cellulase production depends on the moisture level in the solid substrate. 40% moisture content resulted in CMCase production (40.5 U/g) that was higher than obtained at other moisture levels (Figure 1B). Moisture contents less or more than 40% were not suitable for high cellulase production. 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).

Incubation temperature

Temperature strongly affects the SSF process, which varies according to the organism involved. Even slight changes in temperature can affect cellulase production. Presently, the optimal temperature for the highest production of CMCase was 40°C, with decreasing of enzyme production at higher temperatures (Figure 1C). In the previous study, Chahal and Wing (1978) it was stated that, the optimum temperature of thermotolerent *C. cellulolyticum* to produce cellulases is 37°C.

Medium pH

The optimal pH varies with different microorganisms and enzymes. The highest production of CMCase was observed at a wide range of pH 5.0-6.5 (Fig.1D). The influence of pH on cellulase production highlighted the widely-known importance of pH for microbial growth and metabolic activities. Rizk et al. (2007) stated that the hydrogen or hydroxyl ion concentration may have a direct effect on cell, or it may act indirectly by varying the degree of dissociation of substances in the medium. Therefore, the change of pH is also important for the enzyme activity of microorganisms, for the intermediate products and solubility. Moreover, Pamment et al. (1978) found that the optimum pH for CMCase production by *C. cellulolyticum* was 6.0.

Fermentation time

The highest production of enzyme (50.7 U/g) was observed after 4 days of fermentation (Figure 2A). This finding was in accordance with previous studies (Grajek, 1987; Gao et al., 2008; Yousef et al., 2010) who showed that thermophiles or thermotolerent fungi in general, possess a faster rate of enzyme production than mesophiles.

Nitrogen additives

Peptone was the best nitrogen additive which enhanced the CMCase production followed by casein, yeast and beef extract (Figure 2B). On the other hand, the other additives decreased CMCase production. In previous studies, organic nitrogen showed superiority over inorganic nitrogen sources for the production of enzymes (Reid, 1983; Fawzi, 2011).

Metal salts

The result in Figure 2C revealed that, when the solid medium was supplemented with $MgSO_4$ followed by $CuSO_4$ and $MgCl_2$, it produced the highest yield of CMCase (63.4 U/g). It has been established that metal ions significantly affect the progress and efficiency of fermentation, secretion of active enzymes and synthesis of secondary metabolites by microorganisms and their ability to attach to their various substrates by affecting the dynamics of cell membranes, cell viability, permeability, membrane fluidity, stability and signaling systems (Learmonth and Gratton, 2002). Moreover, Fraústo da Silva and Williams (1993) stated that $MgSO_4$ and CuSO4 were essential salts for some organisms. However, presently all other tested metal salts slightly inhibited CMCase production except $ZnSO_4$ which clearly inhibited the enzyme production (Figure 2C). Possible inhibition due to the binding of acid to metal ions affecting the metal bioavailability, which in turn affects efficiencies of metabolic processes, secretion of active enzymes and the ability of the microorganisms to attach to their various substrates (McDonald et al., 1996).

Surfactants

Surfactants are crucial in enhancing microbial growth in SSF by promoting the penetration of water into the solid substrate matrix, leading in an increase of surface area (Singh et al., 1991; Asgher et al., 2006). Presently, amendment of sugarcane bagasse with any of the tested surfactants (Tween 20, Tween 80, Triton X100, EDTA and SDS) enhanced CMCase production. Tween 80 and EDTA were most effective, and resulted in CMCase production of 78~80 U/g (Figure 2D). The stimulatory effect of Tween 80 may be a consequence of its action on cell membranes causing increased in permeability by promoting the release of cell-bound enzymes (Rege

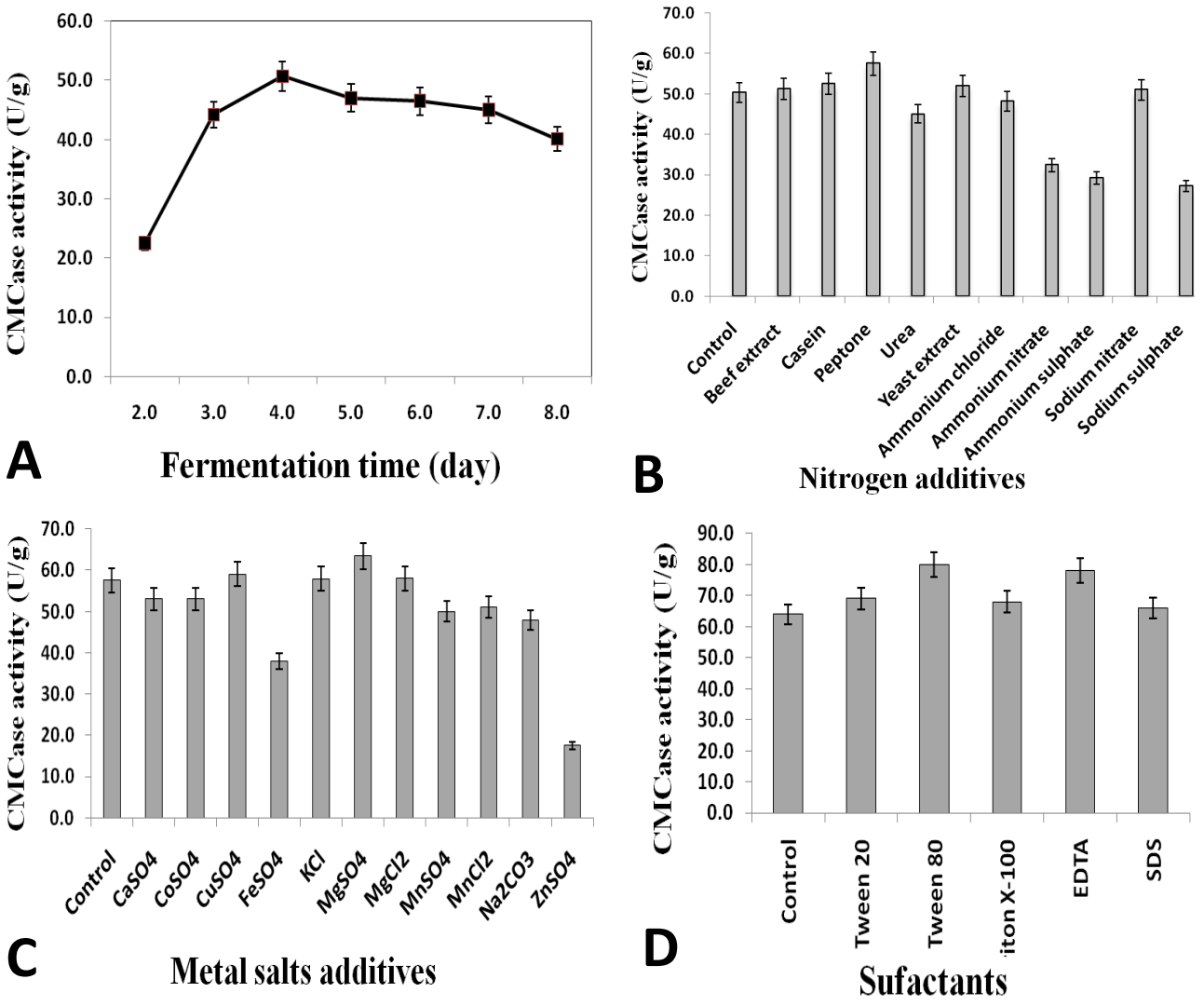

Figure 2. Effect of Fermentation time and medium additives on CMCase production from *C. cellulolyticum*. A, Effect of Fermentation time on CMCase production; B. effect of different nitrogen sources; C, effect of different metal salts; D, effect of some surfactant: (EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate).

et al., 2002). Moreover, the use of Tween 80 is beneficial because it does not denature the enzymes (Shahriarinour et al., 2011).

Characteristics of partially purified carboxymethyl cellulase (CMCase) from wild type and selected mutant

From the partially purification of CMCase of mutant and wild type *C. cellulolyticum* grown in the optimized SSF medium, the fractions possessing the highest spscific activities (data not shown) were used as a partially purified CMCases for the investigation some characteristics of the enzyme. 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 (Figure 3A). Acidic pH optima were reported for CMCase excreted from other fungi (McHale et al., 1981, Beldman et al., 1985). The pH stability exhibited by the mutant CMCase was a wide range between 5.5 to 7.0, but at 5.5 pH for wild type CMCase (Figure 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 (1983) and Gao et al. (2008).

The optimum temperature of partially purified CMCase from the mutant and wild type was 60°C and 50°C respectively (Figure 3C). The optima of mutant CMCase competed favorably with those reported for certain

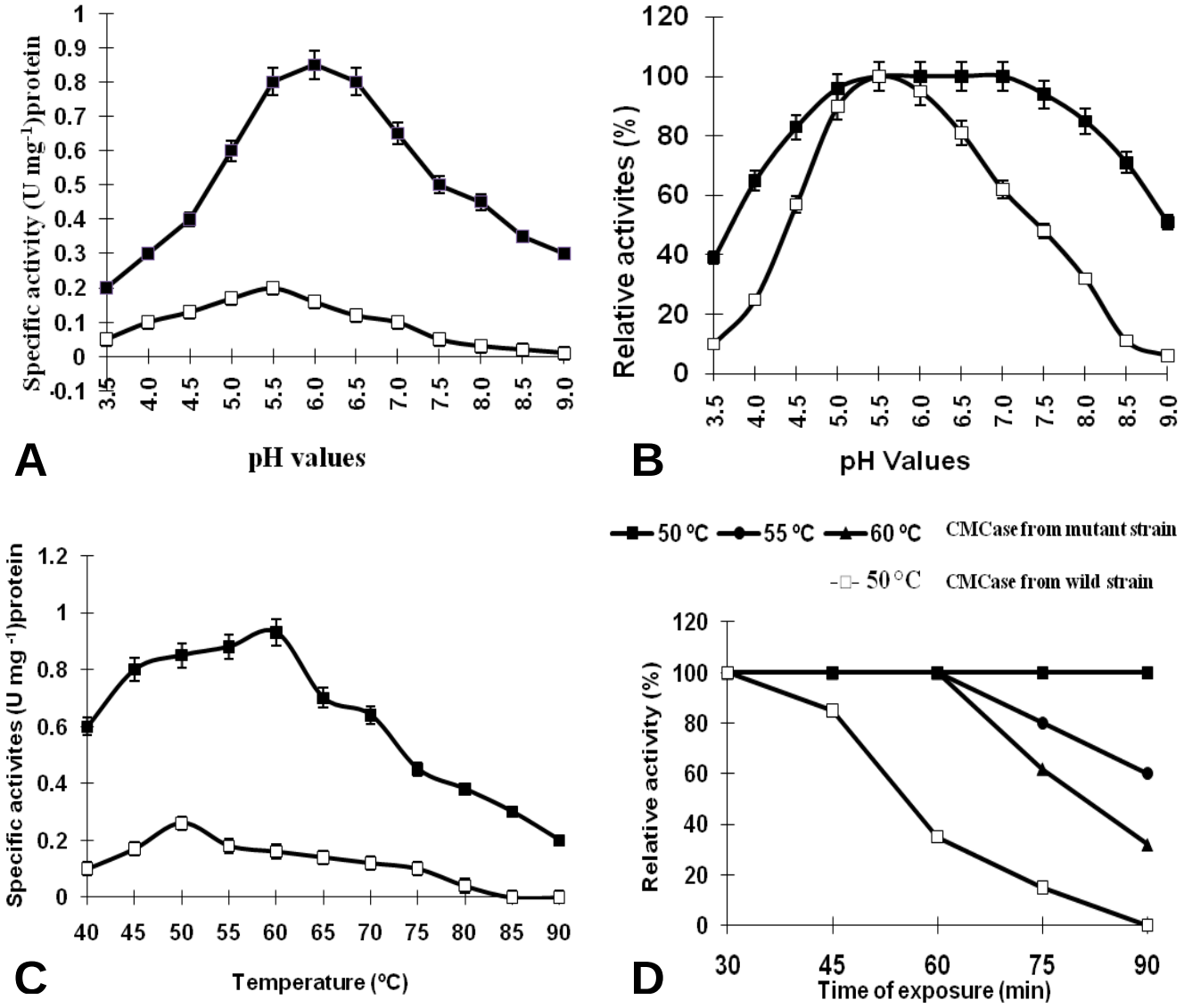

Figure 3. Characterization of partially purified carboxymethyl cellulase (CMCase) of mutant and wild type of *C. cellulolyticum* cultured in optimized solid fermentation medium. A, Effect of different pH values on the specific activity of partially purified CMCases from mutant (-■-) and wild type (-□-); 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.

thermophilic cellulotytic microorganisms (McHale et al., 1981). Mutant CMCase retained its original activity after heating to 60°C for 1 h and to 50°C for 1.5 h (Figure 3D). However, wild type CMCase retained its original activity at 50°C only for 30 min. At the same degree, it lost 65% for 1 h and no activity was recorded after 1.5 h (Figure 3D). Mutant CMCase showed high stability than the wild type CMCase. This result is in agreement to a certain extent with the results obtained by 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).

Conclusion

A mutant fungal strain, *C. cellulolyticum* was created by mutagenesis with Co^{60} γ-rays radiation. From the resulting 15 mutants, the mutant number M7 was selected and cultivated in SSF for the production of CMCase. 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 (20.1 U/g). The partially purified mutant CMCase was more stable than the wild type CMCase.

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