African Journal of Microbiology Research Vol. 6(5), pp. 1079-1084, 9 February, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1550
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Strain improvement and optimization for enhanced production of cellulase in *Cellulomonas* sp. TSU-03

Kanokphorn Sangkharak[1]*, Piyaporn Vangsirikul[2] and Siripa Janthachat[2]

[1]Department of Chemistry, COE of Renewable Energy, Plant Biomolecule Research Unit, Faculty of Science, Thaksin University, Thailand.
[2]Department of Chemistry, Faculty of Science, Thaksin University, Thailand.

Accepted 17 January, 2012

An objective of this study was to develop high cellulase-producing bacteria by mutagenesis and optimization studies. Among 328 **mutant strains of *Cellulomonas* sp. TSU-03, the mutant M23, NTG mutant, gave the highest value of cellulase activity 2008 U/mg protein) followed by mutant M17 (1884 U/mg protein) in CMC medium. The optimum medium and environmental conditions for cellulase production consisted of 4% wastepaper, 1% $NaNO_3$ under cultivation temperature at 35°C with initial pH and agitation speed at 6 and 100 rpm, respectively. *Cellulomonas* sp. strain M23 produced the highest cellular growth (28.09 ± 2.28 g/L) and FPase, CMCase as well as, β-glucosidase activities at 325, 2420 and 152 U/mg protein, respectively. Under optimal condition, the cellulase activity achieved from strain M23 is 1.28- and 1.30-fold higher than cellulase from mutant M17 and wild type, respectively. After being subcultured 12 times, the cellulase production of the mutant M23 was stable. The results suggested that *Cellulomonas* sp. M23 had a good potential for production of cellulase by fermentation using a cultivation medium containing wastepaper as the main substrate**.

Key words: Mutagenesis, NTG, Ultraviolet, Wastepaper.

INTRODUCTION

Cellulose is the most abundant renewable resource on the earth (100 billion dry tons/year). It is the primary product of photosynthesis in the environments (Jarvis, 2003). Cellulase, an enzyme degraded cellulose, are produced by various microorganisms such as bacteria and fungi (Immanuel et al., 2006; Abou-Taleb et al., 2009). Complete enzymatic hydrolysis of cellulase requires synergistic action of three types of enzymes including cellobiohydrolase, endoglucanase (carboxymethyl cellulase, CMCase) and β-glucosidase. Cellulase has extensive applications such as the textile industry, food industry, pulp and paper industries as well as, pharmaceutical applications (Abou-Taleb et al., 2009). However, cost of cellulase in enzymatic hydrolysis is regarded as a major factor.

Many researchers and development attempted to improve cellulase producing-microorganism since the potential to screen and identify over-producing strains remains untapped. Isolation, identification and genetic manipulation of microbes which produce prominent cellulase indicate a promising future for the industrialization application. Our study indicated that *Cellulomonas* sp. strain TSU-03 produced high activity of cellulase (1860.1 U/mg protein) when wastepaper was utilized as sole carbon source (Sangkharak et al., 2011).

Strain improvement for cellulase production via mutagenic agents has attracted great attention owing to their efficiency. The use of different mutagenic agents including ultraviolet (UV), X-rays, gamma radiation, ethyl methane sulfonate (EMS), N-methyl-N′-nitro-N-nitrosoguanidine (NTG) and mustards were demonstrated (Mala et al., 2001; Chen et al., 2008; Vu et al., 2009; Abdel-Aziz et al., 2011; Xu et al., 2011).

*Corresponding author. E-mail: skanokphorn@yahoo.com. Tel: 00 667460 9604.

Streptomyces pseudogriseolus, UV mutant, excreted over-production of xylanase (161% production improved) as compared to the wild type (Abdel-Aziz et al., 2011). Sequential treatment by γ-irradiation, UV and NTG was also utilized for strain improvement in *Aspergillus* sp. The activities of carboxymethyl cellulase, filter paper cellulase and β-glucosidase of cellulase by *Aspergillus* sp. XTG-4 were improved by 2.03-, 3.20- and 1.80-fold, respectively when compared to parent strain (Vu et al., 2009). However, no information has been published on the hyper-production of cellulase by mutagenesis in *Cellulomonas* sp. Therefore, this investigation aims to increase the cellulase production by strain improvement using UV irradiation and NTG.

MATERIALS AND METHODS

Microorganisms and culture medium

The wild type of *Cellulomonas* sp. strain TSU-03 was isolated from agricultural soil, Thailand. This strain produced high activity of cellulase (mainly xylanase and endogluconase) as previously reported (Sangkharak et al., 2011). The culture was maintained on carboxymethyl cellulose (CMC) agar slant in test tube at 35°C, pH 6.0. The CMC medium contained (g/L): CMC 10, $MgSO_4.7H_2O$ 0.2, K_2HPO_4 1, NH_4NO_3 1, $FeCl_3.6H_2O$ 0.05, and $CaCl_2$ 0.02 (Kasana et al., 2008; Lo et al., 2009).

Strain improvement

Mutagenesis by N-methyl-N′-nitro-N-nitrosoguanidine (NTG)

The wide type cell grown in CMC medium at 35°C for 24 h was harvested at logarithmic phase by centrifugation (10,000 × g, 20 min) at 4°C and washed twice with McIlvaine's buffer (containing 0.1 M citric acid and 0.2 M phosphate buffer) pH 5.0. The cell was resuspended in buffer at a concentration of 5.8×10^8 cell/ml. NTG, 100 mg/ml, was added into the cell suspension. After incubation for 1 h at 35°C with rotation speed at 100 rpm, the cell was centrifuged and washed immediately with buffer. The treated sample was transferred into CMC plates (Xu et al., 2011). The plates were strained by Congo red to see the cellulolytic activity after incubated at 35°C for 48 h.

Mutagenesis by ultraviolet (UV) irradiation

The wild type cell was harvested as described earlier. After the cell concentration was determined by counting, cells were spread on CMC medium plates. The plates were placed under a UV lamp (Sylvania G30W) at a distance of 55 cm and were irradiated for various periods of time. Following irradiation, the plates were kept in the dark for 1 h and then were spread onto CMC plates and incubating at 35°C for 48 h, the number of colonies was counted to determine survival rates. Afterward, plates were strained by Congo red.

Screening and selection for the highest cellulase-producing mutant

The mutants were selected from CMC plates after mutagenesis on the basis of clearance zones appearing in detection media. The treated cell was resuspended in CMC medium containing 100 µg/ml of penicillin and incubated for 2 h with mild shaking at 35°C for screening. The screening was repeated until non-mutant cells were killed by penicillin. Thence, cells were centrifuged, washed with McIlvaine's buffer and suspended in buffer at the suitable concentration for spreading onto CMC agar plates. The colonies were then patched onto fresh media plates (17 colonies/plates) to serve as the master plate. The mutants, detected by Congo red, were inoculated into test tube (21 × 200 mm) containing 10 ml of CMC medium. After 60 h of incubation, each sample was collected and measured for enzyme activity. Cells produced the highest enzyme activity than their parent strain that was selected for optimization studies.

Fermentation

Different fermentation runs were conducted using the shake-flask method. To determine the effects of different carbon sources on cellulase production, mutant *Cellulomonas* sp. was grown in optimum medium contained 4% wastepaper as carbon source with initial pH and cultivation temperature at 6 and 35°C according to previous study of Sangkharak et al. (2011). However, the effect of different nitrogen sources and concentrations as well as the effect of agitation speed were identified in this study for increase cellulase production. The medium was supplemented with different nitrogen sources and concentrations ($NaNO_3$, peptone and yeast extract at 0 to 10%). Additionally, the effect of agitation speed (0 to 200 rpm) was also optimized via individual experiments.

Time course on enzyme production under the optimal condition in batch culture

Time course on cellulase production from the selected strain under the optimal condition was studied. The experiments were conducted in a 5 L glass fermentor with three six bladed Rushton turbine impellers (40 mm diameter) and equipped with pH controlled; dissolved oxygen (DO), antifoam and temperature probes connected to the controller. Samples were taken at time interval to determine for growth (DCW) and enzyme activity during 96 h cultivation.

Enzyme assay

Crude enzyme preparation was prepared according to the method of Latifian et al. (2007). Filter paper cellulase (FPase) was determined according to the method of Vu et al. (2010). The FPase was assayed by incubating 1 ml of diluted enzyme solution with acetate buffer (50 mm, pH 5) containing Whatman No.1 filter paper (50 mg). The reaction mixture for FPase was incubated at 50°C for 30 min and the released reducing sugar were then determined by the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). Carboxymethyl-cellulase (CMCase) activity was determined following the method described by Nitisinprasert and Temmes (1991) using a reaction mixture containing 1 ml of enzyme solution with 1 ml of 1% CMC (incubated at 40°C for 30 min) in McIlvaine's buffer (pH 5). The amount of reducing sugar released in the hydrolysis was measured.

The β-glucosidase activity was estimated using ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) as a substrate. An assay mixture (1 ml) consisting of 0.9 ml of ρNPG (1 mm) and 0.1 ml of diluted enzyme was incubated at 50°C for 30 min. The ρ-nitrophenol that was liberated was measured at 420 nm after developing the color with 2 ml of sodium carbonate (2M). One unit of enzyme activity in each case was defined as 1 µmol of glucose or ρ-nitrophenol release per minute.

Analytical method

The bacterial culture broth (5 to 10 ml) was centrifuged at 12846 × g for 10 min at 4°C. The pellet was washed twice with distilled water and then suspended in 5 to 10 ml distilled water. After mixing, growth was monitored by measuring absorbance at 660 nm (Shimizu et al., 1990).

Reducing sugar content in the hydrolysate was determined qualitative by reverse phase-HPLC according to method described by Lo et al. (2009) and Sangkharak et al. (2011). Quantitatively, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard (Ghose, 1987).

RESULTS AND DISCUSSION

Strain improvement by UV and NTG mutagenesis

The wild type *Cellulomonas* sp. strain TSU-03 was subjected to successive mutagenic treatment using UV irradiation and NTG. After strain improvement, 150 and 178 mutant colonies were obtained from UV and NTG treatment, respectively. Based on the ratio of diameter between the clearing zone and colony on the CMC-Congo red medium (Zaldivar et al., 2001; Xu et al., 2011). The cellulase activity of clones that displayed the largest clearing zones was assessed after 60 h of cultivation. The twenty-five best isolates (strain M1 to M25) were selected and cultivated in 10 ml of CMC medium for enzyme production. Mutant strain M23 exhibited the highest cellulase activity at 2008 U/mg protein followed by the mutant strain M17 (1884 U/mg protein) (Table 1). The assayed enzyme activity indicated obvious differences among the mutants of *Cellulomonas* sp. The production of enzyme ranges from 654 to 2008 U/mg protein as compared with CMCase activity at 1860 U/mg protein from the wild type strain. The results conclude that mutagenesis by UV and NTG caused changes in cellulase production or secretion. Therefore, strain M17 and M23 were selected and used throughout this study.

The stability of the mutant *Cellulomonas* sp. strain M23

The stability of mutant *Cellulomonas* sp. M23 for biomass and cellulase production was determined by successive subculturing on CMC agar for over one year. Mutant was subculture every month and evaluated for their ability to stably produce biomass and cellulase activity. The mutant M23 maintained the same production yield after being subcultured 12 times, indicating that the mutant is stably heritable. Mutant *Cellulomonas* sp. M23 was found to continuous produce high production in both growth (20.01 ± 1.65 g/L) and CMCase activity (2008 U/mg protein) on the CMC medium.

Optimization on cellulase production by mutant strain *Cellulomonas* sp. M23

Wastepaper, an abundant biomass found in Thailand were selected and utilized as sole carbon source. From our previous study wastepaper contained high content of cellulose (>88%) in low amount of hemicelluloses and lignin (Sangkharak, 2011; Sangkharak et al., 2011). 4% of wastepaper was suitable for cellulase production by *Cellulomonas* sp. and under optimal condition contained 4% wastepaper with controlled pH at 6 and cultivation temperature at 35°C, *Cellulomonas* sp. TSU-03 (wild type) achieved highest cellulase activity at 1860.1 U/mg protein (Sangkharak et al., 2011). Therefore, carbon source, pH and cultivation temperature were maintained according to our previous study. Additional effect of nitrogen sources and concentrations as well as, effect of agitation speed were determined for growth and cellulase production from *Cellulomonas* sp. mutant M17 and M23.

Effect of different nitrogen sources and concentrations

Effect of various nitrogen sources on the cellular growth and cellulase production from three strain of *Cellulomonas* sp. including wild type and two mutant strains (M17 and M23) were investigated in medium with 4% wastepaper as sole substrate. Sources of nitrogen included NH_4NO_3 (control), $NaNO_3$, peptone and yeast extract at the concentration of 0 to 10 g/L. Data indicated that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase activity. The use of inorganic nitrogen sources responded in the positive cellulase activity than organic ones. Therefore, mutant strain M23 gave the highest biomass (24.32 ± 1.75 g/L) and enzyme activity (FPase, CMCase and β-glucosidase at 267, 2128 and 102 U/mg protein, respectively) when cultivated in the optimum medium with 0.1% $NaNO_3$ as nitrogen source (Table 2).

Inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$, $NH_4H_2PO_4$ as well as organic nitrogen (corn steep liquor and urea) were poor nitrogen sources of cellulase synthesis by *C. flavigena*. The best nitrogen sources of FPase production were $NaNO_3$, KNO_3 and NH_4NO_3 since *C. flavigena* possessed strong nitrate reductase activity which was induced by NO_3 ions to an optimal level and repressed by free NH_4 ions in the growth medium (Rajoka, 2004). Polypeptone was also supported by the maximum production of β-cellobiohydrolase by *C. uda* CB4 (Nakamura and Kitamura, 1988).

Effect of agitation speed

The cultivation of *Cellulomonas* sp. wild type and two mutant strains (M17 and M23) was carried in optimum medium with 4% wastepaper and 1% $NaNO_3$ as carbon and nitrogen source, respectively. pH was controlled at 6.0 at 35°C. Cell growth decreased as the agitation speed increased over 100 rpm which might be due to cell destruction. Therefore, the agitation rate at 100 rpm was most preferred. The maximum biomass (28.00 ± 1.59 g/L)

Table 1. Comparison on carboxymethyl-cellulase (CMCase) activity from wild type and mutant strain of *Cellulomonas* sp. after 60 h of cultivation in CMC medium at 35°C.

Mutant strain	CMCase (U/mg protein)	Mutant strain	CMCase (U/ mg protein)	Mutant strain	CMCase (U/ mg protein)
M01	887	M10	665	M19	990
M02	1020	M11	1652	M20	1003
M03	654	M12	1087	M21	1546
M04	992	M13	1100	M22	1054
M05	1001	M14	897	M23	2008
M06	1176	M15	901	M24	1789
M07	891	M16	1454	M25	991
M08	880	M17	1884	wild type	1860
M09	1224	M18	1039		

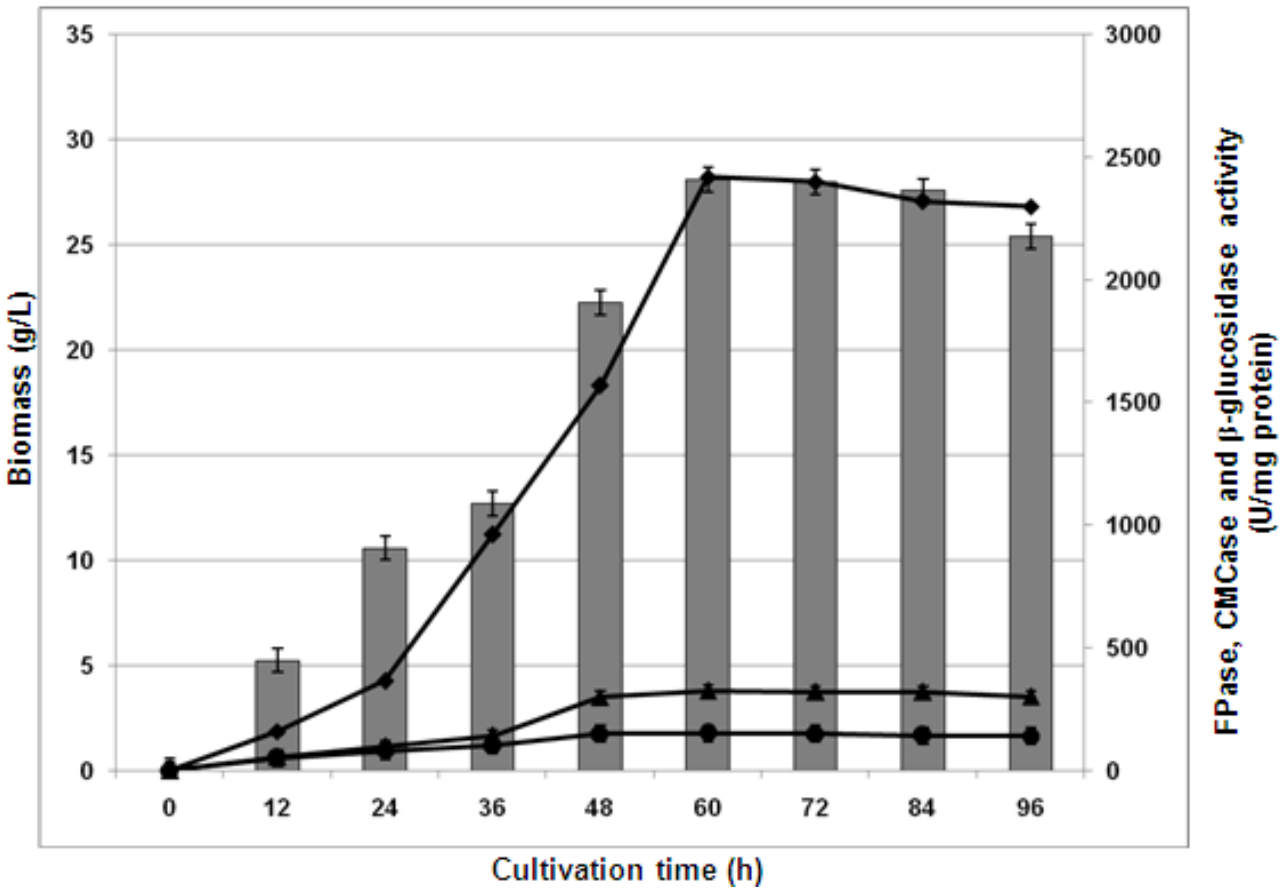
Table 2. Effect of nitrogen sources and concentrations on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23 cultivation in medium containing 4% wastepaper as carbon source.

Nitrogen source	*Cellulomonas* sp.											
	Mutant strain M17				Mutant strain M23				Wild type			
	Biomass (g/L)	Cellulase activity (U/mg protein)			Biomass (g/L)	Cellulase activity (U/mg protein)			Biomass (g/L)	Cellulase activity (U/mg protein)		
		FPase	CMCase	β-glucosidase		FPase	CMCase	β-glucosidase		FPase	CMCase	β-glucosidase
NH_4NO_3 (control)	19.51	102	1884	65	22.01	188	2008	87	19.04	98	1860	54
$NaNO_3$	21.15	115	1992	74	24.32	267	2128	102	21.25	120	1998	81
Peptone	11.74	10	248	28	12.21	45	980	25	10.56	15	457	25
Yeast extract	11.92	12	336	31	14.47	50	1012	35	11.18	33	554	33

and cellulase activity (FPase, CMCase and β-glucosidase at 281, 2254 and 132 U/mg protein, respectively) by *Cellulomonas* strain M23. Dissolved oxygen showed that increased agitation speeds gave the increase of dissolved oxygen. This indicated that agitation speed could elevate dissolved oxygen levels as agitation seems to play a role in addition to that of maintaining oxygen tension, possibly by improving mass transfer between the medium and cells (Slodki and Cadmus, 1978). The results showed that 0, 100 and 200 rpm of agitation speeds maintained dissolved oxygen over 25, 65 and 80%, respectively.

Time course on cellulase production under optimal condition in batch culture

Among three strains of *Cellulomonas* sp. including one strain of wild type and two mutant (M17 and M23), mutant M23 yielded the highest biomass and enzyme activity. Therefore, *Cellulomonas* sp. strain M23, NTG mutant, was selected and studied for cellulase production in fermentor. *Cellulomonas* sp. M23 was cultivated in the optimal medium supplemented with 4% wastepaper and 1% $NaNO_3$; pH was controlled at 6.0 and incubation at 35°C for 96 h. The cultivation was performed in a 5 L fermentor with agitation speed of 100 rpm. The results are given in Figure 1. The specific growth rate (μ) was 0.29 h^{-1}. Cellular growth was 28.09 ± 2.28 g/L, FPase, CMCase and β-glucosidase activities were 325, 2420 and 152 U/mg protein, respectively. These values of productivity were 1.2-folds higher than



Figures 1. Growth and cellulase activity of *Cellulomonas* sp. M23 on optimal medium contained wastepaper (carbon source), 40 g/L; $NaNO_3$ (nitrogen source), 10 g/L at 35°C under controlled pH (6.0) conditions and agitation speed at 100 rpm. ■ biomass, ▲ FPase activity, ♦ CMCase activity, ● β-glucosidase activity.

those cultivated in un-modified medium. The results suggested that *Cellulomonas* sp. M23 had a good potential for production of cellulase by fermentation using a cultivation medium containing CMC as the main substrate. The CMCase activity (2420 U/mg protein) achieved from this strain is 1.28 and 1.30-fold higher than cellulase from mutant M17 (1884 U/mg protein) and wild type (1860 U/mg protein), respectively.

Conclusion

The improvement of cellulase production from *Cellulomonas* sp. TSU-03 was attempted by the mutagenesis and optimization study. *Cellulomonas* sp. strain M23, a prominent strain, producing a high level of cellulase was selected from 328 mutant strains after UV irradiation and NTG treatment. The highest value of cellulase activity 2008 U/mg protein) was achieved by mutant M23 followed by mutant M17 (1884 U/mg protein) in CMC medium.

The commercial use of cellulase is dependent on the following factors (i) high titer ad good enzymatic activity, (ii) low production cost and (iii) feasible mass production. Under the optimum medium and environmental conditions for cellulase production consisted of 4% wastepaper, 1% $NaNO_3$ under cultivatation temperature at 35°C with initial pH and agitation speed at 6 and 100 rpm, respectively. *Cellulomonas* sp. strain M23 produced the highest cellular growth (28.09 ± 2.28 g/L) and FPase, CMCase as well as, β-glucosidase activities at 325, 2420 and 152 U/mg protein, respectively. Under optimal condition, the cellulase activity achieved from strain M23 is 1.28- and 1.30-fold higher than cellulase from mutant M17 and wild type, respectively. The results suggested that *Cellulomonas* sp. M23 had a good potential for production of cellulase by fermentation using a cultivation medium containing wastepaper as the main substrate.

ACKNOWLEDGEMENT

This work was supported by Research and Development Institute, Thaksin University.

REFERENCES

Abdel-Aziz MS, Talkhan FN, Fadel M, AbouZied AA, Abdel-Razik AS (2011). Improvement of xyalanse production from Streptomyces pseudogriseolus via UV mutagenesis. Aust. J. Basic Appl. Sci., 5: 1045-1050.

Abou-Taleb KAA, Mashhoor WA, Nasr SA, Sharaf MS, Abdel- Azeem HHM (2009). Nutritional and environmental factors affecting cellulase production by two strains of cellulolytic Bacilli. Aust. J. Basic Appl. Sci., 3: 2429-2436.

Chen XE, Feng XB, Xia WS (2008). Strain improvement and optimization of the media composition of chitosananse-producing fungus *Aspergillus* sp. CJ 22-326. Afr. J. Biotechnol., 7: 2501-2508.

Ghose TK (1987). The measurment of cellulase activities. Pure Appl. Chem., 59: 257–268.

Immanuel G, Dhanusa R, Prema P, Palavesam A (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. Int. J. Environ. Sci. Tech., 3: 25-34.

Jarvis M (2003). Cellulose stacks up. Nature, 426: 611-612.

Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A (2008). A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. Curr. Microbiol., 57: 503–507.

Latifian M, Hamidi-Esfahani Z, Barzegar M (2007). Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresour. Technol., 98: 3634–3637.

Lo YC, Saratale GD, Chen WM, Bai MD, Chang JS (2009). Isolation of cellulosee-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. Enzyme Microb. Tech., 44: 417–425.

Mala JGS, Kamini NR, Puvanakrishnan R (2001). Strain improvement of *Aspergillus niger* for enhanced lipase production. J. Gen. Appl. Microbiol., 47: 181-186.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426–428.

Nakamura K, Kitamura K (1988). Cellulases of *Cellulomonas uda*. Methods Enzymol., 160: 211-216.

Nitisinprasert S, Temmes A (1991). The characteristics of a new non-spore-forming cellulolytic mesophilic anaerobe strain CMC126 isolated from municipal sewage sludge. J. Appl. Bateriol., 71: 154–161.

Rajoka MI (2004). Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena*. Electron. J. Biotechn., 7: 256-263.

Sangkharak K (2011). Optimization studies on enzymatic hydrolysis for ethanol production by simultaneous saccharification and fermentation from wastepaper. Waste Manage. Res., 29: 1134-1144.

Sangkharak K, Vangsirikul P, Janthachat S (2011). Isolation of novel cellulase from agricultural soil and application for ethanol production. Int. J. Adv. Biotechnol. Res., 2: 230-239.

Shimizu H, Shioya S, Suga KI (1990). Cultivation condition for production of biodegradable poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus* H16 and *Myoplans ruba* R14 with respect to the dissolved oxygen tension in ammonium limited batch cultures. Eur. J. Appl. Microbiol. Biotechnol., 7: 1-10.

Slodki ME, Cadmus MC (1978). Production of microbial polysaccharides. Adv. Appl. Microbiol., 23: 19–54.

Vu VH, Pham TA, Kim K (2010). Improvement of a fungal strain by repeated and sequential mutagenesis and optimization of solid-state fermentation for the hyper-production of rawstarch-digesting enzyme. J. Microbiol. Biotechnol., 20: 718-726.

Vu VH, Pham TA, Kim K. (2009). Fungal strain improvement for cellulase production using repeated and sequential mutagenesis. Micobiol., 37: 267-271.

Xu H, Jia S, Liu J (2011). Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin. Afr. J. Biotechnol., 10: 779-788.

Zaldivar M, Velasquez JC, Contreras I, Perez LM (2001). *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. Electron. J. Biotechnol., 4:1-9.