รายงานวิจัยฉบับสมบูรณ์

การปรับปรุงสายพันธุ์แบคทีเรียผลิตเซลลูเลสและสภาวะที่เหมาะสมต่อการผลิตเซลลูเลสจากเชื้อสายพันธุ์กลาย

Strain Improvement of Cellulase – Producing Bacteria and Optimization Study on Cellulase Production from Mutant Strain

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สนับสนุนโดย งบประมาณเงินแผ่นดิน

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มหาวิทยาลัยทักษิณ

กิตติกรรมประกาศ

ขอขอบพระคุณมหาวิทยาลัยทักษิณ และสถาบันวิจัยและพัฒนา ม.ทักษิณ สำหรับเงินสนับสนุนตลอดโครงการในการทำวิจัย เรื่องการปรับปรุงสายพันธุ์แบคทีเรียผลิตเซลลูเลสและสภาวะที่เหมาะสมต่อการผลิตเซลลูเลสจากเชื้อสายพันธุ์กลายจนกระทั่งงานวิจัยนี้สำเร็จลุล่วงไปได้ด้วยดี

ขอขอบพระคุณอาจารย์ และบุคลากรประจำสาขาวิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยทักษิณ ที่ได้ให้การสนับสนุน อำนวยความสะดวกในการทำวิจัยในครั้งนี้

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หัวหน้าโครงการวิจัย

บทคัดย่อ

ชื่อโครงการวิจัย การปรับปรุงสายพันธุ์แบคทีเรียผลิตเซลลูเลสและสภาวะที่เหมาะสมต่อการผลิตเซลลูเลสจากเชื้อสายพันธุ์กลาย

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งานวิจัยนี้มีวัตถุประสงค์เพื่อเพิ่มประสิทธิภาพการผลิตเซลลูเลสโดยการพัฒนาสายพันธุ์จุลินทรีย์ *Cellulomonas* sp. TSU-03 ซึ่งเป็นสายพันธุ์ที่คัดแยกได้จากดินและมีประสิทธิภาพในการผลิตเซลลูเลสสูง รวมถึงการเพิ่มผลผลิตของเอนไซม์โดยการหาสภาวะที่เหมาะสมในการผลิตเซลลูเลส และการพัฒนาสายพันธุ์จุลินทรีย์ด้วยเทคนิคทางพันธุวิศวกรรม จากการพัฒนาสายพันธุ์ด้วยวิธีการกลายพันธุ์โดยใช้สารเคมีและการใช้รังสียูวี จะได้เชื้อสายพันธุ์กลายทั้งหมด 328 สายพันธุ์ ซึ่งในจำนวนดังกล่าว พบว่าเชื้อพันธุ์กลาย สายพันธุ์ M27 ที่เกิดการกลายพันธุ์โดยใช้ NTG เป็นสายพันธุ์ที่ให้ผลผลิตของเซลลูเลสสูงที่สุด เท่ากับ 2.93±0.11 ยูนิตต่อมิลลิลิตร ตามด้วยสายพันธุ์ M17 ที่ให้ผลผลิต 2.57±0.14 ยูนิตต่อมิลลิลิตร เมื่อทำการเลี้ยงในอาหาร CMC

เมื่อทำการพัฒนาสายพันธุ์จุลินทรีย์เพื่อให้ได้ผลผลิตเซลลูเลสที่สูงขึ้นด้วยวิธีพันธุวิศวกรรม โดยทำการคัดลอกยีนเอนโดกลูคาเนส (Cen_{sp}) ที่ควบคุมการผลิตเซลลูเลสจากเชื้อ *Cellulomonas* sp. ผลการทดลองพบว่ายีนที่คัดลอกได้มีความเหมือนกับยีนเอนโดกลูคาเนส (*Cen*) จากเชื้อจุลินทรีย์ *Cellulomonas fimi* ถึง 100 เปอร์เซ็นต์ แต่เมื่อนำยีนดังกล่าวไปทำการศึกษาการแสดงออกใน *E. coli* และ *Cellulomonas* sp. พบว่ายีนดังกล่าวมีการ

แสดงออกได้ต่ำและให้ผลผลิตของเอนไซม์ที่ไม่แตกต่างจาก *Cellulomonas* sp. TSU-03 (สายพันธุ์ดั้งเดิม) ดังนั้นในการศึกษาในขั้นตอนต่อไปจึงเลือกเฉพาะจุลินทรีย์พันธุ์กลาย M17 และ M23 เท่านั้นในการทดลอง

อาหารและสภาวะที่เหมาะสมในการผลิตเซลลูเลส ประกอบไปด้วย CMC ปริมาณ 10 กรัมต่อลิตร และ $NaNO_3$ ปริมาณ 1 กรัมต่อลิตร ภายใต้สภาวะเพาะเลี้ยงที่อุณหภูมิ 45^{o}C ค่าความเป็นกรดและด่างของอาหาร เท่ากับ 6 และความเร็วรอบในการหมุนเหวี่ยงเท่ากับ 100 รอบต่อนาที ภายใต้สภาวะการเพาะเลี้ยงดังกล่าว จุลินทรีย์ *Cellulomonas* sp. สายพันธุ์ M27 ให้ผลผลิตเซลล์สูงสุดเท่ากับ 7.11±1.28 กรัมต่อลิตร และให้กิจกรรมของเอนไซม์ FPase, CMCase และ β-glucosidase เท่ากับ 3.25±0.20, 4.51±1.15, 1.52±0.40 ยูนิตต่อมิลลิลิตร ตามลำดับ กิจกรรมของเซลลูเลสที่ได้พบว่ามีค่าสูงกว่าค่าที่ได้จากเชื้อพันธ์กลาย M17 ถึง 1.2 เท่า และสูงกว่า 2.8 เท่าเมื่อเปรียบเทียบกับผลผลิตจากเชื้อสายพันธุ์ดั้งเดิม ซึ่งแสดงให้เห็นว่า เชื้อสายพันธุ์กลาย *Cellulomonas* sp. M23 เป็นเชื้อที่มีศักยภาพในการผลิตเซลลูเลส และให้ผลผลิตของเซลลูเลสที่มีกิจกรรมของเอนไซม์ที่สูงในอาหารที่มี CMC เป็นองค์ประกอบ

An objective of this study was to develop a high cellulase production by improve the prominent cellulase producing bacteria, increased enzyme production by optimization studies and strain improvement by genetic engineering. Among 328 mutant strains of *Cellulomonas* sp. TSU-03, the mutant strain M23, NTG mutant, gave the highest value of cellulase activity (2.93±0.11 U/mL) followed by mutant M17 (2.57±0.14 U/mL) in CMC medium.

Strain improvement for higher production of cellulase from *Cellulomonas* sp. TSU-03 was conducted by cloning of gene encoding the endoglucanase of *Cellulomonas* sp. (Cen_{Csp}) through complementation to cellulase accumulation of *Cellulomonas* sp. (wild type) was investigated. The Cen_{Csp} sequence of DNA from *Cellulomonas* sp. was analyzed and the fragment showed 100% identity with *Cen* gene from *Cellulomonas fimi* (accession number 15823). Cloning was confirmed by enzymatic studies and results showed that cellulase activity was low expression in *E. coli* and *Cellulomonas* sp. Therefore, mutant M17 and M23 (NTG mutant) were selected and used throughout this study.

The optimum medium and environmental for cellulase production consisted of 10 g/L CMC, 1 g/L $NaNO_3$ under cultivatation temperature at 45^{o}C with initial pH and agitation speed at 6 and 100 rpm, respectively. *Cellulomonas* sp. strain M23 produced the highest cellular growth (7.11±1.28 g/L) and FPase, CMCase as well as β-glucosidase activities at 3.25±0.20, 4.51±1.15, 1.52±0.40 U/mL, respectively. The cellulase activity achieved from strain M23 is 1.2 and 2.8 folds higher than cellulase from mutant M17

(2.50±1.02 U/mL) and wild type (1.12±0.50 U/mL), respectively. 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.

Keywords: β-glucosidase, Cellulase, *Cellulomonas* sp., CMCase, FPase

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1. 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 (carboxymethycellulase, 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.

Numerous researchers attempted to improve cellulase producing-microorganism since the potential to screen and identify novel species with vastly superior production capacity 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 (1,860.1 U/mg protein). Therefore, this investigation aims to increase the cellulase production by strain improvement and optimization methods as well as genetic engineering method was also applied to improve the accumulation of cellulase in this study.

2. LITERATURE REVIEW

1. Introduction

Cellulose, a polysaccharide made up from D-glucose subunits in a polymerization degree of 10,000 or even higher, is considered to be the primary product of photosynthesis and the most abundant renewable carbon resource in nature (Jarvis, 2003; Zhang, 2004; Li *et al*., 2011). Because cellulose can be utilized to produce ethanol, it is a promising alternative energy source for the production of fossil fuels (Sangkharak *et al*., 2011). Cellulose is degraded by cellulases to reducing sugars and fermented by yeast or bacteria to ethanol (Duff and Murray, 1996; Kim *et al*., 2009).

Cellulase are produced by various microorganisms including *Aspergillus* sp., *Bacillus* sp., *Chrysosporium* sp., *Fusarium* sp. *Phanerochaete* sp., *Sclerotium* sp., *Schizophyllum* sp. and *Trichoderma* sp. (Kim *et al*., 2009) as well as novel strain of *Cellulomonas* sp. which exhibited high activity of cellulase (Sangkharak *et al*., 2011). Economic analyses have indicated that the production cost of cellulase is still the major cost factor in the hydrolysis of cellulosic materials to fermentable sugars. It is therefore imperative to improve the production of cellulase in order to make the process more economically viable (Gomes *et al*., 1992; Adsul *et al*., 2007). A reduction in the cost of cellulase production, an improvement in cellulase activity and an increase in sugar yields are all vital to reducing the processing costs of bioethanol from cellulosic substrates (Zhang *et al*., 2006). During ethanol production from lignocellulosics, cellulase play a very important role in the cellulose digestion process and so far the cost of cellulases is very expensive

due to the large amounts required for cellulose digestion (Sun and Cheng, 2002; Schell *et al*., 2003). Therefore, the improvement of microbial strains for the over-production of cellulases has attracted attention in the commercial fermentation process (Kim *et al*., 2009).

2. Strain improvement

The commercial use of cellulases is dependent on the following factors (i) high titer ad good enzymatic activity, (ii) low production cost and (iii) feasible mass production. Owing to their inherent control system, microorganisms usually produce commercially important metabolites in very low concentrations and, although the yield may be increased by optimizing the cultural conditions, productivity is controlled ultimately by the organism's genome. Strain improvement is an essential and important part of process development of a biotechnology process. The overall is to reduce costs by developing strains with increased productivity, ability to use cheaper or alternative substrates, or other unique and desirable characteristics. Therefore, the organism's genome must be modified and this may be achieved in three ways: by (i) randome selection, (ii) classical strain improvement by mutation and selection as well as (iii) the use of recombination (Stanbury *et al*., 2003).

2.1 Random screening

Random screening has been used reliably for many years for improvement first in penicillin production and then in a variety of other fermentations. It still plays a central role in many fermentation industrials today. Random screening has benefited from advances in many fields which is making it more likely to obtain improved mutants (Sawangsaeng, 2004).

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzyme capable of completely hydrolyzing crystalline cellulose in vitro. Fungi are the mail cellulose-producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity (Table 1) Microorganisms of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use. Microorganisms of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase and exo-β-glucanase, but only low levels of β-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-β-glucanase and β-glucosidase with low levels of exo-β-glucanase production.

Table 1 Representative cellulase-producing microorganisms

Acremonium cellulotylicus	*Irpex lacteus*	*Sporotrichum cellulophilum*
Aspergillus acculeatus	*Pennicillin funmiculosum*	*Talaromyces emersonii*
Aspergillus fumigates	*Phanerochaete chrysosporium*	*Thermoactinomyces* sp.
Aspergillus niger	*Ruminococcus albus*	*Thermomonospora curvata*
Cellulomonas sp.	*Schizophyllum commune*	*Thielavia terrestris*
Clostridium thermocellum	*Sclerotium rolfsii*	*Trichoderma koningii*
Fusarium solani	*Streptomyces* sp.	*Trichoderma reesei*
		Trichoderma viride

2.2 Mutagenesis

Each time a microbial cell divides there is a small probability of an inheritable change occurring. A strain exhibiting such a changed characteristic is termed a mutant and the process giving rise to it, a mutation. Mutagenesis is the source of all genetic variations, but no single mutagenic treatment will give all possible types of mutation. In molecular biology and genetics, mutations are changes in a genomic sequence: the DNA sequence of a cell's genome or the DNA or RNA sequence of a virus. They can be defined as sudden and spontaneous changes in the cell.There are two major types of mutagens (a) Physical mutagen such as UV, X-rays and gamma radiation and (b) Chemical mutagen such as ethyl methane sulfonate (EMS), nitrosomethyl guanidine (NTG) and mustards, as well as errors that occur during meiosis or DNA replication (Bertram, 2000; Burrus and Waldor, 2004; Aminetzach *et al*. 2005). They can also be induced by the organism itself, by cellular processes such as hypermutation.

Mutation can result in several different types of change in sequences; (DNA) these can have no effect, alter the product of a gene, or prevent the gene from functioning properly or completely. The type of mutations induces in any cell type depends on the follow factors:

(1) The type of DNA damage caused by the mutagen. Most mutagens produce more than one type of DNA damage. Some mutagens produce more of one type of DNA damage than other

(2) The cell’s DNA repair mechanisms: DNA repair systems may non-mutagenic (error-free) or mutagenic (error-prone). Some mechanisms

include photoreactivation, excision repair, recombination repair and SOS repair

The use of different mutagenic agents for strain improvement was demonstrated by Perekh *et al*. (2000). Additionally, treatment of Fusarium oxysporum with ultraviolet (UV) followed by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was used to improve carboxymenthyl cellulase (CMCase) production (Kuhad *et al*., 1994). Moreover, Chand *et al*. (2005) used simultaneous treatment with NTG, ethidium bromide and UV or NTG combined with ethidium bromide to create mutant fungi that produced more CMCase and filter paper cellulase (FPase) than wild type fungi (Vu *et al*., 2010). The wide type strain of filamentous fungi, *Trichoderma* TL-124 produces extracellular cellulase. The strain TL-124 was subjected to successive mutagenic treatments with UV irradiation, low-energy ion beam implantation, atmospheric pressure non-equilibrium discharge plasma (APNEDP), and NTG to generate about 3000 mutants. Among these mutants, *T. viride* N879 strain exhibited the greatest relevant activity: 2.38-fold filter paper activity and 2.61-fold carboxymethyl cellulase, 2.18-fold β-glucosidase, and 2.27-fold cellobiohydrolase activities, compared with the respective wild-type activities, under solid-state fermentation using the inexpensive raw material wheat straw as a substrate.

Cellulase production was also improved by sequential treatments by two repeated rounds of γ-irradiation of Co^{60}, UV treatment and four repeated rounds of treatment with NTG. The best mutant strain, *Aspergillus* sp. XTG-4, was selected after screening and the activities of carboxymethyl cellulase,

filter paper cellulase and β-glucosidase of the cellulase were improved by 2.03-, 3.20-, and 1.80-folds, respectively, when compared to the wild type strain. After being subcultured 19 times, the enzyme production of the mutant *Aspergillus* sp. XTG-4s was stable (Vu *et al*., 2010).

Protoplast fusion is the other method to improvement of industrially important microorganisms instant of UV and NTG. Protoplast fusion technology, a viable option for strain improvement in higher basidiomycetes was applied for improvement in the lignolytic activities of a white-rot fungus *Pleurotus ostreatus*. The two mono-caryotic parent strains of *P. ostreatus* – POM_1 (UV irradiated mutant) and POM_2 (X-ray irradiated mutant) were isolated and used for protoplast isolation and fusion. In malt extract broth after 14 days of incubation the maximum enzyme activity was recored (116 U/ml) in the culture filtrate of POM_2 (Vijaya *et al*., 2005).

2.3 Recombination

Recombination is a process which helps to generate new combinations of genes that were originally present in different individuals. Compared with the use of mutation techniques for the improvement of industrial strains the use of recombination was fairly limited in the early years of improvement programmed. However, techniques are now widely available which allow the use of recombinant as a system of strain improvement.

Two strategies are available for improving the properties of individual cellulase components: (i) rational design and (2) directed evolution.

2.3.1 Rational design

Rational design is the earliest approach to protein engineering, was introduced after the development of recombinant DNA methods and site-directed mutagenesis more than 20 years ago, and is still widely used. This strategy requires detailed knowledge of the protein structure, of the structural causes of biological catalysis or structure-based molecular modeling, and of the ideally structure-function relationship. The process of rational design involves (1) choice of a suitable enzyme, (2) identification of the amino acid sites to be changed, based usually on a high resolution crystallographic structure, and (3) characterization of the mutants (Fig. 1).

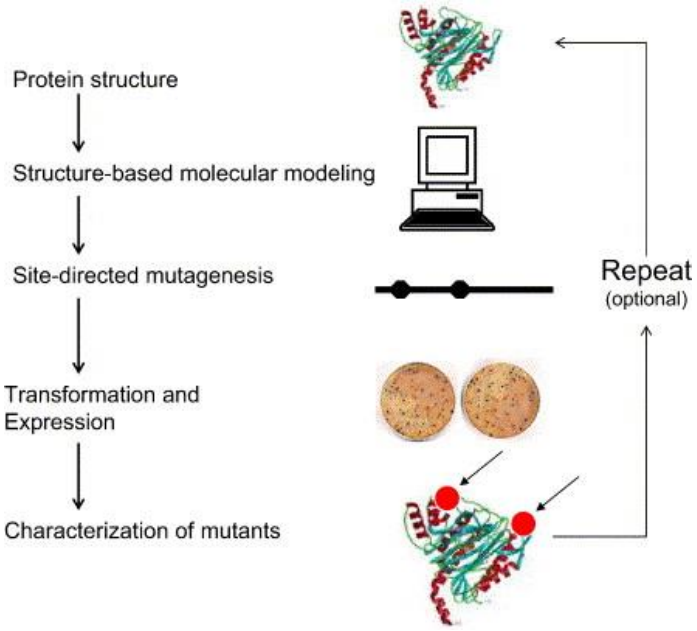

Figure 1 Scheme of rational protein design

Source : Zhang *et al*. (2006)

Several excellent reviews summarize numerous studies using site-directed mutagenesis for investigating cellulase mechanisms and improving enzyme properties (Schulein, 2000; Wither, 2001; Wilson, 2004). Not

surprisingly, few researchers using site-directed mutagenesis have reported successful examples of significantly higher activity cellulase mutants on insoluble substrates (Zhang *et al*., 2000; Escovar-Kousen *et al*., 2004). One clear example, however, is the report by Baker and his co-worker of a 20% improvement in the activity on microcrystalline cellulose of a modified endoglucanase Cel5A from *Acidothermus cellulolyticus* (Baker *et al*., 2005). However, today there are no general rules for site-directed mutagenesis strategies for improving cellulase activity on solide cellulase substrates and it remains in a trial-and-test process (Zhang *et al*., 2006).

2.3.2 Direct evolution

The great advantage of directed evolution is that it is independent of knowledge of enzyme structure and of the interactions between enzyme and substrate. The greatest challenged of this method is developing tools to correctly evaluate the performance of mutants generated by recombinant DNA techniques (Fig. 2).

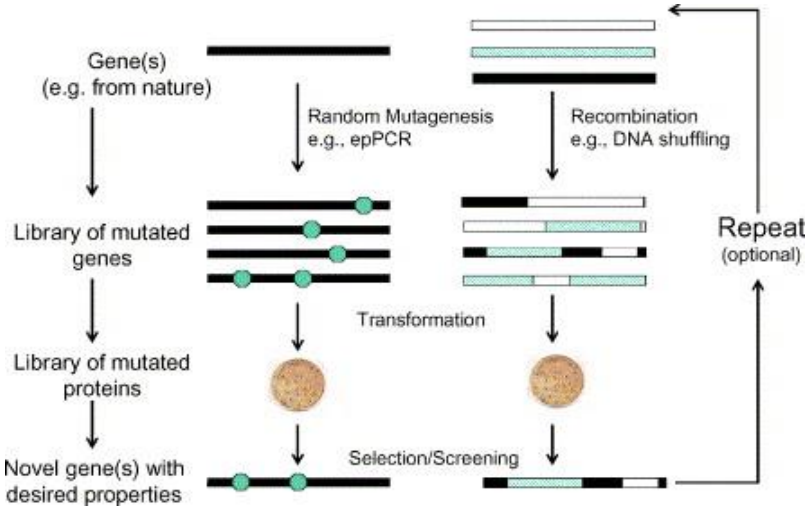

Figure 2 Scheme of directed protein evolution

Source: Zhang *et al*. (2006).

T. reesei is a well-known cellulase producer and widely applied in enzyme industry. To increase its ability to efficiently decompose cellulose, the beta-glucosidase activity of its enzyme cocktail needs to be enhanced. Cellulase activity in *T. reesei* was improved by ligate a beta-glucosidase I coding sequence from *Penicillium decumbens* with the cellobiohydrolase I (cbhI) promoter of *T. reesei* and introduced into the genome of *T. reesei* strain Rut-30 by *Agrobacterium*-mediated transformation. In comparison to that from the parent strain, the beta-glucosidase activity of the enzyme complexes from two selected transformant increases 6- and 8-folds and their filter paper activity (FPAs) was enhanced by 30% on average (Ma *et al*., 2011). Takashima *et al*. (1998) was examined the expression system of *T. reesei* cellulase genes using *Aspergillus oryzae* as a host. The expression of *T. reesei* cellulase genes were regulated under the control of *A. oryzae* Take-amylase promoter and the cellulase genes were highly expressed when maltose was used as a main carbon source for inducer. In a recent example, after DNA family shuffling, a β-glycosidase mutant was found to display lactose hydrolysis rates 3.5-fold and 8.6-fold higher than the parental *Pyrococcus furious* CelB and *Sulfolobus solfataricus* LacS, respectively (Kaper *et al*., 2002).

3. Conclusion and future

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). Thus, microorganisms are capable of producing a wide range of products, a range which has been increased by the techniques of random screening, mutation and recombinant technology. In additional, improved productivity may be achieved by the optimization of cultural conditions and DNA technology of the producer cells. However, to achieve a high-efficient production and a successful commercial process, the improvement may focus on improve at genetic level using chemical engineering expertise as it does on that of microbiology and genetics.

3. MATERIALS AND METHODS

1. Microorganisms and culture medium

The wild type of *Cellulomona*s 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^{o}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, $CaCl_2$ 0.02 (Kasana *et al*., 2008; Lo *et al*., 2009).

2. Strain improvement and screening on mutant producing the highest cellulase production

2.1 Strain improvement by mutagenesis

2.1.1 Mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (NTG)

The wide type cell grown in CMC medium at 35^{o}C for 24 h was harvested at logarithmic phase by centrifugation (10,000 *x g*, 20 min) at 4^{o}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 x 10^8 cell/mL. N-methyl-N'-nitro-N-nitrosoguanidine (NTG), 100 mg/ml, was added into the cell suspension. After incubation for 1 h at 35^{o}C, 100 rpm, the cell was centrifuged, washed immediately with buffer. The treated sample was transfered into CMC medium, for further studies (modified from Xu *et al*., 2011).

2.1.2 Mutagenesis by Ultraviolet (UV) irradiation

The wild type cell was harvested as described above. 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 incubated at 35°C for 2 days, the number of colonies were counted to determine survival rates.

2.1.3 Preliminarey screening for the highest cellulase producing mutant by mutagenesis

The treated cell was resuspended in CMC medium containing 100 μg/mL of penicillin, and incubated for 2 h with mild shaking at 35^{o}C. The treatment 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. For the preliminary selection of mutants, CMC medium supplemented with 0.02% congo red was used.

2.2 Strain improvement by double gene dosage

2.2.1 Cloning of endoglucanase gene (*cen*A) from *Cellulomonas* sp.

Primers for amplification of *cen*A genes were designed from highly conserved amino acid sequences of known endoglucanase from *Cellulomonas fimi* (accession number M15823) and employed in PCR. Total genomic DNA of mutant was used after partial restriction with *Bam*HI as template and incubated for 30 cycles with designed primers from the downstream and

upstream regions of the gene to amplify *cen*A$_{Cf}$: *cen*A(Forward) 5'-ATAGAATTCTATGGGACTGCTGCAAACC-3' and c*en*A(Reverse) 5'-ATAGAATTCTTATCAGCATTCTGCCCCA-3'. The PCR protocol included the following steps: 30 second incubation at 95^{o}C for denaturation, 1 min at 58^{o}C for annealing and 2 min 10 second at 72^{o}C for extension. For all PCRs, a Platinum *Pfx* DNA polymerase kit (Invitrogen Life Technologies, Carlsbad, Calif.) was employed. PCR products of about 1.1 kbp were purified with a NucleoTrap kit (Macherey-Nagel GmbH) and were then ligated to *Eco*RV restricted pSK^{-} and pBBR122 DNA by employing T4 DNA ligase (MBI Fermentas GmbH). The ligation products were then transformed into *E. coli* strains BL21, respectively, by using standard methods (Sambrook *et al*., 1989), and recombinant strains of *E. coli* harboring the hybrid plasmids pSK::*cen*A$_{Csp}$ and pBBR122::*cen*A$_{Csp}$, respectively were selected (Table 2).

Table 2 Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant markers and characteristics	Reference or source
***E. coli* :**		
BL21(DE3)	B strain F^- *omp*T *hsd*R17 (r^- m^-) *gal* *dcm*(DE3)	Sambrook *et al*., 1989
Plasmids:		
pBBR122	Km^r, *lacPOZ*' mob^+, broad host range	Kovach *et al*., 1995
pBluescriptSK$^-$	Amp^r, *lacPOZ*', T7 and T3 promoter	Stratagene, San Diego, Calif.
pBBR122:c*en*A_{Csp}	pBBR122 harbouring 1.1 kb PCR product from *Cellulomonas* sp. strain TSU-03 DNA	This study
pSK:: *cen*A_{Csp}	pSK$^-$ harbouring a 1.1-kb PCR product from *Cellulomonas* sp. strain TSU-03 DNA	This study
Bacteria		
Cellulomonas sp. TSU-03	Wild type	This study

2.2.2 Heterologous expression of the *Cellulomonas* sp. endoglucanase gene in wild type

From *E. coli* BL21 the genomic library was mobilized into the *Cellulomonas* sp. (wild type) by electroporation (Kawagishi *et al*., 1994). For this, the recombinant mutants were transferred onto CMC agar plates plus

kanamycin (150 mg/L) to screen for recombinants containing cellulase activity.

2.3 Selection of mutant producing the highest cellulase production

The mutants were inoculated into test tube (21 x 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 was selected for optimization studies.

3. 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 CMC medium which CMC was supplemented with cheap carbon sources (1% rice straw and wastepaper). Rice straw and wastepaper used in medium preparation for fermentation was treated according to the method of Sangkharak (2011). Fermentation medium was prepared in a 250-ml flask when CMC medium was supplemented with different carbon sources at 35^{o}C 48 h. The media were autoclaved for 30 min at 121^{o}C. To determine the effects of different nitrogen sources on cellulase production, the 0.1% NH_4NO_3 was substituted with different nitrogen sources ($NaNO_3$, peptone and yeast extract). Different carbon and nitrogen sources were chosen as test substrates on the basis of literature data and their availability. Finally, initial pH (4.0-7.0), growth temperature (35-65^{o}C) and rotation speed (0-200 rpm) were optimized via individual experiments.

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

The selected strain was cultivated under optimal cultivation condition for 96 h. The kinetic parameter was determined as followed (Prasertsan *et al*., 1993; Doelle, 1997).

Specific growth rate (μ)

$$\mu = (\ln X - \ln X_0)/\Delta t \qquad (h^{-1})$$

When X = final biomass concentration (g/L)

X_0 = initial biomass concentration (g/L)

Δt = elapsed time (h)

5. Enzyme assay

Crude enzyme preparation was prepared according to the method of Latifian *et al*. (2007). Filter paper cellulase (FPase) (total cellulase) 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^{o}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 to 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^{o}C for 30 min) in McIlvaine's buffer (pH 5). The amount of reducing sugar released in the hydrolysis was measured. One unit of FPase

and CMCase activity was expressed as 1 μmol of glucose liberated per mL enzyme per minute.

The β-glucosidase (β-D-glucoside, glucohydrolase) 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^{o}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.

6. Analytical method

The bacterial culture broth (5-10 mL) was centrifuged at 12846 *x g* for 10 min at 4^{o}C. The pellet was washed twice with distilled water and then suspended in 5-10 mL distilled water. After mixing, growth will be 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). For the quantitatively, reducing sugars were estimated colorimetrically with dinitrosalicylic reagent method, using glucose as standard (Ghose, 1987).

4. RESULTS AND DISCUSSION

1. Strain improvement by mutagenesis

The wild type *Cellulomonas* sp. strain TSU-03 was subjected to successive mutagenic treatment using NTG and UV irradiation. After strain improvement, 150 and 178 mutant colonies were obtained from NTG and UV 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 twenty-five best isolates (strain M1-25) were selected and cultivated in 10 mL of CMC medium for enzyme production. The cellulase activity of clones that displayed the largest clearing zones was assessed after 60 h of cultivation. The most promising strain was subjected to the next mutagenesis treatment. After four mutagenic steps, mutant strain M23 exhibited the highest filter paper cellulase (FPase) activity at 2.93±0.11 U/mL followed by the mutant strain N17 (2.57±0.14 U/mL) (Table 3). The assayed protein concentrations indicated obvious differences among the mutants of *Cellulomonas* sp. The protein concentration in the crude enzyme preparation from the mutants ranges from 0.012 to 0.075 mg/mL, compared with 0.048 mg/mL in the preparations from the wild type strain. The results conclude that mutagenesis by UV and NTG caused changes in protein production or secretion (Prabavathy *et al*., 2006; Xu *et al*., 2011). However, various factors such as the presence of non-protein components in solutions or non-cellulase proteins in the preparations may interfere with the determination of protein concentration (Zaldivar *et al*., 2001; Xu *et al*., 2011).Therefore, strain M17 and M23 were selected and used throughout this study.

Table 3 Comparison on filter paper cellulase (FPase) activity from wild type and mutant strain of *Cellulomonas* sp. strain TSU-03 after 60 h of cultivation in CMC medium at 35°C.

Mutant strain	FPase (U/mL)	Mutant strain	FPase (U/mL)	Mutant strain	FPase (U/mL)
M01	1.02±0.10	M10	2.00±0.10	M19	1.17±0.10
M02	1.51±0.08	M11	2.21±0.08	M20	1.25±0.10
M03	2.00±0.11	M12	1.14±0.10	M21	1.68±0.08
M04	1.10±0.09	M13	1.10±0.12	M22	1.28±0.40
M05	1.00±0.20	M14	1.17±0.07	M23	2.93±0.11
M06	1.45±0.10	M15	1.58±0.10	M24	1.74±0.15
M07	1.05±0.11	M16	2.20±0.01	M25	1.00±0.08
M08	1.14±0.21	M17	2.57±0.14	**wild type**	**1.01±0.10**
M09	2.04±0.10	M18	2.01±0.10		

2. Strain improvement by double gene dosage

2.1 Cloning of the cellulase gene of *Cellulomonas* sp. DNA sequence analysis

PCR of the cellulase gene of *Cellulomonas* sp. strain TSU-03 (*cen*$A_{Csp.}$) were transformed and screened for presence of the *cen*A_{Csp} in *E. coli*. A plasmid (pBBR122:*cen*A_{Csp}) contained a 1.1-kbp *Bam*HI genomic *Cellulomonas* sp. DNA fragment, which was sequenced and exhibited sequence homologies to *cen*A_{Cfimi} (Fig. 3). A BLAST alignment revealed very high homology with the cellulase gene from *Cellulomonas fimi* (GenBank accession no.15823, BLAST score 236, 100% Identity).

```
   1 GGATCCGGAC GGTGGGCGTC GTGGCCGACA CCGACGCGCT GGAGACGACC TTCGCGGACG
  61 TCGCGGACCT CGCGCGGCAG TGCCGGTTCG GCGACTGCCG GCACGAGCGG GAGCCGGGGT
 121 GCGCGGTGCG GGCGGCCGTC GAGTCGGGCG ACCTGCCGGC CCGGCGGCTG GACTCGTGGC
 181 GGCGCCTGGA GCGCGAGGCG GCCTACCAGG CACGGCGCAG CGACGGCGGC TGGCCGCGGA
 241 GGAGCGCGCA CGCTGGAAGA AGATCACCAA GGAGTACCAG CGGGGGATGC GCGGGCCGGG
 301 GCGTCCGCGG AGCTGACGGG CCCGGGAGGC CCGCAGCCGG GCGGTGGGGA GTCCGCTCGG
 361 CGCCAGCGGG TGTCGAAGCG ACGGGTCGAA GCGCGCCAAC GTCGCCCGAT CCGGAACTGA
 421 AGCGATTAGG AAATCCTCAT CCGCTCGCGC CGTGGGGCAT TCGTCGGGTT TCCTCGTCGG
 481 GACCCGCACG AGCGTGCCAC GAGGCCCGAA CCCAGGGAGC TCCTTGATGT CCACCCGCAG
 541 AACCGCCGCA GCGCTGCTGG CGGCCGCGGC CGTCGCCGTC GGCGGTCTGA CCGCCCTCAC
 601 CACCACCGCC GCGCAGGCGG CTCCCGGCTG CCGCGTCGAC TACGCCGTCA CCAACCAGTG
 661 GCCCGGCGGC TTCGGCGCCA ACGTCACGAT CACCAACCTC GGCGACCCCG TCTCGTCGTG
 721 GAAGCTCGAC TGGACCTACA CCGCAGGCCA GCGGATCCAG CAGCTGTGGA ACGGCACCGC
 781 GTCGACCAAC GGCGGCCAGG TCTCCGTCAC CAGCCTGCCC TGGAACGGCA GCATCCCGAC
 841 CGGCGGCACG GCGTCGTTCG GGTTCAACGG CTCGTGGGCC GGGTCCAACC CGACGCCGGC
 901 GTCGTTCTCG CTCAACGGCA CCACCTGCAC GGGCACCGTG CCGACGACCA GCCCCACGCC
 961 GACCCCGACG CCGACGACCC CCACGCCGAC GCCGACCCCG ACCCCCACCC CCACGCCGAC
1021 GGTCACGCCG CAGCCGACCA GCGGCTTCTA CGTCGACCCG ACGACGCAGG GCTACCGCGC
1081 GTGGCAGGCC GCGTCCGGCA
```

Figure 3 Nucleotide sequence of 1.1-kb region containing the cellulose gene (*cenA*$_{Csp}$) (given in bold) from *Cellulomonas* sp. strain TSU-03.

Alignment and identities of amino acid sequence of the *cen*A gene from *Cellulomonas* sp. TSU-03 with *cen*A gene from *Cellulomonas fimi* (GenBank accession M15823) was shown in Figure 4.

```
Score =  236 bits (510),  Expect = 0.0
Identities = 96/96 (100%), Positives = 96/96 (100%), Gaps = 0/96 (0%)
Frame = +3/+3

Query  3    IRTVGVVADTDALETTFADVADLARQCRFGDCRHEREPGCAVRAAVESGDLPARRLDSWR  182
            IRTVGVVADTDALETTFADVADLARQCRFGDCRHEREPGCAVRAAVESGDLPARRLDSWR
Sbjct  3    IRTVGVVADTDALETTFADVADLARQCRFGDCRHEREPGCAVRAAVESGDLPARRLDSWR  182

Query  183  RLEREAAYQARRSDGGWPRRSAHAGRRSPRSTSGGC  290
            RLEREAAYQARRSDGGWPRRSAHAGRRSPRSTSGGC
Sbjct  183  RLEREAAYQARRSDGGWPRRSAHAGRRSPRSTSGGC  290
```

Figure 4 Alignment and identities of amino acid sequence of the cellulase gene (*Cen*A$_{Csp}$) from *Cellulomonas* sp. TSU-03 with *Cen*A gene from *Cellulomonas fimi* (GenBank accession M15823).

2.2 Heterologous expression of the *Cellulomonas* sp. cellulase gene in *E. coli*

DNA fragments comprising only *cen*A$_{Csp}$ were obtained by PCR. These PCR products were inserted into the *Eco*RV restriction sites of plasmids pSK$^-$ and pBBR122 and transformed into *E. coli* strain BL21(DE3) and the cells of the recombinant strains were analyzed for cellulase activity.

The recombinant *E. coli*, which described above did not confer the ability of enzyme activity and cellulase activity was observed with cells harboring only the vector. Cells harboring only *cen*A$_{Csp}$ had very low enzyme activity (0.8 U/mL) in the relative with low amount of protein concentration. To substantiate this observation the enzyme activity in recombinant *E. coli* harboring pSK$^-$::*cen*A$_{Csps}$ or pBBR122::*cen*A$_{Csp}$ and in the untransformed *E. coli* (BL21) host was assayed after about 60 h of growth. Both revealed similar activity levels. While the cellulase activity was not determined in the untransformed *E. coli* host, it was 0.8 U/mL in the recombinant cells. The result was different from recombinant recombinant strain of *E. coli* harboring only the beta-glucosidase activity from *Pennicillium decumbens* ligated with the cellobiohydrolase I (cbhI) promoter of *Trichoderma reesei*. The mutants increased 6- to 8- fold and their filter paper activity (FPAs) was enhanced by 30% of average (Ma *et al*., 2011). These results may be due to the lack of efficient promoter to express cellulase gene. Therefore, the disable of exoglucanase as well as β-glucosidase may play the important role in cellulase biosynthesis. If *E. coli* harbored a second plasmid encoding cellulase enzyme

complex from *Cellulomonas* sp., the activity of cellulase would be increase dramatically.

2.3 Expression of *Cellulomonas* cellulase in wild type

Recombinant of *Cellulomonas* sp. was constructed by transformed *E. coli* BL21 pBB122::*cen*$A_{Csp.}$ To the natural cellulase producer *Cellulomonas* sp. TSU-03. This is a first report on the transformation of *cen*$A_{Csp.}$ plasmid into *Cellulomonas* sp. (wild type) with the expectation to increase cellulase production. Analyses of these recombinant *Cellulomonas* sp. revealed high cellulase activity in crude extracts (1.02 U/mL). However, the enzyme activity of the recombinant *Cellulomonas* sp. cells was not significantly higher than those from the natural cellulase producer *Cellulomonas* sp.TSU-03 (1.10 U/mL) cultivated in CMC medium. It was concluded that the recombinant strains conferred low level of plasmid stability. The low stability was in accordance with no increase of cellulase production in the recombinant strains.

The results revealed that the potential of cellulase production of *Cellulomonas* sp. was still limited. Perhaps the recombinant method seems not to be a good method to increase the production of cellulase by *Cellulomonas* sp. Therefore, *Cellulomonas* sp. strain M17 and M23, NTG-mutant, were selected and used throughout this study due to their highest activity of cellulase compared with the other strains.

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

3.1 Effect of different carbon sources

Rice straw and wastepaper, an abundant biomass found in Thailand were selected and utilized as sole carbon source. From our previous study rice straw and wastepaper contained high content of cellulose (>88%) in low amount of hemicelluloses and lignin (Sangkharak, 2011; Sangkharak *et al*., 2011). Therefore, effect of carbon sources on growth and cellulase production from *Cellulomonas* sp. mutant M17 and M23 in carboxymethylcellulose (CMC) medium were studied where CMC was substituted by rice straw and wastepaper at 1% w/v.

The strain M23 could grew on all carbon sources tested and using 1% of CMC gave the maximum cell growth (6.54±1.10 g/L) and FPase activity (2.93±0.11 U/mL) after 48 h cultivation (Table 4). These values were followed, in descending order by rice straw and wastepaper. Medium containing wastepaper as the substrate yielded the lowest biomass and cellulase activity. Rajoka (2004) reported that *Cellulomonas flavigena* NIAB 441 had a shorter period and more specific growth rate when grown on monosaccharides than those on disaccharides and polysaccharides. However, cellulosic substrate including cellulose, CMC and kallar grass induced high level of enzyme activity. This result was agreed with the data from *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C23 using CMC (1%, w/v) as a carbon source which gave the highest FPase activity (1.31 and 1.41 U/mL) compared with the alternative carbon sources such as glucose,

cellobiose and cellulose (Abou- Taleb *et al*., 2009). As well as the result of Narasimha *et al*. (2006) and Niranjane *et al*. (2007) which indicated that CMC was the best carbon source followed by cellulose for cellulase production. A highest production of cellulase when CMC was utilized as carbon source may be as a result of induction of the enzyme, since cellulose is a universal inducer of cellulase synthesis (Abou- Taleb *et al*., 2009).

Table 4 The effect of carbon sources on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23

Carbon source	***Cellulomonas* sp.**					
	Mutant strain M17		**Mutant strain M23**		**Wild type**	
	Biomass (g/L)	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**
CMC (control)	5.98±1.01	2.57±0.14	6.54±1.10	2.93±0.11	5.02±0.87	1.01±0.10
Rice straw	5.05±0.59	2.20±0.96	6.34±1.09	2.55±1.00	4.50±0.50	0.92±0.21
Wastepaper	4.00±1.00	1.02±0.54	6.02±1.15	2.09±1.00	4.06±0.68	0.80±0.12

Many research and developments attempted to study the effect of different concentrations of CMC for cellulase production. The result clearly stated that 1% of CMC gave the highest activity of cellulase by *B. alcalophilus* S39 and *B. amyloliquefaciens* C23 (Abou- Taleb *et al*., 2009). The result is similar with other literature (Fukumori *et al*., 1985; Kawai *et al*., 1988; Shikata *et al*., 1990) where the highest cellulase activity was detected in cultures contained 1% (w/v) CMC as sole substrate.

3.2 Effect of different nitrogen sources

Effect of various nitrogen sources on the cellular growth and cellulase production from three strain of *Cellulomonas* sp. including wild type and mutant strain M17 and M23 cultivating in CMC medium were investigated. Sources of nitrogen included NH_4NO_3 (control), $NaNO_3$, peptone and yeast extract at the concentration of 1 g/L (Table 5). Data indicated that the supplementation of organic and inorganic nitrogen sources stimulated the cellulase activity. Using of inorganic nitrogen sources responded in the positive cellulase activity more than organic ones. Therefore, mutant strain M23 gave the highest biomass (6.98±1.21 g/L) and cellulase activity (3.15±1.02 U/mL) when cultivated in the optimum medium with 0.1% $NaNO_3$ as nitrogen source.

Table 5 Effect of nitrogen sources on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23 cultivation in CMC medium containing 1% CMC as carbon source

Nitrogen source	***Cellulomonas* sp.**					
	Mutant strain M17		**Mutant strain M23**		**Wild type**	
	Biomass (g/L)	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**
NH_4NO_3 (control)	5.98±1.01	2.57±0.14	6.54±1.10	2.93±0.11	5.02±0.87	1.01±0.10
$NaNO_3$	6.10±1.40	2.59±1.45	6.98±1.21	3.15±1.02	5.28±1.14	1.12±0.56
Peptone	5.54±1.01	2.05±0.58	6.02±1.54	2.20±1.21	5.00±1.22	1.01±0.26
Yeast extract	5.84±1.00	2.21±1.10	6.34±1.30	2.40±0.98	5.21±1.54	1.10±0.24

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 the maximum production of β-cellobiohydrolase by *C. uda* CB4 (Nakamura and Kitamura, 1988).

3.3 Effect of initial pH

Effect of initial pH on cellulase production was investigated by adjusting the pH of the optimal culture medium at 4.0, 5.0, 6.0 and 7.0. It was indicated that the highest productivity of biomass (6.98±0.15 g/L) and FPase activity (3.16±0.20 U/mL) were achieved at pH 6.0 followed by the production of biomass (6.74±0.11 g/L) and cellulase activity (3.01±0.22 U/mL) obtained at pH 7.0 (Table 6) by mutant M23. Cellulase yield by *Cellulomonas* sp. appear to depend on pH value. The result was agreed with the data from our previous study which wild type strain of *Cellulomonas* sp. achieved the highest productivity at pH 6.0 as well as enzyme activity decreased significantly under acidic (pH 2.0-5.0) and alkali conditions (pH 8.0-10.0) (Sangkharak *et al*., 2011).

The significant effect of pH on cellulase production during batch experiments was therefore highlighted. The hydrolysis of substrates by cellulolytic enzyme occurred in the pH range of 4.0-9.0. Therefore, the optimum pH at 6.0-7.0 was reported for induced the production of cellulolytic enzyme by *Cellulomonas*, *Bacillus* and *Micrococcus* sp. (Immanuel *et al*., 2006; Sangkharak *et al*., 2011).

Table 6 Effect of initial pH on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23 cultivation in CMC medium containing 1% CMC and 0.1% $NaNO_3$ as carbon and nitrogen source, respectively

Initial pH	*Cellulomonas* sp.					
	Mutant strain M17		Mutant strain M23		Wild type	
	Biomass (g/L)	FPase (U/mL)	Biomass (g/L)	FPase (U/mL)	Biomass (g/L)	FPase (U/mL)
4	5.00±1.00	1.23±0.21	6.23±1.05	3.1±0.95	5.12±1.10	1.02±0.54
5	5.12±1.11	1.98±0.24	6.55±1.01	3.11±0.11	5.21±1.24	1.1±0.09
6 (control)	6.1±0.85	2.59±0.05	6.98±0.15	3.16±0.20	5.28±1.02	1.12±0.24
7	5.92±1.00	2.24±0.22	6.74±0.11	3.01±0.22	5.12±1.11	1.00±0.05

3.4 Effect of incubation temperature

Temperature is also a significant factor that influences the cellulase yield (Abou- Taleb *et al*., 2009). *Cellulomonas* sp. M23 possessed the highest biomass (6.98-6.99 g/L) and cellulase activity (3.12-3.16 U/mL) at 35-45^{o}C (Table 7). The results from the other strains of *Cellulomonas* sp. exhibited similar trends with those from mutant strain M23 when cultivated in the optimal medium. *Cellulomonas* sp. M17 yielded much higher concentration of biomass (6.10-6.33 g/L) and enzyme activity (2.54-2.59 U/mL) than those of the wild type (5.28-5.30 g/L, 1.10-1.12 U/mL of enzyme activity).

The effect of temperature on biomass and enzyme activity at various temperatures ranging from 20-100^{o}C was also investigated by *Cellulomonas* sp. (wild type). The wild type strain could grow and produce high activity of xylanase and endoglucanase in the temperature range of 30-70^{o}C (Sangkharak

et al., 2011). These results are close to those of Immanuel *et al*. (2006) who found that the optimum temperature for endoglucanase produced by *Cellulomonas, Bacillus* and *Micrococcus* sp. was 40^{o}C at neutral pH. The highest cellulase activity was obtained at temperatures 30-45^{o}C for *Bacillus* sp. (Ray *et al*., 2007; Abou- Taleb *et al*., 2009) and 30-35^{o}C for *Pesudomonas fluorescence* (Bakare *et al*., 2005).

Table 7 Effect of incubation temperature on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23 cultivation in CMC medium (pH 6.0) containing 1% CMC and 0.1% $NaNO_3$ as carbon and nitrogen source, respectively.

Incubation temperature	***Cellulomonas* sp.**					
	Mutant strain M17		**Mutant strain M23**		**Wild type**	
	Biomass (g/L)	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**
25	5.50±1.20	2.14±0.89	5.20±1.10	2.54±1.05	4.82±1.02	1.00±0.54
35 (control)	6.10±1.00	2.54±0.25	6.98±1.00	3.12±0.95	5.28±1.04	1.10±0.69
45	6.33±1.10	2.59±1.00	6.99±1.54	3.16±0.58	5.30±1.54	1.12±0.56
55	6.01±1.41	2.34±0.44	6.24±1.05	2.99±0.45	5.01±1.23	1.10±0.50
65	6.00±2.02	2.20±0.12	6.21±0.95	2.98±0.98	5.00±1.17	1.01±0.24

3.5 Effect of agitation speed

The cultivation of *Cellulomonas* sp. wild type and two mutant strains (M17 and M23) was carried in optimum medium, controlled pH at 6.0 and maintained incubation temperature at 45^{o}C. Cell growth decreased as the agitation speed increased over 100 rpm which might be due to the cell destruction (Table 8). Therefore, the agitation rate at 100 rpm was most

preferred. The maximum biomass and FPase activity were observed at 7.05±1.59 g/L and 3.21 U/mL, respectively by *Cellulomonas* strain M23. Dissolved oxygen showed that increase 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.

Table 8 Effect of agitation speed on growth and cellulase production by *Cellulomonas* sp. (wild type) and the mutant strain M17 and M23 cultivation in CMC medium at 45^{o}C (pH 6.0) containing 1% CMC and 0.1% $NaNO_3$ as carbon and nitrogen source, respectively.

Agitation speed	***Cellulomonas* sp.**					
	Mutant strain M17		**Mutant strain M23**		**Wild type**	
	Biomass (g/L)	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**	**Biomass (g/L)**	**FPase (U/mL)**
0	4.02±1.14	1.59±0.65	6.59±2.04	2.59±1.01	3.54±0.94	1.05±0.68
100 (control)	6.34±1.59	2.50±1.02	7.05±1.69	3.21±1.00	5.28±2.21	1.12±0.50
200	5.11±2.01	2.38±1.04	6.68±1.05	3.05±1.55	5.11±1.58	1.10±0.21

The highest cellulase activity by *B. amyloliquefaciens* UMAS 10002 were 2.97 and 2.89 IU/mL at the range of shaking rate of 100-200 rpm (Khan *et al*., 2006). The similar data was observed by *B. alcalophilus* S39 and *B. amyloliquefaciens* C23 which produce maximum biomass and enzyme activity at 150-200 rpm (Abou- Taleb *et al*., 2009).

4. 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, pH was controlled at 6.0 and incubation at 45^{o}C for 96 h. The cultivation was performed in a 250 mL shaken flask with agitation speed of 100 rpm. The results were given in Figure 5 and kinetic parameter was also calculated. The specific growth rate (μ) was 0.07 h^{-1}. Cellular growth was 7.11±1.28 g/L, FPase, CMCase and β-glucosidase activities were 3.25±0.20, 4.51±1.15, 1.52±0.40 U/mL, respectively. These values of productivity were 1.1 folds higher than those cultivated in un-modified CMC 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 cellulase activity (3.25±0.20 U/mL) achieved from this strain is 1.2 and 2.8 folds higher than cellulase from mutant M17 (2.50±1.02 U/mL) and wild type (1.12±0.50 U/mL), respectively.

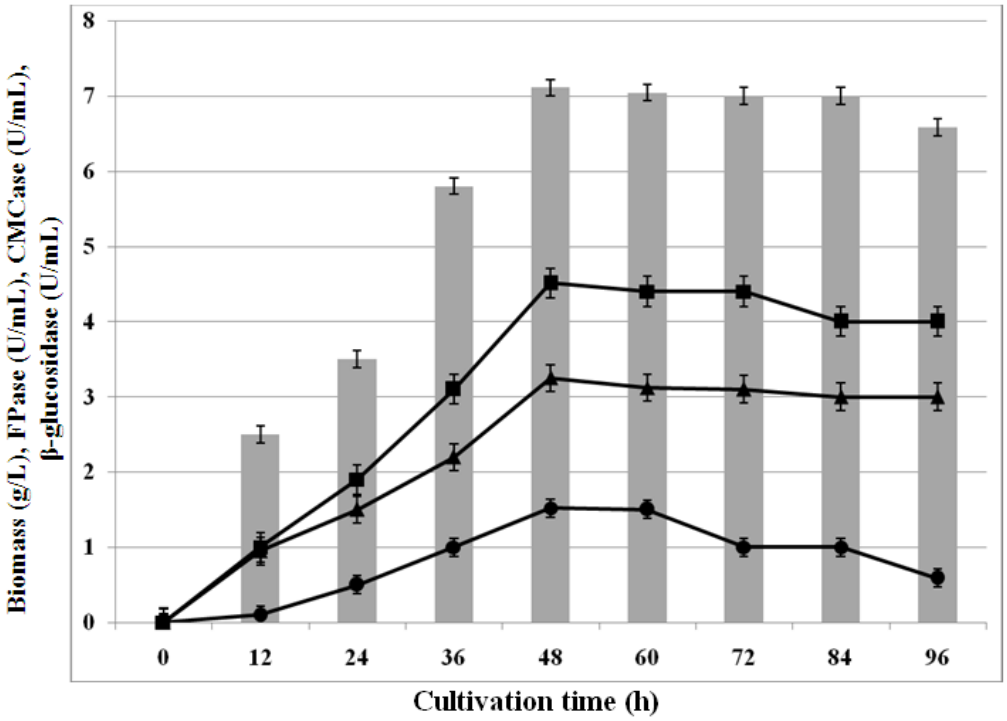

Figure 5 Growth and cellulase activity of *Cellulomonas* sp. M23 on optimal medium contained 10 g/L CMC (carbon source), 0.1 g/L $NaNO_3$ (nitrogen source) at 45^{o}C under controlled pH (6.0) conditions and agitation speed at 150 rpm. ▒ biomass, ■ CMCase activity, ▲ FPase activity and ● β-glucosidase activity.

5. CONCLUSION

An objective of this study was to develop a high cellulase production by improve the prominent cellulase producing bacteria, increased enzyme production by optimization studies and strain improvement by genetic engineering. Among 330 mutant strains of *Cellulomonas* sp. TSU-03, the mutant strain M23, NTG mutant, gave the highest value of cellulase activity (2.93±0.11 U/mL) followed by mutant M17 (2.57±0.14 U/mL) in CMC medium.

Strain improvement for higher production of cellulase from *Cellulomonas* sp. was conducted by cloning of gene encoding the endoglucanase of *Cellulomonas* sp. (Cen_{Csp}) through complementation to cellulase accumulation of *Cellulomonas* sp. (wild type) was investigated. The Cen_{Csp} sequence of DNA from *Cellulomonas* sp. was analyzed and the fragment showed 100% identity with *Cen* gene from *Cellulomonas fimi* (accession number 15823). Cloning was confirmed by enzymatic studies and results showed that cellulase activity was low expression in *E. coli* and *Cellulomonas* sp. Therefore, mutant M17 and M23, NTG mutant, were selected and used throughout this study.

The optimum medium and environmental for cellulase production consisted of 10 g/L CMC, 1 g/L $NaNO_3$ under cultivatation temperature at 45^{o}C with initial pH and agitation speed at 6 and 100 rpm, respectively. Cellulomonas sp. strain M23 produced the highest cellular growth (7.11±1.28 g/L) and FPase, CMCase as well as β-glucosidase activities at 3.25±0.20, 4.51±1.15, 1.52±0.40 U/mL, respectively. The cellulase activity achieved

from strain M23 is 1.2 and 2.8 folds higher than cellulase from mutant M17 (2.50±1.02 U/mL) and wild type (1.12±0.50 U/mL), respectively. 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.

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