African Journal of Biotechnology Vol. 11(6), pp. 1433-1442, 19 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3158
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Enhanced cellulose production by ultraviolet (UV) irradiation and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis of an *Acetobacter* species isolate

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Accepted 7 December, 2011

Cellulose-producing bacteria were isolated from 14 bacterial cellulose (BC) factories in Thailand, and subjected to ultraviolet (UV) irradiation and/or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis for strain improvement. BC yields of the NTG mutant NU4-NTG30-51 were 54.68% higher than that of the reference strain *Acetobacter xylinum* ATCC 10245 and 43.69% higher than that of the parent strain NU4 ($P < 0.01$). BC yields of the UV mutant NU4-UV40-07 were 50.59% higher than that of *A. xylinum* and 39.60% higher than that of NU4. Glucose consumption of these mutants was also higher than that of NU4. However, crystallinity of cellulose produced by NU4-NTG30-51 was higher than that produced by NU4-UV40-07. Combining UV irradiation and NTG did not result in BC yields higher than those of the mutants generated with a single mutagen. The mutant strains were then tested for reversion. Our results demonstrate that the BC yields of these mutants remained higher than that of the reference strain *A. xylinum* ATCC10245, and did not revert to the same level as the parent strain. The isolate NU4 and the two mutants were identified as *Acetobacter* species.

Key words: Acetobacter xylinum, bacterial cellulose, strain improvement, UV irradiation (UV), N-methyl-N'-nitro-N-nitrosoguanidine (NTG).

INTRODUCTION

Acetobacter xylinum is a gram-negative, rod-shaped, aerobic, non-pathogenic bacterium that secretes bacterial cellulose (BC) in the form of a pellicle on the surface of liquid culture (Ross et al., 1991; McKenna et al., 2009). Over the past decade, this organism has been widely used as a model for basic and applied research on BC biosynthesis. *A. xylinum* produces pure cellulose without impurities, such as lignin, hemicellulose or pectin, and its crystallinity and degree of polymerization are higher than those of plant cellulose (Ross et al., 1991). Consequently, BC from *A. xylinum* is an attractive material for various applications (Jonas and Farah, 1998; Chawla et al., 2009). *A. xylinum* utilizes a range of carbon sources (e.g. glucose, fructose, sucrose, galactose, invert sugar, glycerol, inositol, mannitol, ethanol and methanol) (Sameshima et al., 1991; Shoda and Sugano, 2005); however, the BC yield of *A. xylinum* is low, and commercial carbon sources such as glucose are relatively expensive. Attempts to overcome these limitations include optimization of culture conditions and medium components, screening for more productive strains, use of shaking flasks or stirred-tank reactors, comparing surface versus submerged fermentation, optimization of batch culture, use of an airlift reactor or rotating disk reactor and genetic modification (Tonouchi et al., 1995; Vandamme et al., 1998; Chawla et al., 2009). In addition, the supplements endoglucanase, pyruvate, ethanol, lactate and sodium alginate have been added to the cell culture medium to boost BC production (Matsuoka et al., 1996; Naritomi et al., 1998a, b; Zhou et al., 2007) and a synthetic

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medium (KC medium) was developed (Premjet et al., 1994a, b, 1996). The use of sugar cane molasses, a by-product of sugar cane processing, as the sole carbon source in KC medium reduced cost and improved BC yields (Premjet et al., 2003, 2007); however, this cellulose is not suitable for consumption.

In Thailand, bacterial cellulose is cut into small cubes for use in desserts, health foods, salads and fruit cocktails, and is known as "*Vhun-sawan*". In most Thai factories, *A. xylinum* is cultured using the traditional static culture in a tray containing coconut water, which results in a BC sheet on the surface of the liquid medium. However, low productivity and high cost of production are problems. Karaman and Medicherla (2010) proposed strain improvement as an important step in improving productivity and cost-effectiveness. In the present study, we therefore aimed to generate improved strains of *Acetobacter* through random mutagenesis by ultraviolet (UV) irradiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) or a combination of these techniques. BC yields from several mutant strains were determined and compared with that of the parent strain.

MATERIALS AND METHODS

Sample collection and reference strain

To screen BC producers, we collected 100 waste water samples from various sites around 14 BC factories (designated as Factories A to N) located in Lopburi province, which has long produced BC for use in desserts: Factory A (n = 8), B (n = 10), C (n = 4), D (n = 6), E (n = 12), F (n = 6), G (n = 10), H (n = 5), I (n = 7), J (n = 5), K (n = 7), L (n = 5), M (n = 5) and N (n = 10).

A. xylinum ATCC 10245 was used as the reference strain which was kindly provided by the Wood Chemistry Laboratory, Kochi University, Japan. The reference strain was grown on Hestrin-Schramm (HS) agar slants at 29°C for three days, and then was stored at 10°C.

Chemicals

NTG (Sigma-Aldrich Chemicals) was kindly provided by Associate Professor Dr. Hunsa Punnapayak, Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. All other reagents and chemicals were purchased from Merck and Sigma-Aldrich Chemicals in Thailand.

Culture media and conditions

Isolation medium consists of (w/v): 2% yeast extract, 2% peptone, 2% $CaCO_3$, 0.01% cycloheximide, 15% agar powder and 2% absolute ethanol (v/v) (pH 5). The HS liquid medium, which was used as the standard culture medium for cellulose production, consists of (w/v): 0.5% yeast extract, 0.5% peptone, 0.115%, citric acid, 0.27% Na_2HPO_4 and 2% glucose (pH 6). Corn steep liquor-glucose (CSLG) liquid medium consists of mg/L: 14.7 $CaCl_2 \cdot 2H_2O$, 3.6 $FeSO_4 \cdot 7H_2O$, 2.42 $Na_2MoO_4 \cdot 2H_2O$, 250 $Mg_2SO_4 \cdot 7H_2O$, 1.73 $ZnSO_4 \cdot 7\ H_2O$, 1.39 $MnSO_4 \cdot 5H_2O$, 0.05 $CuS_4O \cdot 5H_2O$, 2.0 inositol, 0.4 niacin, 0.4 pyridoxine HCl, 0.4 thiamine HCl, 0.2 Ca pantothenate, 0.2 riboflavin, 0.2 aminobenzoic acid, 0.002 folic acid, 0.002 biotin, 1.0 g/L KH_2PO_4, 3.3 g/L $(NH_4)_2\ SO_4$, 20 g/L corn steep liquor and 40 g/L glucose (pH 6). For solid culture media, 1.5% (w/v) agar powder was added to the liquid media. All media were sterilized at 121°C, 15 psi for 15 min.

BC production

A. xylinum ATCC 10245, parental strains and mutant strains were grown 24 h on an HS agar slant, and then, one loop-full was inoculated into 30 ml HS medium in a 125 ml Erlenmeyer flask. The bacteria were grown in static culture in the dark at 29°C for seven days.

Treatment of BC pellicles

BC pellicles produced by *A. xylinum* ATCC 10245 and all mutant strains were washed with distilled water and treated with 1% NaOH at 90°C for 15 min. After cooling, the pellicles were neutralized with 1% acetic acid, thoroughly washed with distilled water, and air-dried on glass plates. The BC pellicles were then dried at 105°C and were weighed. These procedures were performed in triplicate for each culture, and the three BC yields were averaged.

The BC yields were expressed as relative weight percentages calculated by the following equation:

$$\text{BC yield (\%)} = (B/A) \times 100,$$

where, B and A are dry weight (mg) of BC produced by test strains and *A. xylinum* ATCC 10245 in 30 ml HS medium in static culture, respectively.

Isolation of cellulose-producing strains

To isolate cellulose-producing strain, 0.1 ml of each wastewater sample was inoculated into 10 ml HS medium in 18 (Ø) × 150 mm tubes and was mixed with vortex mixer for 1 to 2 s. Each sample was serially diluted, and then 0.1 ml of each dilution (to 10^{-3} dilution) was spread on isolated medium agar plate and was incubated at 29°C. We selected bacteria forming white or cream-colored colonies that produced visible clear zones on the agar plate. To produce pure cultures of cellulose-producing strains, well-isolated colonies were streaked onto HS agar plates; this procedure was repeated at least three times. Individual colonies were each streaked onto to two HS agar slants and incubated at 29°C for three days. One agar slant was stored at 10°C, and the other one was used for experimentation.

Preparation of starting culture

The isolate that produced the highest BC yields was used as the parent strain for subsequent mutagenesis. This strain was inoculated into 30 ml HS medium in a 125 ml Erlenmeyer flask and was grown in static culture at 29°C for 24 h. The culture was then diluted with fresh medium to obtain approximately 10^3 cells/ml. This parent strain liquid culture was used as the starting strain for strain improvement by UV irradiation and NTG mutagenesis.

Mutagenesis by UV irradiation

The starting strain (0.1 ml, approximately10^3 cell/ml) was spread onto a CSLG agar plate and was incubated at 29°C for 12 h. The bacterial colonies were then exposed to UV irradiation in a laminar flow cabinet (Holten HB 2448), using a 30 W germicidal lamp

(G30T8, Sankyo Denki), which emits about 90% of its radiation at 254 to 255 nm. Bacterial plates were placed 30 cm away from the center of germicidal lamp for 0, 20, 40, 60 or 80 min (each exposure time, n = 3). After UV irradiation, plates were incubated overnight in the dark at 29°C to prevent photoreactivation. Dose-survival curves of the parental strain were plotted, and mutants were isolated from plates showing 5 to 10% survival. Individual large and white or cream-colored colonies were selected and streaked onto a fresh HS agar plate. These cultures were subsequently used as inoculum for BC production in HS medium. Among these selected strains, the mutant with the highest BC yield was selected for further mutagenesis by NTG.

Mutagenesis by NTG

CSLG agar medium was melted in 250 ml of Erlenmeyer flasks (100 ml/flask) in a 60°C water bath and was cooled to 45 to 50°C. Various concentrations of NTG (1 ml) were then added (10, 20, 30, 40, 50, 60 and 70 µg/ml in Tris-maleate buffer (pH 6) consisting of 0.6% Tris-maleate, 0.58% maleic acid, 0.1% $(NH_4)_2SO_4$, 0.025% $FeSO_4$, 0.01% $MgSO_4$ and 0.0005% Ca $(NO_3)_2$), and the flasks were gently swirled by hand for 10 s. Then, this medium was poured into 80 mm Petri dishes (15 ml/dish) and was allowed to solidify overnight at room temperature. The starting strain (1 ml, approximately 10^3 cells/ml) was spread onto the CSLG agar plates containing NTG and was incubated in dark at 29°C for seven days (three plates per NTG concentration). Dose-survival curves were plotted, and mutants were isolated from plates showing 5 to 10% survival. Individual large and white or cream-colored colonies were streaked onto fresh HS agar slants and were incubated for 24 h. These cultures were subsequently used as inoculum for BC production in HS medium. Among the selected strains, the NTG mutant that produced highest BC yield was selected for further mutagenesis by UV irradiation.

Characterization of cellulose-producing strains

Pure cultures of the test strains were identified at the genus level. The morphologic, physiologic and biochemical properties of the strains were examined by standard procedures as described in Bergey's Manual of Determinative Bacteriology and compared with the reference strain *A. xylinum* ATCC 10245.

Determination of strain productivity

The sugar consumed during BC production was determined by high performance liquid chromatography (HPLC; Agilent 1100, Agilent Technologies Co). At one, two, three, four, five, six and seven days after the start of incubation, 1 ml culture medium was filtered through 0.2 µm Millipore filter. Then, 20 µl of the sample solution was loaded onto an HPLC column (Zorbax Carbohydrate Analysis Column, 4.6 × 150 mm, 5 µm particle size, Agilent Technologies Co), using a refractive index detector (HP 1100 RID) at 30°C. The mobile phase consisted of acetonitrile and water (75:25, v/v), and the flow rate was 2.0 ml/min. The amount of sugar consumed was compared with the amount of BC produced to estimate production efficiency.

X-Ray diffraction

After washing with distilled water, the BC pellicle was treated with 1% NaOH at 90°C for 15 min. After cooling, the pellicles were neutralized with 1% acetic acid and thoroughly washed with distilled water. The pellicles were then air-dried on a glass plate and pressed into a thin, flat layer. X-ray diffractograms were recorded with a Philips PW3040/60 X'pert PRO automatic diffractometer using reflection geometry and Ni-filtered CuKα radiation (λ = 0.154 nm) operated at 40 kV and 30 mA. The scanning was made through 2θ (5 to 40°) with a scanning speed of 10° min^{-1}. The crystallinity index was calculated as $I_{002}/(I_{002} + I_{002B})$, and the crystallite orientation calculated as I_{101}/I_{002} as described by Isogai and Usuda (1989), where I_{002} is the intensity of the peak at 2θ = 22.7° corresponding to the 002 plane and I_{002B} is the height of the slope line at the peak.

Experimental design

Experiments were carried out using a completely randomized design (CRD). Results were compared by analysis of variance followed by Duncan's multiple range test using SPSS software (version 10.0). $P < 0.01$ was considered significant. The mean and standard deviations of cellulose (dry weight) were calculated from at least two independent experiments.

RESULTS AND DISCUSSION

Screening of cellulose-producing strains collected from waste water samples

A total of 14 cellulose-producing strains were isolated from waste water samples of the four factories: strains NU1 and NU2 (Factory A), NU3 to NU6 (Factory B), NU7 to NU10 (Factory C) and NU11 to NU14 (Factory D) (Table 1). Waste water samples taken from isolate cellulose producing bacteria were not heat treated before drainage. The number of cellulose-producing strains was low when compared with that reported by other studies.

Several studies have reported that cellulose-producing bacteria are widely distributed in the nature and can be isolated from plant juices, rotting fruits and vegetables. However, fruits are the best source of cellulose-producing bacteria and that cellulose-producers could not be isolated from soil samples (Toyosaki et al., 1995; Seto et al., 1997; Kojima et al., 1997; Son et al., 2002).

To determine the most productive strain, the cellulose production of each isolate was compared with that of the reference strain *A. xylinum* ATCC 10245. All isolates produced cellulose pellicles, although the yield and texture varied among the strains. Most of the isolated strains produced tough pellicles, whereas slimy pellicle was produced by five isolates. As shown in Table 1, relative BC yields of most isolates ranged from 59.75 ± 0.42 to 82.62 ± 0.61%; however, the yields of two isolates were less than 50% of the reference strain (NU2, 45.66 ± 0.05%; NU5, 47.03 ± 0.27%). The isolate NU4 produced the highest BC yield, 110.99 ± 93% of the cellulose produced by *A. xylinum* ATCC 10245 ($P < 0.01$). We therefore used NU4 as the parent strain for strain improvement by UV irradiation and NTG mutagenesis.

Strain improvement by UV irradiation

To improve productivity, the NU4 colonies on agar plates

Table 1. Cellulose production of isolates from factory waste water.

Source	Isolated	Relative yield (%)[1]	Colony color	Cellulose texture	Final pH
Factory A	NU1	59.75 ± 0.42 ^{h}	W	Slimy	3.46
	NU2	45.66 ± 0.05^{j}	W	Slimy	3.28
	NU3	100.87 ± 0.51^{b}	W	Tough	3.65
Factory B	NU4	110.99 ± 0.93^{a}	W	Tough	3.79
	NU5	47.03 ± 0.27^{i}	W	Slimy	3.31
	NU6	82.62 ± 0.50^{c}	W	Tough	3.48
	NU7	74.09 ± 0.35^{e}	W	Tough	3.43
Factory C	NU8	77.39 ± 0.45^{d}	W	Tough	3.53
	NU9	65.04 ± 0.72^{g}	W	Slimy	3.53
	NU10	82.19 ± 0.61^{c}	W	Tough	3.73
	NU11	60.20 ± 0.57^{h}	W	Slimy	3.55
Factory D	NU12	71.39 ± 0.53^{f}	W	Tough	3.56
	NU13	76.46 ± 0.47^{d}	W	Tough	3.46
	NU14	70.32 ± 0.16^{f}	W	Tough	3.58
A. xylinum ATCC 10245[2]		100 ± 0.34^{b}	W	Tough	3.54

[1]Data are expressed as mean ± SD (n = 3). Results with different subscripts within a column are significantly different from each other (P < 0.01; Duncan's multiple range test). [2]*A. xylinum* ATCC 10245 was used as the reference culture. W, white.

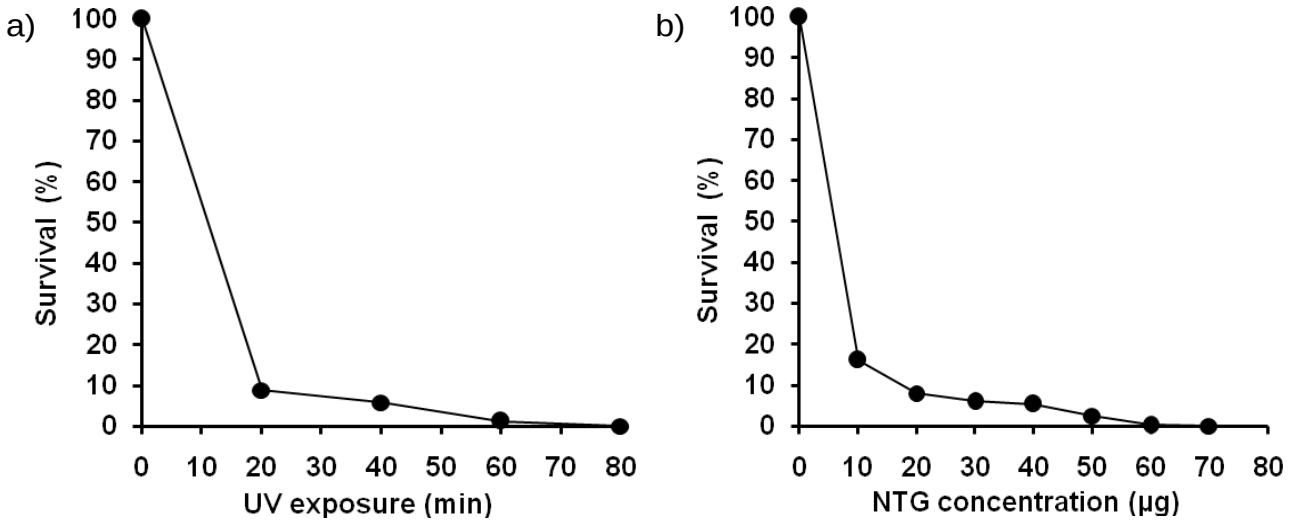

Figure 1. Dose-survival curve of parental NU4 cells after (a) UV irradiation and (b) NTG treatment. NTG, Nitrosoguanidine.

were exposed to UV radiation as mentioned in the method. It has been reported that plates showing a 1% survival rate are appropriate for selecting the UV mutants (De Wulf et al., 1996). However, in the present study, the number of mutants in these plates was low (38 colonies). Stephen and David (1986) reported that most mutants exhibiting improved yields were obtained from plates showing about 10% survival.

Survival of NU4 cells gradually decreased with increasing UV exposure time. The exposure time of 80 min was the lethal dose (0% survival); however, survival was 8.53% after 20 min (n = 256), 5.80% after 40 min (n = 174) and 1.27% after 60 min (n = 38) for a total of 468 isolated mutants (Figure 1a). The mutant colonies varied in both size and morphology. In the present study, *A. xylinum* ATCC 10245 and NU4 gave rise to small,

Table 2. Cellulose production of mutant strains generated by ultraviolet (UV) irradiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) or both.

Relative yield (%)	UV irradiation (n)	NTG (n)	UV irradiation followed by NTG (n)	NTG followed by UV irradiation (n)
49 - 59	11	6	-	-
60 - 79	45	44	9	7
80 - 99	42	55	21	14
100 - 119	40	75	42	33
120 - 139	3	12	5	4
140 - 159	1	3	-	-
Total	142	195	77	58

Table 3. Cellulose production and final pH of mutants generated by ultraviolet (UV) irradiation.

Isolate[2]	Relative yield (%)[1]	Final pH
NU4[3]	110.99 ± 0.93^{i}	3.46
UV20-05	127.55 ± 0.59^{c}	3.50
UV20-84	111.08 ± 0.52^{i}	3.93
UV40-07	150.59 ± 0.19^{a}	4.03
UV40-11	130.15 ± 0.17^{b}	3.89
UV40-21	116.16 ± 0.37^{g}	3.94
UV40-22	123.77 ± 0.48^{d}	3.95
UV40-27	110.81 ± 0.45^{i}	3.69
UV40-43	119.42 ± 0.53^{e}	3.68
UV40-44	114.54 ± 0.13^{h}	3.75
UV40-54	117.31 ± 0.30^{f}	3.89
UV40-55	115.49 ± 0.42^{g}	3.55

[1]Data are expressed as mean ± SD (n = 3). Results within a column with different subscripts are significantly different from each other ($P < 0.01$; Duncan's multiple range tests). [2]Isolate name refers to UV exposure time (min) and colony number (e.g. UV 20-05 is colony number 5 isolated from a plate exposed to UV irradiation for 20 min). [3]Parent strain selected from Factory B.

rough and raised colonies. Although, mutant colonies were small/smooth, large/smooth, raised or flat, colony morphology was not considered when screening mutants. However, colonies exhibiting rapid growth when compared with the parent strain have been reported to produce large amounts of BC (Saxena and Brown, 1989). Therefore, 142 colonies that were up to 2-fold larger than the parent strain were selected; they consisted of 87 colonies resulting from 20 min UV irradiation and 55 colonies resulting from 40 min irradiation.

Cellulose production of these large mutant colonies was evaluated after culture in HS medium and when compared with that of the reference strain. Table 2 groups these mutants according to relative BC yield: 40 to 59, 60 to 79, 80 to 99, 100 to 119, 120 to 139 or 140 to 150%. The BC yield of most mutant strains ranged from 43.08 ± 0.34 to 98.97 ± 0.16% of the yield of *A. xylinum* ATCC 10245 (100 ± 0.34%). As shown in Table 3, two mutants achieved BC yields approximately equal to that of the parent strain NU4 (110.81 ± 0.45 and 111.0.8 ± 0.52%). In addition, BC yields of nine mutants were significantly higher than that of the parent strain ($P < 0.01$); most of these mutants were generated by 40 min UV irradiation. BC production was highest in NU4-UV20-05 (127.55 ± 0.59%), NU4-UV40-011 (130.15 ± 0.17%) and NU4-UV40-07 (150.59 ± 0.19%). De Wulf et al. (1996) reported that 20 min UV irradiation of *A. xylinum* resulted in 1% survival; this method produced one mutant strain defective in ketogluconate synthesis, but its BC production was about two-fold that of the parent strain. In the present study, NU4-UV40-07 had the highest BC yield (150.59 ± 0.19%), suggesting that a 40 min UV irradiation was the optimum dose for improving BC production.

Strain improvement by NTG mutagenesis

To boost productivity, NU4 cells were exposed to NTG. Survival decreased with increasing NTG concentrations, and 70 µg/ml NTG was lethal (0% survival). Survival was 16.17% with 10 µg/ml NTG (n = 485), 8% with 20 µg/ml (n = 240), 6% with 30 µg/ml (n = 180), 5.33% with 40 µg/ml (n = 160), 2.50% with 50 µg/ml (n = 75) and 0.2% with 60 µg/ml (n = 6) for a total of 1146 mutants (Figure 1b). Similar to the results of UV irradiation, these mutant colonies varied in size. Using 5 to 10% survival as a criterion for choosing the plates from which mutants were selected, 656 mutant colonies were obtained. Of these mutants, 195 gave rise to colonies that were up to 2-fold larger than those of the parent strain; they consisted of 86 mutants generated by 20 µg/ml NTG, 61 mutants generated by 30 µg/ml NTG and 48 mutants generated from 40 µg/ml NTG treatment. Table 2 groups these mutants according relative BC yield: 40 to 59, 60 to 79, 80 to 99, 100 to 119, 120 to 139 or 140 to 150%. Of the 195 mutants, the BC yields of 98 mutants were lower than that of the reference strain, ranging from 43.34 ± 0.37 to 98.71 ± 0.24%. BC yields of seven mutants were similar to that of the parent strain (110.54 ± 0.55 to 111.74 ± 0.56%). BC yields ranging from 112.22 ± 0.21 to 154.68 ± 0.39% of the parent strain were produced by 42

Table 4. Cellulose yield and final pH of mutants generated by NTG.

Isolate[1]	Relative yield (%)[2]	Final pH
NU4[3]	110.99 ± 0.93[k]	3.46
NTG20-03	122.63 ± 0.33[h]	3.78
NTG20-06	121.26 ± 0.32[j]	3.50
NTG20-26	120.98 ± 0.29[j]	3.28
NTG20-45	121.28 ± 0.32[j]	3.11
NTG20-53	129.55 ± 0.27[d]	3.86
NTG20-63	124.35 ± 0.46[g]	4.02
NTG20-68	122.42 ± 0.28[hi]	3.93
NTG30-07	129.02 ± 0.14[d]	3.89
NTG30-18	142.30 ± 0.53[b]	3.94
NTG30-32	131.23 ± 0.53[c]	3.84
NTG30-35	120.68 ± 0.17[j]	3.69
NTG30-40	121.53 ± 0.50[ij]	3.68
NTG30-51	154.68 ± 0.39[a]	4.07
NTG40-24	125.87 ± 0.53[f]	3.89
NTG40-28	127.02 ± 0.32[e]	3.55
NTG40-42	125.91 ± 0.27[f]	4.03

[1]Isolate name refers to NTG concentration and colony number (e.g. NTG20-03 is colony number 3 isolated from a plate containing 20 µg/ml NTG).[2]Data are expressed as mean ± SD (n = 3). Results within a column with different subscripts are significantly different from each other (P < 0.01; Duncan's multiple range tests). [3]Parent strain selected from Factory B.

mutants; thus, the proportion of NTG mutants with BC yields exceeding that of the parent strain appeared to be higher than the proportion of highly productive mutants obtained by UV irradiation. The mutants producing BC amounts that were significantly higher (P < 0.01) than that of the parent strain were: NU4-NTG30-51 (154.68 ± 0.39%), NU4-NTG30-18 (142.30 ± 0.53%), NU4-NTG30-32 (131.23 ± 0.53%), NU4-NTG20-53 (129.55 ± 0.27%) and NU4-NTG30-07 (129.02 ± 0.73%) (Table 4). The mutant NU4-NTG30-51 generated by 30 µg/ml NTG was the most efficient producer of cellulose, suggesting that this dose was optimal for improving BC yields. Ishigawa et al. (1995) used 10 µg/ml NTG to improve productivity of *A. xylinum* by mutagenesis and produced a strain in which cell growth and BC yields were up to 40% higher than that of the parent strain.

Effected of combination of mutagens

Next, we evaluated whether NTG mutagenesis followed by UV irradiation or UV irradiation followed by NTG mutagenesis could further improve BC yields. NU4-UV40-07 was exposed to 30 µg/ml NTG, producing 58 mutants. NU4-NTG30-51 was subjected to UV irradiation for 15 min, but no cells survived. The UV exposure was decreesed to 5 min to obtain a survival rate between 5 and 10%, which produced 77 mutants. We found that combination treatment did not improve productivity; BC yields of all mutants generated by a combination of mutagens were lower than that of NU4-UV40-07 and NU4-NTG30-51 (Table 2). Ingle and Erickson (1978) reported that seq-uential exposure to different mutagens (UV irradiation and NTG mutagenesis) was essential to improve the yields of bacteria. The combined treatment of physical and chemical mutagens would be expected to increase yield if they produced synergistic effects. However, if they alter the same locus or if the effects of these mutagens are independent, then undesirable results may occur (Girija and Dhanavel, 2009). In the present study, neither NTG mutagenesis followed by UV irradiation nor UV irradiation followed by NTG improved BC yields when compared with that of a single mutagen.

Reversion of BC-producing of mutant strains

Cook and Colvin (1980) found that a cellulose-deficient mutant of *A. xylinum* reverted to wild type after sub-culturing five times in liquid medium. Similarly, Valla and Kjosbakken (1982) reported that cellulose-deficient mutants of *A. xylinum* obtained by spontaneous mutation or induced by ethyl methanesulfonate or nitrous acid reverted to wild type when grown in static culture. To determine whether our mutant strains were susceptible to spontaneous reversion, NU4-UV40-07 and NU4-NTG30-51 were cultured on HS agar slants at 29°C and subcultured weekly for three months. The mutant strains were then tested for BC production by culturing in 30 ml HS medium for seven days. We found that the BC yields of these mutants remained higher than that of the reference strain *A. xylinum* ATCC10245 and did not revert to the same level as the parent strain (Figure 3).

Consumption of glucose in culture medium

As shown in Figure 2, glucose consumption of the mutants NU4-UV40-07 and NU4-NTG30-51 was higher than that of the parent strain NU4. However, the pattern of glucose consumption of NU4 was similar to that of NU4-NTG30-51: glucose consumption was low during the first two days, increased rapidly until day five, and then declined again. After the seven-day incubation, total glu-cose consumption of NU4 and NU4-NTG30-51 was 84.67 and 91.70%, respectively (Table 5).

In contrast, mutant NU4-UV40-07 and reference strain *A. xylinum* ATCC 10245 both consumed glucose quickly, almost depleting the glucose by day four of incubation. After the seven-day incubation, NU4-UV40-07 had con-sumed 100% of the glucose and *A. xylinum* ATCC 10245 had consumed 97.45% (Table 5). Masaoka et al. (1993) reported that BC yield was associated with the level of glucose consumption. However, in the present study, the highest glucose consumption did not result in the highest BC yield. Isolate NU4 consumed the least glucose (84.67%), but its BC productivity was 110.99%. In contrast,

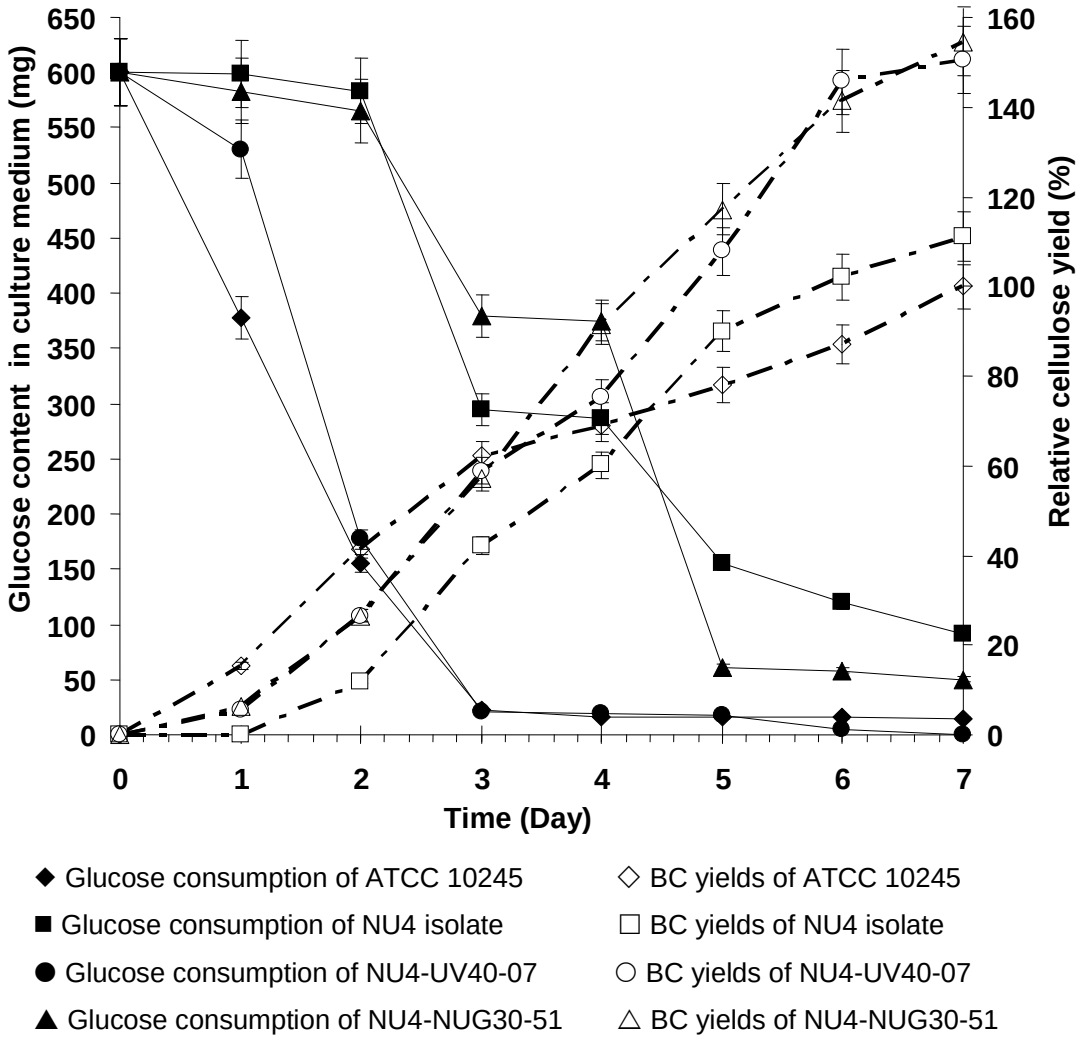

Figure 2. Glucose consumption and cellulose yield of *A.xylinum* ATCC 10245, isolate NU4, mutants NU4-UV40-07 and NU4-NUG30-51.

Table 5. Cellulose production efficiencies of *A. xylinum* ATCC 10245, isolate NU4 and two mutants.

Strain	Relative yield (%)[1]	Glucose Consumption (%)	BC Yield (%)[2]	Production Efficiency (%)[3]	Final pH
Reference strain ATCC 10245	100	97.45	5.59	5.74	3.62
Parent strain NU4	110.99	84.67	6.21	7.33	3.65
NU4-UV40-07	150.59	100	8.42	8.42	4.01
NU4-NTG30-51	154.68	91.70	8.65	9.43	4.07

[1]33.56 mg/30 ml = 100%. [2]Calculated from pellicle dry weight (mg) and weight of 2% glucose (600 mg) in the culture medium. [3]Calculated from pellicle dry weight (mg) and weight of glucose consumed.

glucose consumption of *A. xylinum* ATCC 10245 was relatively high (97.45%), but its BC productivity was the lowest (100%).

Production efficiencies of the mutants NU4-NTG30-51 (9.43%) and NU4-UV40-07 (8.42%) were higher than that of NU4 (7.33%) and *A. xylinum* ATCC 10245 (5.74%) (Table 5). Sameshima et al. (1991) reported that cellulose production of *A. xylinum* ATCC 10245 was decreased by increasing the initial glucose concentration, and found that the highest BC production was obtained with 1% glucose. In present study, the highest BC yield was achieved with 2% glucose. Masuoka et al. (1993) demonstrated

that glucose concentrations from 0 to 2% did not affect the rate of BC production. Moreover, it has been reported that BC production efficiency was dependent on the carbon source and *Acetobacter* strain (Sameshima et al., 1991; Pourramezan et al., 2009). Glycerol was found to produce the highest BC yields and resulted in the highest production efficiency (28.70%) (Sameshima et al., 1991; Premjet et al., 1994a). In contrast, efficiency of BC production of *A. xylinum* ATCC 10245 using glucose substrate was 8.7% (Sameshima et al., 1991). In this work, *A. xylinum* ATCC 10245 has the production efficiency to be only 5.74%. The results show that production efficiency of mutant NU4-UV40-07 (8.42%) was equivalent to that reported by previous studies using glucose, and the efficiency of mutant NU4-NTG30-51 (9.43%) was slightly higher.

Preventing the pH decrease in static culture, primarily caused by oxidation of glucose to gluconic acid during BC production, is not easy to manage in static culture (Ishikawa et al., 1995; Dudman, 1995). Ishikawa et al. (1995) reported that BC yields of *A. xylinum* were correlated with cell growth. Accordingly, decreasing the culture pH not only affects cell growth, but also affects BC production of *A. xylinum* (Seto et al., 1997). The final pH in the culture medium of mutant NU4-NTG30-51 (4.07) and NU4-UV40-07 (4.01) was slightly higher than that of the parent strain NU4 (3.65) and *A. xylinum* ATCC 10245 (3.62) (Table 5). This result suggests that mutagenesis by UV irradiation or NTG altered the oxidation reaction and gluconic acid production of these mutants, which may have promoted cell growth and BC production.

X-ray diffractograms of BC

BC produced by wild type *A. xylinum* in HS liquid medium normally consists of highly crystalline microfibrils of cellulose I. However, a mutant strain of *A. xylinum* was reported to have produced cellulose II (Kuga et al., 1993; Tokoh et al., 1998). To determine whether the structure of cellulose produced by the two mutants was different from that of the parent strain, X-ray diffractograms were taken of the BC pellicles produced by *A. xylinum* ATCC 10245, NU4 and the mutants NU4-UV40-07 and NU4-NTG30-51. The X-ray diffractograms revealed two main peaks corresponding to the 101 and 002 crystallographic planes at 14.4 and 22.7°, identified as crystalline microfibrils of cellulose I (Figure 3). These results confirm that the BC microstructure produced by NU4-UV40-07 and NU4-NTG30-51 were not markedly different from that of the parent strain or *A. xylinum* ATCC 10245.

Crystallinity and crystallite orientation of the cellulose produced by these strains are summarized in Table 6. The crystallinity of BC pellicles produced by NU4-UV40-07 (75.53%), NU4-NTG30-51 (80.53%) and NU4 (78.81%) were lower than that of *A. xylinum* ATCC 10245 (81.03%). Crystallinity of the cellulose produced by NU4-UV40-07 (75.53%) was slightly lower than that of the parent strain NU4 (78.81%), whereas the crystallinity of cellulose produced by NU4-NTG30-51 (80.53%) was higher, suggesting that UV irradiation and NTG mutagenesis exerted opposite effects on loci associated with cellulose synthesis.

Normally, crystallite orientation values are not associated with crystallinity, because the arrangement of the ribbon-like microfibrils in the cellulose pellicle is affected by the drying method. Crystallinity and crystallite orientation of BC pellicles treated with 1% NaOH are higher than those of BC pellicles dried with acetone (Premjet et al., 1996). In the present study, BC pellicles were treated with 1% NaOH and dried on a glass plate. The crystallite orientation values of cellulose produced by mutants NU4-NTG30-51 (0.49) and NU4-UV40-07 (0.32) were smaller than those of isolate NU4 (0.64) and *A. xylinum* ATCC 10245 (0.84). Thus, mutagenesis by UV irradiation or NTG not only increased cellulose production, but altered cellulose crystallinity and crystallite orientation.

Pure cultures of isolate NU4 and mutants NU4-UV40-07 and NU4-NTG30-51 were identified at the genus level according to Bergey's Manual of Determinative Bacteriology and related works (Holt et al., 1994; Kojima et al., 1998; Tanasupawat et al., 2009). These strains were gram-negative, rod-shaped, catalase-positive, cellulose producers, producers of acetic acid from ethanol, oxidezers of acetate and lactate to CO_2 and H_2O and gelatin liquefaction-negative. Therefore, these strains were tentatively identified as *Acetobacter* species of the family of Acetobacteraceae.

Uridine diphosphoglucose (UDP-glucose) is the immediate sugar nucleotide precursor of cellulose synthesis in *A. xylinum*. The metabolic pathway from glucose to cellulose in *A. xylinum* consists of: (1) phosphorylation of glucose by glucokinase, (2) isomerization of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase, (3) synthesis of UDP-glucose by UDP-glucose-pyrophosphorylase and (4) polymerization of UDP-glucose into β-1,4-glucan chains by membrane-bound cellulose synthase, which is activated by the cyclic dinucleotide c-di-GMP (Ross et al., 1991).

Our results show that strain improvement of the *Acetobacter* isolate NU4 was achieved with NTG mutagenesis and UV irradiation. The mutant NU4-NTG30-51 generated by 30 µg/ml NTG produced the highest cellulose yield (154.68 ± 0.39% of the reference strain, $P < 0.01$), and the mutant NU4-UV40-07 generated by 40 min UV irradiation produced a slightly lower cellulose yield (150.59 ± 0.19% of the reference strain). Furthermore, the cellulose yields of these mutants were 43.69 and 39.60% higher, respectively, than that of the parent strain NU4. The production efficiency of NU4 (7.33%) was increased to 9.43% by NTG mutagenesis and 8.42% by UV irradiation. Mutagenesis also altered cellulose crystallinity and crystallite orientation. However, the combination of NTG mutagenesis and UV irradiation

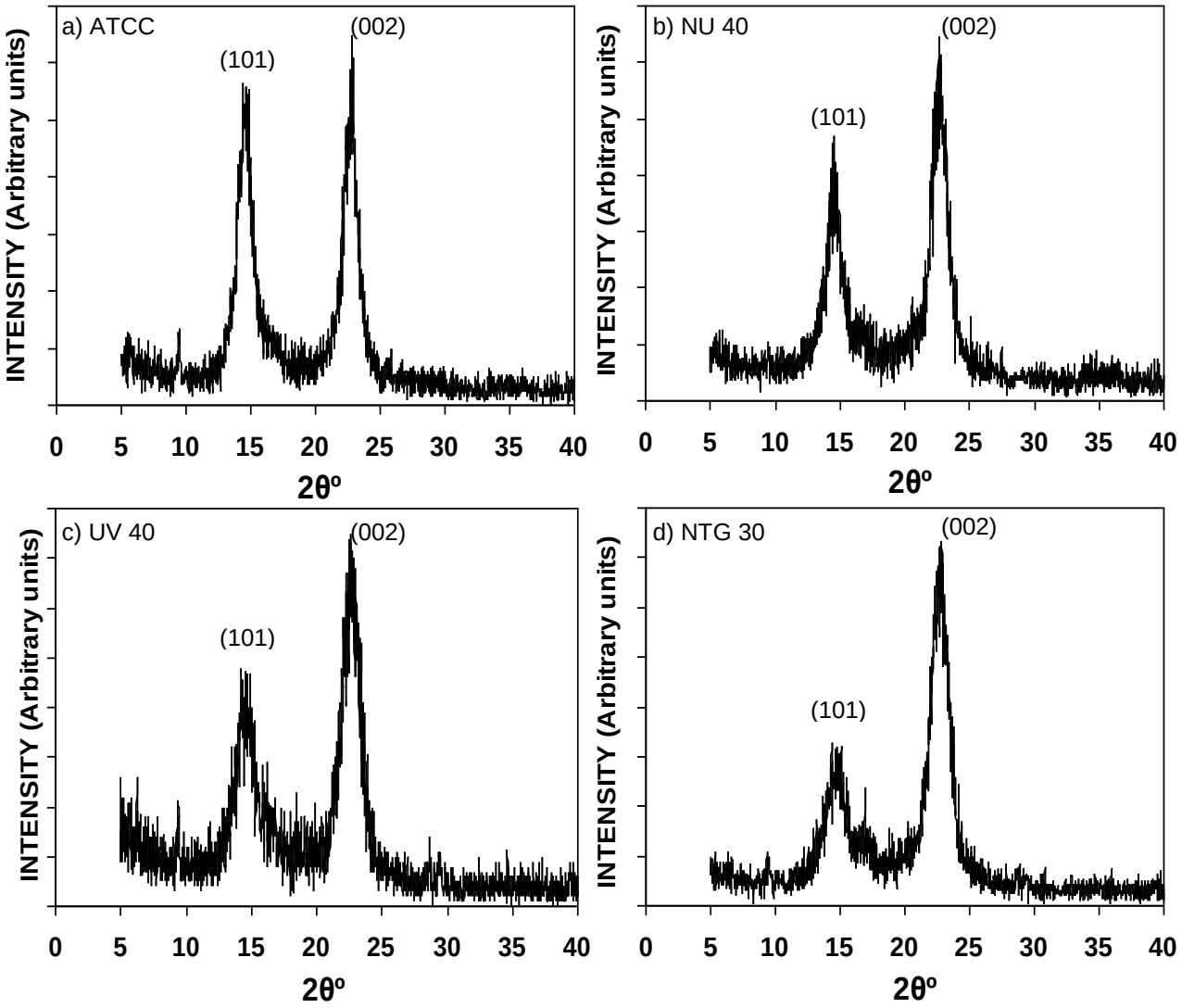

Figure 3. X-ray diffractograms of cellulose pellicles produced by (a) reference strain *A. xylinum* ATCC 10245, (b) *Acetobacter* isolate NU4, (c) mutant NU4-UV40-07 and (d) mutant NU4-NTG30-51.

Table 6. Crystallinity and crystallite orientation of BC pellicle produced by *A. xylinum* ATCC 10245, isolate NU4 and two mutants.

***A. xylinum* strain**	**Thickness (mm)**	**Crystallinity (%)**	**Crystallite orientation**
ATCC 10245	0.035	81.03	0.84
NU4[1]	0.037	78.81	0.64
NU4-UV40-07	0.040	75.53	0.49
NU4-NTG30-51	0.045	80.53	0.32

[1]Parent strain from Factory B.

produced mutants with BC yields that were slightly higher than that of the parent strain, but were not higher than those of NTG30-51 or NU4-UV40-07, which were generated by a single mutagen. These high yielding mutants could be used to reduce the cost of BC production using traditional processes.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of the National Research Council of Thailand (AR14/2547). We are grateful to both the former advisor, Associate Professor Dr. Hunsa Punnapayak, for kindly providing *N*-

methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and Emeritus Professor Dr. Kazuhiko Sameshima, Wood Chemistry Laboratory, Kochi University, Japan for kindly providing *Acetobacter xylinum* ATCC 10245 and for his invaluable suggestions.

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