

*Maejo Int. J. Sci. Technol.* **2013**, 7(01), 70-82

## *Maejo International Journal of Science and Technology*

ISSN 1905-7873

Available online at [www.mijst.mju.ac.th](http://www.mijst.mju.ac.th)

*Full Paper*

### **Identification and biocellulose production of *Gluconacetobacter* strains isolated from tropical fruits in Thailand**

**Amornrat Suwanposri<sup>1</sup>, Pattaraporn Yukphan<sup>2</sup>, Yuzo Yamada<sup>3</sup> and Daungjai Ochaikul<sup>1,\*</sup>**

<sup>1</sup> Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Chalongkrung Rd. Ladkrabang, Bangkok, 10520, Thailand

<sup>2</sup> BIOTEC Culture Collection (BCC), Bioresources Technology Unit, National Centre for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Phaholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand

<sup>3</sup> Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

\* Corresponding author, e-mail: [kodaungj@kmitl.ac.th](mailto:kodaungj@kmitl.ac.th)

*Received: 28 January 2012 / Accepted: 19 January 2013 / Published: 1 February 2013*

---

**Abstract:** Two hundred and four strains of biocellulose (BC)-producing *Gluconacetobacter* strains were isolated from 48 rotten tropical fruits collected in Thailand. Twenty-nine representative isolates were selected from each of the 16 isolation sources and identified by morphological, physiological and biochemical characteristics and 16S rRNA gene sequence analysis. The selected 29 isolates were divided into seven subgroups within the *Gluconacetobacter xylinus* group of the genus *Gluconacetobacter* and identified as *Gluconacetobacter oboediens* (subgroup I, five isolates), *Gluconacetobacter rhaeticus* (subgroup II, one isolate), *Gluconacetobacter hansenii* (subgroup III, seven isolates), *Gluconacetobacter swingsii* (subgroup IV, two isolates) and *Gluconacetobacter sucrofermentans* (subgroup V, two isolates). The remaining isolates were grouped into subgroups VIa (three isolates) and VIb (nine isolates). All the isolates were cultured in Hestrin-Schramm (HS) medium statically at 30°C for 7 days to determine cellulose production capability. Of the 29 isolates, isolate PAP1 (subgroup VIb, unidentified) gave the highest yield (1.15 g/L) of BC. However, the BC yield increased threefold (3.5 g/L) when D-glucose in HS medium was replaced by D-mannitol.

**Keywords:** biocellulose-producing bacteria, *Gluconacetobacter*, tropical fruit, 16S rRNA gene sequence analysis

---

## INTRODUCTION

Bacterial cellulose or biocellulose (BC) is an extracellular cellulose naturally produced by many species of microorganisms. BC has been considered as an alternative biomaterial since it possesses superior qualities to other cellulose. BC exhibits many unique characteristics which are different from those of other plant celluloses, such as high water-holding capacity (over 100 times of its weight), high degree of crystallinity, great elasticity, high tensile strength, non-drying state, excellent biocompatibility and high purity, because it is free from other contaminating components such as hemicelluloses, lignins or waxy aromatic substances [1-3]. These distinct physical and mechanical qualities have made BC more attractive than other materials well known as alternative materials in food, biomedical and other industries. For food applications, BC has been used as raw materials for nata de coco, which is a popular dessert in Philippines and other countries, and a dietary drink called Kombucha or Manchurian tea. In biomedical applications, BC is ideal for wound-healing dressing, micro blood vessels and scaffolds for tissue engineering of cartilage and bone [4-5]. In other applications, BC has potential for producing banknote and Bible paper, high performance speaker diaphragms, electronic paper displays, flexible display screens, paint thickeners, make-up pads and anti-aging cosmetics [2, 6-8].

Members of the genus *Gluconacetobacter* are divided into two groups, viz. the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group [9]. The former group consists of the non-nitrogen fixers such as *G. liquefaciens* and *G. sacchari* and the nitrogen fixers such as *G. diazotrophicus*, *G. azotocaptans* and *G. johannae*. The latter group consists of the non-BC producers such as *G. hansenii* and *G. europaeus*, and the BC producers such as *G. xylinus*, *G. nataicola* and *G. rhaeticus*. *G. xylinus* (formerly *Acetobacter xylinum*) are the most common species, many strains of which are high cellulose producers. These cellulose-producing bacteria are commonly found in natural sources such as flowers, vegetables, nuts, sugar cane and, in particular, rotten fruits [10-12]. Industrial production of BC using these bacteria is traditionally achieved by using a static cultivation method. BC is produced as white pellicle at the air-liquid interface of a liquid medium. However, this method requires a long cultivation time and large area while in shaking or agitated culture, non-BC producing mutants are produced [13]. Therefore, the improvement of static fermentation process, optimisation of culture condition and isolation of highly effective BC-producing strains are desirable.

Thailand is a country with relatively high humidity and high temperature and has a range of indigenous fruits that might be a rich source of BC-producing bacteria. This study is aimed at the isolation, identification and production of cellulose from *Gluconacetobacter* strains isolated from tropical fruits in Thailand.

## MATERIALS AND METHODS

### Isolation of *Gluconacetobacter* Strains

BC-producing *Gluconacetobacter* isolates in this study were isolated from 48 rotten tropical fruits collected in Thailand using the modification method described by Park et al. [12]. Firstly, 10 g of each rotten fruit was transferred into 90 mL of a modified Hestrin-Schramm (HS) medium in a 250-mL flask containing 2.0% D-glucose (w/v), 0.5% peptone (w/v), 0.5% yeast extract (w/v), 0.27% Na<sub>2</sub>HPO<sub>4</sub> (w/v), 0.12% citric acid (w/v), 0.2% acetic acid (v/v), 0.5% ethanol (v/v) and 0.01% cycloheximide (w/v) [14]. The flask with rotten fruit and liquid medium was then incubated statically at 30°C for 7 days. After incubation, the flask with white pellicle covering the surface of

the liquid medium was selected. The culture broth of the selected flask was serially diluted with 0.85% NaCl (w/v) and 0.1 mL of each dilution was spread on GEY agar, which was comprised of 2.0% D-glucose, 1.0% yeast extract, 5% ethanol and 0.3% CaCO<sub>3</sub>. The agar plates were incubated at 30°C until colonies were formed. The colonies with a clear zone around were selected and transferred to vials containing 5 mL of HS medium and then incubated at 30°C for 3-7 days. Subsequently, only the vials with white pellicle on the surface were collected for further purification. The pellicles were confirmed by boiling with 0.5N NaOH for 15 min., since they might not be cellulose.

### **Selection of *Gluconacetobacter* Isolates**

The BC-producing isolates with the highest and the lowest yields were selected from each fruit on the basis of BC thickness, yield and appearance. A single colony of each BC-producing isolate was transferred into 5 mL of HS medium in a vial and incubated statically at 30°C for 7 days. The resulting pellicle was harvested and washed three times with distilled water. Subsequently, BC appearance was observed by the naked eye and the thickness was measured with a vernier. The pellicle was then purified by heating with 2% NaOH at 121°C for 15 min. to remove bacterial contaminants and other residues. Finally, the purified cellulose was dried at 80°C in a hot air oven to constant weight.

### **Identification of *Gluconacetobacter* Strains**

Morphological, physiological and biochemical characteristics of the selected isolates were determined using the method described by Asai et al. [15], Sokollek et al. [16] and Tortora et al. [17]. All the selected cellulose-producing bacteria were examined for 16S rRNA gene sequence analysis according to the method described by Yukphan et al. [18]. A specific fragment for 16S rRNA gene-coding regions was amplified using PCR amplification. Two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3'; positions 9-27) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'; positions 1509-1492) were used. The positions in the rRNA gene fragment were based on the *Escherichia coli* numbering system (accession number V00348 [19]). The purified 16S rRNA genes from positions 9 to 1509 (approximately 1,500 bases) were sequenced by using four primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 27-46), 800R (5'-TAC CAG GGT ATC TAA TCC-3'; position 800-783), 518F (5'-CCA GCA GCC GCG GTA ATA CG-3'; position 518-537) and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'; position 1492-1471). A phylogenetic tree for 1,280 bases was constructed by the neighbour-joining method of Saitou and Nei [20] using MEGA programme (version 4.0) [21] after multiple alignments of the sequences obtained with CLUSTAL W [22]. The distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura [23]. The bootstrap values at branching points in the phylogenetic tree were calculated with 1,000 replications [24]. A 16S rRNA gene sequence similarity between the type strain of *Gluconacetobacter* species and an isolate was calculated for 1,390 bases.

### **BC Production by *Gluconacetobacter* Strains**

To investigate BC-producing capacity, one loop of a cellulose-producing isolate was transferred to 100 mL of HS medium in a 250-mL flask and incubated at 30°C for 48 hr as starter culture. Ten millilitres of the culture was then added to 90 ml of HS medium in a 250-mL flask and incubated at 30°C for 7 days. The resulting pellicle was harvested, washed three times with distilled

water and purified by heating with 2% NaOH at 121°C for 15 min. The purified cellulose pellicle was dried at 80°C in a hot air oven to constant weight.

## RESULTS AND DISCUSSION

### Identification of *Gluconacetobacter* Strains

From the 48 rotten fruits collected, 2,500 bacterial isolates were obtained as BC-producing candidates. They were then examined for BC production using a modified HS medium. As a result, 204 isolates from 16 fruits were BC-producing bacteria (Table 1). The most efficient isolates were from the governor's plum (*Flacourtia indica*) with 25 isolates, approximately 1.00% of the total 2,500 isolates and the least was from lady finger's banana (*Musa acuminata*) with one isolate, approximately 0.04% of the total isolates.

**Table 1.** BC-producing bacterial isolates

Isolation source and code	BC-producing isolate (% <sup>a</sup> )	Selected isolate	Subgroup
Beleric myrobalan ( <i>Terminalia bellerica</i> ), BEL	3 (0.12)	BEL1	III ( <i>G. hansenii</i> )
		BEL2	III ( <i>G. hansenii</i> )
Fetid passion flower ( <i>Passiflora foetida</i> ), FET	15 (0.60)	FET4	III ( <i>G. hansenii</i> )
		FET8	V ( <i>G. sucrofermentans</i> )
		GOV9	I ( <i>G. oboediens</i> )
Governor's plum ( <i>Flacourtia indica</i> ), GOV	25 (1.00)	GOV15	I ( <i>G. oboediens</i> )
		GRA2	VIb (unidentified)
Grape ( <i>Vitis vinifera</i> ), GRA	11 (0.45)	GRA8	VIb (unidentified)
		JAV1	VIb (unidentified)
Java plum ( <i>Syzygium cumini</i> ), JAV	3 (0.12)	JAV3	VIb (unidentified)
		LAD1	III ( <i>G. hansenii</i> )
Lady's finger banana ( <i>Musa acuminata</i> ), LAD	1 (0.04)	LAD1	III ( <i>G. hansenii</i> )
Lychee ( <i>Litchi chinensis</i> ), LYC	15 (0.60)	LYC7	V ( <i>G. sucrofermentans</i> )
		LYC8	III ( <i>G. hansenii</i> )
		MAM2	VIb (unidentified)
Mamao ( <i>Antidesma thwaiteaianum</i> ), MAM	4 (0.16)	MAM4	I ( <i>G. oboediens</i> )
Mangosteen ( <i>Garcinia mangostana</i> ), MAG	23 (0.92)	MAG6	VIa (unidentified)
		MAG15	VIb (unidentified)
		PAP1	VIb (unidentified)
Papaya ( <i>Carrica papaya</i> ), PAP	2 (0.08)	PAP1	VIb (unidentified)
Rambutan ( <i>Nephelium lappaceum</i> ), RAM	20 (0.80)	RAM1	II ( <i>G. rhaeticus</i> )
		RAM4	VIb (unidentified)
		SPO4	I ( <i>G. oboediens</i> )
Sapodilla ( <i>Manikara achras</i> ), SPO	23 (0.92)	SPO15	IV ( <i>G. swingsii</i> )
Star fruit ( <i>Averrhoa carambola</i> ), STA	21 (0.85)	STA5	III ( <i>G. hansenii</i> )
Sugar apple ( <i>Annona squamosa</i> ), SUG	20 (0.80)	SUG5	VIa (unidentified)
		SUG8	VIa (unidentified)
		WAT11	I ( <i>G. oboediens</i> )
Water melon ( <i>Citrullus lanatus</i> ), WAT	15 (0.60)	WAT14	IV ( <i>G. swingsii</i> )
		WIL2	III ( <i>G. hansenii</i> )
Wild lemon (unknown species), WIL	3 (0.12)	WIL3	VIb (unidentified)
		Total	204 (8.16)

<sup>a</sup>Percentage of BC-producing bacteria from a total of 2,500 isolates

From the 204 BC-producing isolates, 29 isolates were selected as representative BC-producing strains and divided into seven subgroups based on morphological, physiological, biochemical characteristics and 16S rRNA gene sequences (Table 2 and Figure 1). Colonies of the 29 isolates on HS agar plates after 48-hr growth were pale yellow, smooth, viscous, convex, dense, with circular or irregular shape and entire or undulating margin. All the isolates were Gram-

negative, rod-shaped or short rod and occurred singly or in pairs. The morphological results obtained are congruent with Dellaglio et al. [25], who isolated *Gluconacetobacter* strains from Italian apple fruit.

All the BC-producing isolates showed catalase-positive reactions and growth at pH 3.0-7.0. They grew slowly at pH 3.0, 3.5 and 4.0, but the growth was better at pH 4.5-7.0. Growing in different carbon sources indicated that all the isolates could not grow on sorbitol or methanol medium but grew well on glucose or sucrose medium (data not shown). Testing for acid production in different carbon sources indicated that all the isolates produced acid from D-glucose and D-sorbitol, but 9 out of 29 isolates also produced acid from D-arabinose, L-rhamnose and L-sorbose. From the different phenotypic characteristics obtained, the isolates were grouped into seven subgroups (Table 2).

Subgroup I contains five isolates, GOV9, GOV15, MAM4, SPO4 and WAT11, and is not identified as *G. intermedius* but as *G. oboediens*. The calculated 16S rRNA gene sequence similarities of these isolates are in the range of 99.6-99.7% of the type strain. According to Lisdiyanti et al. [29], *G. intermedius* is a later subjective synonym of *G. oboediens*, although the isolates first constituted a cluster along with the type strain of *G. intermedius* (Figure 1). They were isolated from governor's plum, mamao and water melon.

Subgroup II contains only one isolate, RAM1, and is identified as *G. rhaeticus*. The calculated 16S rRNA gene sequence similarity of the isolate is 99.9% of the type strain. It was isolated from rambutan.

Subgroup III contains seven isolates, BEL1, BEL2, FET4, LAD1, STA5, WIL2 and LYC8, and is identified as *G. hansenii*. The calculated 16S rRNA gene sequence similarities of these isolates are in the range of 99.6-99.8% of the type strain. They were isolated from beleric myrobalan, fetid passionflower, lady's finger banana, star fruit, wild lemon and lychee. Although isolate LYC8 is located in the cluster of *G. kombuchae* KG3<sup>T</sup> (AY4688433), the isolate is identified as *G. hansenii*, as suggested by Cleenwerck et al. [30], who reported that *G. kombuchae* is a later subjective synonym of *G. hansenii*.

Subgroup IV contains two isolates, SPO15 and WAT14, which are located within the same cluster as *G. swingsii* and *G. europaeus* in the phylogenetic tree (Figure 1). On the basis of the growth on 30% D-glucose (w/v) with/without 0.2% acetic acid (v/v) [31], the isolates are identified as *G. swingsii*, not as *G. europaeus*, since they show the same results as the former but not as the latter (Table 2). The two isolates have 99.9% 16S rRNA gene sequence similarity to the type strain of *G. swingsii* and were isolated from sapodilla and water melon.

Subgroup V contains two isolates, LYC7 and FET8, and are identified as *G. sacrofermentans*, to the type strain of which the calculated 16S rRNA gene sequence are 100% similar. They grew on all the different carbon sources tested and were isolated from lychee and fetid passion flower.

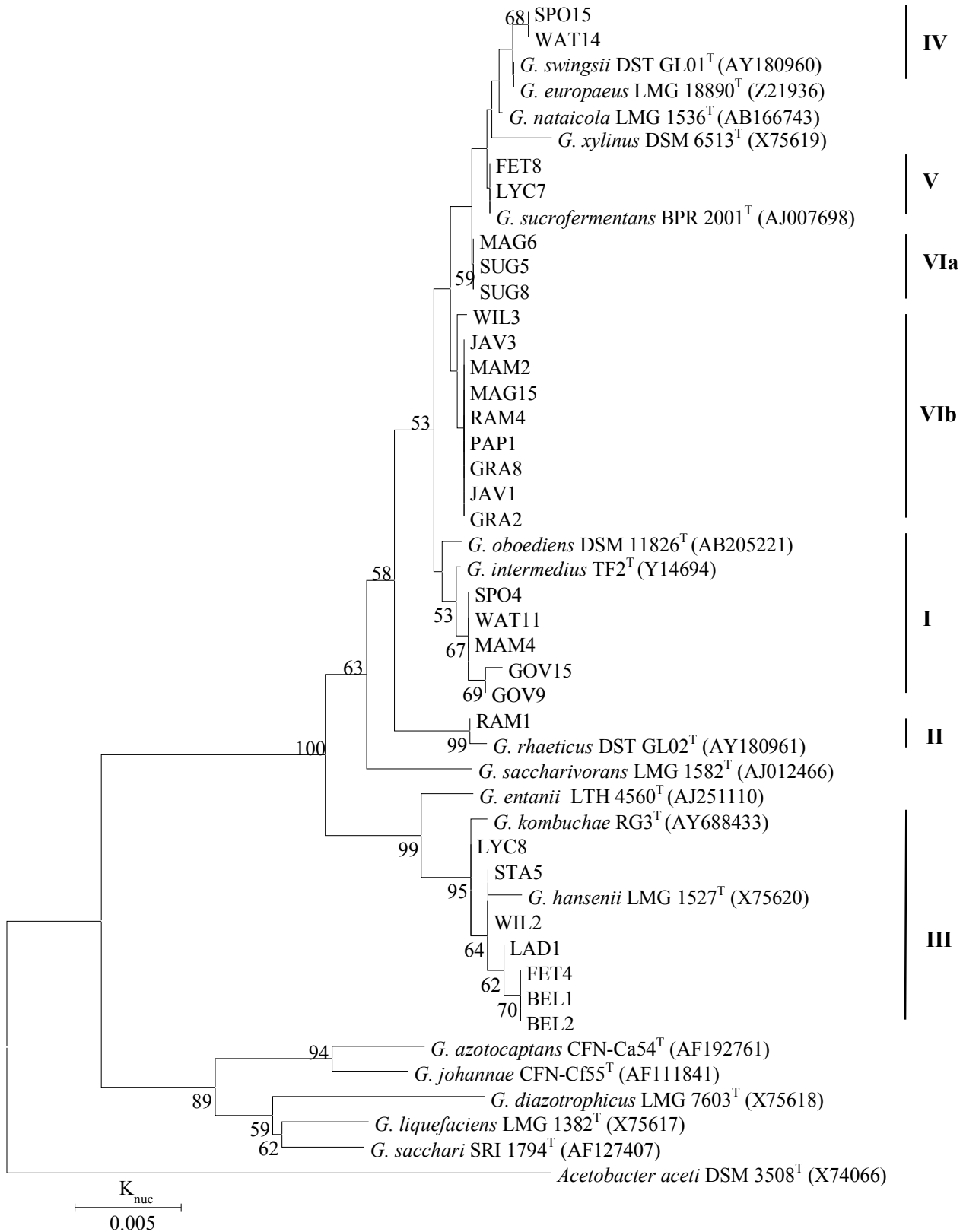
Subgroup VI is divided into two subgroups, VIa and VIb. Subgroup VIa contains three isolates, MAG6, SUG5 and SUG8, and subgroup VIb contains nine isolates, GRA2, GRA8, JAV1, JAV3, MAG15, MAM2, PAPI, RAM4 and WIL3, all of which are located in different phylogenetic positions from any other known species of the genus *Gluconacetobacter* in the 16S rRNA gene sequence phylogenetic tree and assumed to constitute new species. They were isolated from grape, java plum, mangosteen, mamao, papaya, rambutan, sugar apple and wild lemon.

**Table 2.** Different phenotypic characteristics of selected isolates

Characteristic	Subgroup I						Subgroup II		Subgroup III						Subgroup IV				Subgroup V			Subgroup VIa			Subgroup VIb											
	GOV9	GOV15	MAM4	SPO4	WAT11	<i>G. oboediens</i> LMG 18849 <sup>1a</sup>	RAM1	<i>G. rhaeticus</i> LMG 22126 <sup>1b</sup>	BEL1	BEL2	FET4	LAD1	LYC8	STA5	WIL2	<i>G. hansenii</i> NBRC 14820 <sup>1c</sup>	SPO15	WAT14	<i>G. swingsii</i> LMG 22125 <sup>1b</sup>	<i>G. europaeus</i> LMG 18890 <sup>1d</sup>	LYC7	FET8	<i>G. sucrofermentans</i> BPR2001 <sup>1e</sup>	MAG6	SUG5	SUG8	GRA2	GRA8	JAV1	JAV3	MAG15	MAM2	PAP1	RAM4	WIL3	
Growth on 30% D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	nr	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth without acetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	nr	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid production from																																				
D -glucose	+	+	+	+	+	nr	+	nr	+	+	+	+	+	+	+	+	+	+	nr	nr	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D -arabinose	-	-	-	-	-	nr	+	nr	-	+	-	-	-	-	-	nr	-	-	nr	nr	-	-	nr	-	-	-	-	+	-	-	-	-	-	-	-	-
D -sorbitol	+	+	+	+	+	nr	+	nr	+	+	+	+	+	+	+	-	+	+	nr	nr	+	+	nr	+	+	+	+	+	+	+	+	+	+	+	+	+
L-rhamnose	-	-	-	-	-	nr	-	nr	+	+	-	+	-	+	+	nr	+	+	nr	nr	-	-	nr	-	-	-	-	-	-	+	-	-	+	-	-	-
L-sorbose	-	-	-	-	-	nr	-	nr	-	+	-	-	-	-	-	-	-	-	nr	nr	-	-	nr	-	-	-	-	+	-	-	-	-	+	-	-	+
Growth on different carbon																																				
ethanol	+	+	-	+	+	-	+	+	+	+	-	+	+	+	-	-	-	+	+	-	+	+	+	-	-	+	-	+	-	-	-	-	+	+	+	-
sucrose	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	-	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	-
sorbitol	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	v	+	+	+	-	+	w	nr	+	+	+	+	+	+	+	+	+	+	+	+	-
D-mannitol	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	w	nr	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = positive; - = negative; w = weakly positive; v = very weakly positive; nr = not reported

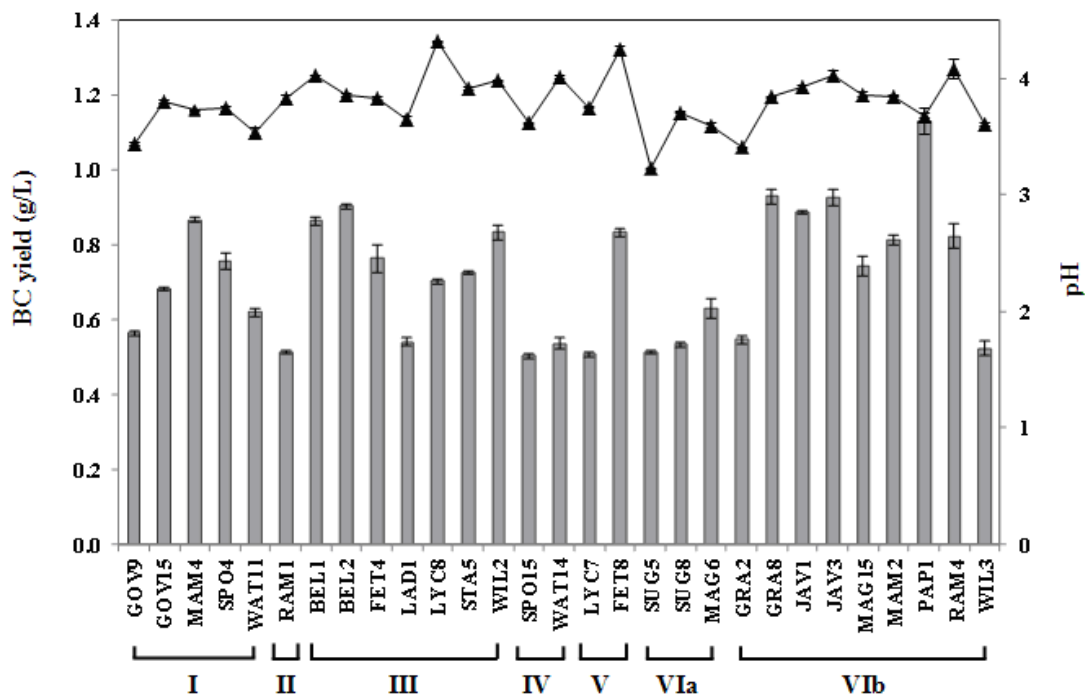
The data of the reference strains were cited from <sup>a,d</sup>Sokollek et al. [16], <sup>b</sup>Dellaglio et al. [25], <sup>c</sup>Gosselé et al. [26] and Navarro et al. [27] and <sup>e</sup>Toyosaki et al. [28]



**Figure 1.** Phylogenetic relationships of BC-producing isolates. The numerals at the branching points indicate bootstrap values (%) derived from 1,000 replications. Only values greater than 50% are indicated.

### BC Production by BC-Producing Isolates

All 29 isolates were cultivated in the standard HS medium under static condition with D-glucose as sole carbon source. After 48 hr, all isolates produced white gelatinous sheet at the air-liquid interface position of the medium. The results obtained are the same as those of Jagannath et al. [32]. The level of BC production ranged from 0.5 to 1.15 g/L, when the isolates were incubated statically at 30°C for 7 days (Figure 2). The lowest yield of 0.50 g/L was found in isolate SPO15 obtained from sapodilla and identified as *G. swingsii*. The highest yield of 1.15 g/L was found in isolate PAP1, isolated from papaya, and grouped into subgroup VIb and unidentified. The pH of the culture filtrates was 3.2-4.3.

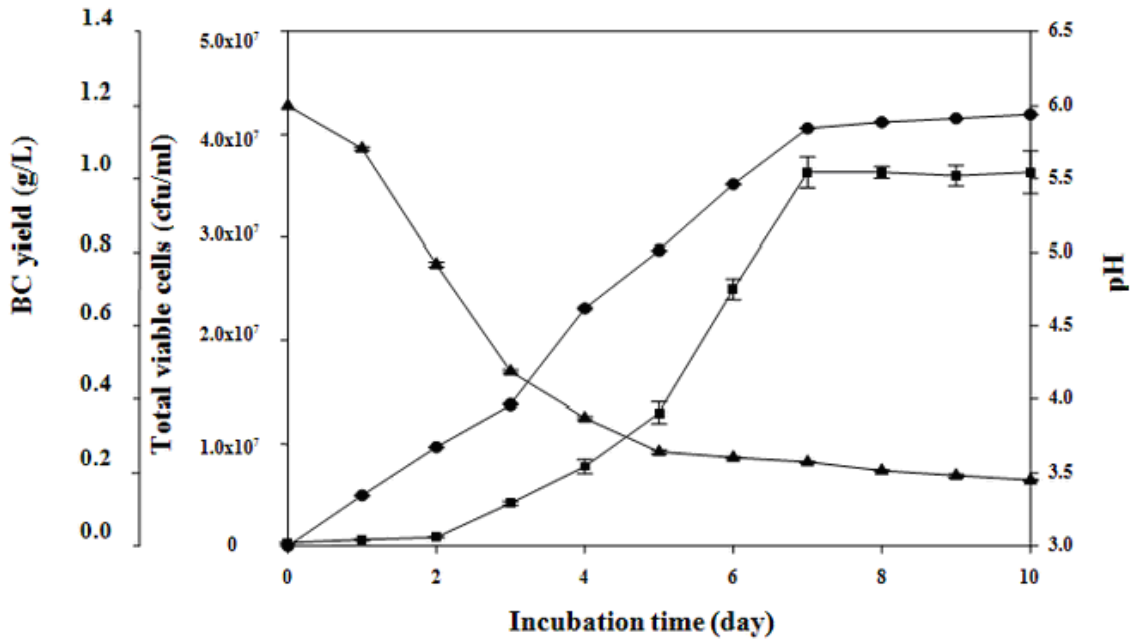


**Figure 2.** BC production by selected BC-producing isolates (▲ = pH). All data are means  $\pm$  1 SD of triplicate analyses.

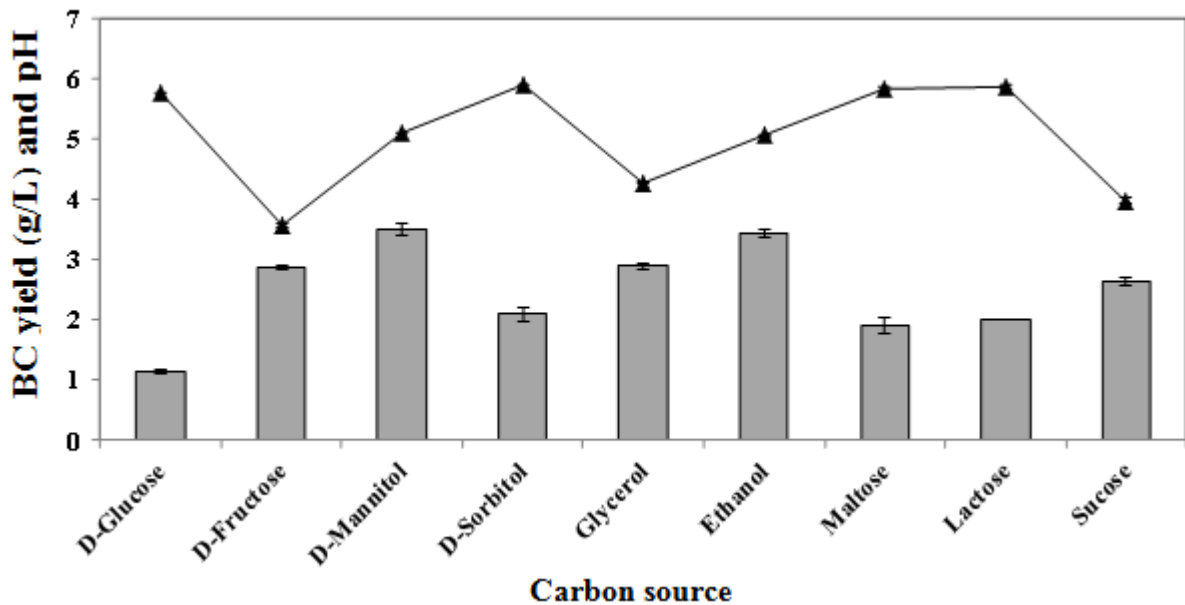
Isolate PAP1 with the highest BC-production capability was selected and examined for viable cell count, BC production and pH change during cultivation in standard HS medium at 30°C for 10 days. As shown in Figure 3, the viable cells of isolate PAP1 increase exponentially after a 2-day lag period. The total viable cells increase rather slowly in the first and second days and then increase rapidly from the third day. The production of BC also increases rapidly from the third day, indicating that BC production by isolate PAP1 is growth-associated.

To investigate the effect of carbon sources on BC production, isolate PAP1 was incubated in standard HS medium, in which D-glucose, the original carbon source, was replaced by different carbon sources, i.e. D-fructose, D-mannitol, D-sorbitol, glycerol, ethanol, maltose, lactose and sucrose, at the concentration of 2.0% (w/v or v/v) (Figure 4). When D-mannitol is used as carbon source, isolate PAP1 produces BC with the highest yield of 3.5 g/L.





**Figure 3.** BC production by isolate PAP1: ● = BC yield (g/L); ▲ = pH; ■ = total viable cells (cfu/mL). All data are means  $\pm$  1 SD of triplicate analyses.



**Figure 4.** Effect of different carbon sources on BC production by isolate PAP1 (▲ = pH). All data are means  $\pm$  1 SD of triplicate analyses.

A number of BC production studies have been reported. Keshk and Sameshima [33] mentioned that *Acetobacter xylinum* (= *G. xylinus*) ATCC 10245 gave 1.15 g/L of BC when cultivated in HS medium under static condition for 7 days. The amounts of BC produced in the present study appear to correspond to their results. Nguyen et al. [34] characterised the cellulose production by a *G. xylinus* strain isolated from Kombucha and found that this bacterium produced  $0.28 \pm 0.01$  g/L of BC in HS medium when statically incubated at 30°C for 7 days. Under the same

condition, Park et al. [12] also reported that 0.35 g/L cellulose was produced by a *G. hansenii* strain isolated from rotten apple. In the present study, isolate PAP1, which was isolated from rotten papaya, produces a large amount of BC (1.15 g/L) in HS medium.

It is well known that *G. xylinus*, a Gram-negative acetic acid bacterium, has long been used as a model organism for the study of BC biosynthesis, since it can utilise a wide range of substrates such as 5- or 6-carbon monosaccharides (e.g. D-glucose, D-fructose and D-xylose), oligosaccharides (e.g. sucrose), polysaccharides (e.g. starch), sugar alcohols (e.g. glycerol, D-mannitol and D-sorbitol), aliphatic alcohol (e.g. glycerol and ethanol), and industrial wastes including sugar cane molasses, coconut water, pineapple water and hydrolysed konjac powder to generate high amounts of cellulose [33-37].

In the present study, isolate PAP1 shows the capability of utilising a wide variety of carbon sources for BC production and D-mannitol seems to be the most suitable carbon source. D-Mannitol is probably transformed to D-fructose and then metabolised to BC. Under the experimental condition, D-gluconic acid was not produced during fermentation, so the pH remained stable [38]. Non-production of D-gluconic acid is assumed to give an optimal condition in cell growth and BC production. These results are in good agreement with previous reports that BC production by *Gluconacetobacter* strains and *G. xylinus* isolated from Kombucha culture produce the highest yields in a medium containing D-mannitol [34]. However, the capability of certain carbon source for BC production also seems to depend on the bacterial strain concerned. For example, *G. xylinus* ATCC 10245 and *Gluconacetobacter* sp. RKY5 isolated from persimmon vinegar give the highest BC yields (1.33 g/L and 2.45 g/L respectively) in glycerol [39, 40], and *G. sacchari* isolated from Kombucha gives the highest production (2.70 g/L) of BC in D-glucose [41]. The results obtained therefore seem to demonstrate that the factors affecting BC production are bacterial strain and carbon source.

Phylogenetic analysis based on 16S rRNA gene sequences shows that all the 29 BC-producing isolates belong to the *G. xylinus* group but not to the *G. liquefaciens* group, and are divided into seven subgroups (Figure 1). In the present study, the BC-producing bacterial isolates are identified as *G. oboediens* (subgroup I), the type strain of which does not produce BC; *G. rhaeticus* (subgroup II) and *G. hansenii* (subgroup III), the type strain of which does not produce BC; *G. swingsii* (subgroup IV) and *G. sucrofermentans* (subgroup V). However, it is remarkable in the present study that any strains assigned to *G. xylinus* were not isolated from tropical fruits collected in Thailand, suggesting that the species distribution might be rare in a tropical country. This phenomenon is in good accord with previous work on the diversity of acetic acid bacteria in Indonesia, Thailand and Philippines, where no *G. xylinus* strains are isolated from tropical fruits or flowers [42].

The isolates in the remaining subgroups VIa and VIb, are not identified. From the phylogenetic data obtained, it is obvious that these isolates constitute new species, which will be presented elsewhere.

## CONCLUSIONS

The present study has demonstrated that tropical fruits collected in Thailand are a rich source of BC producers and isolate PAP1 of subgroup VIb is the most effective BC-producing *Gluconacetobacter* strain with the highest BC yield of 1.15 g/L in standard HS medium at static condition. In addition, D-mannitol is the most suitable carbon source for BC production by isolate

PAP1 with 3.5 g/L of BC. To reduce the production cost, however, optimisation of the culture condition and use of alternative cheaper carbon sources such as by-products or wastes from agricultural industry are desirable.

#### ACKNOWLEDGEMENTS

The authors are grateful to the National Science and Technology Development Agency, Pathumthani, Thailand for supporting this work and also to Sakaerat Environmental Research Station, Sakaerat Biosphere Reserves, Nakhon Ratchasima, Thailand for providing tropical fruit samples.

#### REFERENCES

1. P. Ross, R. Mayer and M. Benziman, "Cellulose biosynthesis and function in bacteria", *Microbiol. Mol. Biol. Rev.*, **1991**, 55, 35-58.
2. J. Shah and R. M. Brown, "Towards electronic paper displays made from microbial cellulose", *Appl. Microbiol. Biotechnol.*, **2005**, 66, 352-355.
3. M. Shoda and Y. Sugano, "Recent advances in bacterial cellulose production", *Biotechnol. Bioproc. Eng.*, **2005**, 10, 1-8.
4. A. Svensson, E. Nicklasson, T. Harrah, B. Panilaitis, D. L. Kaplan, M. Brittberg and P. Gatenholm, "Bacterial cellulose as a potential scaffold for tissue engineering of cartilage", *Biomaterials*, **2005**, 26, 419-431.
5. D. Klemm, D. Schumann, U. Udhardt and S. Marsch, "Bacterial synthesized cellulose-artificial blood vessels for microsurgery", *Prog. Polym. Sci.*, **2001**, 26, 1561-1603.
6. M. Iguchi, S. Yamanaka and A. Budhiono, "Bacterial cellulose-a masterpiece of nature's arts", *J. Mater. Sci.*, **2000**, 35, 261-270.
7. E. J. Vandamme, S. De Baets, A. Vanbaelen, K. Joris and P. De Wulf, "Improved production of bacterial cellulose and its application potential", *Polym. Degrad. Stabil.*, **1998**, 59, 93-99.
8. A. N. Nakagaito, M. Nogi and H. Yano, "Displays from transparent films of natural nanofibers", *Mater. Res. Soc. Bull.*, **2010**, 35, 214-218.
9. Y. Yamada and P. Yukphan, "Genera and species in acetic acid bacteria", *Int. J. Food. Microbiol.*, **2008**, 125, 15-24.
10. T. Tsuchida and F. Yoshinaga, "Production of bacterial cellulose by agitation culture systems", *Pure Appl. Chem.*, **1997**, 69, 2453-2458.
11. A. Seto, Y. Kojima, N. Tonouchi, T. Tsuchida and F. Yoshinaga, "Screening of bacterial cellulose-producing *Acetobacter* strains suitable for sucrose as a carbon source", *Biosci. Biotechnol. Biochem.*, **1997**, 61, 735-736.
12. J. K. Park, Y. H. Park and J. Y. Jung, "Production of bacterial cellulose by *Gluconacetobacter hansenii* PJK isolated from rotten apple", *Biotechnol. Bioproc. Eng.*, **2003**, 8, 83-88.
13. S. Valla and J. Kjosbakken, "Isolation and characterization of a new extracellular polysaccharide from a cellulose-negative strain of *Acetobacter xylinum*", *Can. J. Microbiol.*, **1981**, 27, 599-603.
14. B. Hestrin and M. Schramm, "Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose", *Biochem. J.*, **1954**, 58, 345-352.

15. T. Asai, H. Iizuka and K. Komagata, "The flagellation and taxonomy of the genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains", *J. Gen. Appl. Microbiol.*, **1964**, 10, 95-126.
16. S. J. Sokollek, C. Hertel and W. P. Hammes, "Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations", *Int. J. Syst. Bacteriol.*, **1998**, 48, 935-940.
17. G. J. Tortora, B. R. Funke and C. L. Case, "Microbiology: An Introduction", 7<sup>th</sup> Edn., Benjamin Cummings Publishing, San Francisco, **2001**, pp.69-70.
18. P. Yukphan, T. Malimas, M. Takahashi, W. Potacharoen, T. Busabun, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen and Y. Yamada, "Re-identification of *Gluconobacter* strains based on the restriction analysis of 16S-23S rDNA internal transcribed spacer regions", *J. Gen. Appl. Microbiol.*, **2004**, 50, 189-195.
19. J. Brosius, T. J. Dull, D. D. Sleeter and H. F. Noller, "Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*", *J. Mol. Biol.*, **1981**, 148, 107-127.
20. N. Saitou and M. Nei, "The neighbor-joining method: A new method for reconstructing phylogenetic trees", *Mol. Bio. Evol.*, **1987**, 4, 406-425.
21. K. Tamura, J. Dudley, M. Nei and S. Kumar, "MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0", *Mol. Biol. Evol.*, **2007**, 24, 1596-1599.
22. J. D. Thomson, D. G. Higgins and T. J. Gibson, "CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice", *Nucleic Acids Res.*, **1994**, 22, 4673-4680.
23. M. Kimura, "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences", *J. Mol. Evol.*, **1980**, 16, 111-120.
24. J. Felsenstein, "Confidence limits on phylogenies: An approach using the bootstrap", *Evolution*, **1985**, 39, 783-791.
25. F. Dellaglio, I. Cleenwerck, G. E. Felis, K. Engelbeen, D. Janssens and M. Marzotto, "Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit", *Int. J. Syst. Evol. Microbiol.*, **2005**, 55, 2365-2370.
26. F. Gosselé, J. Swings, K. Kersters, P. Pauwels and J. De Ley, "Numerical analysis of phenotypic features and protein gel electrophoregrams of a wide variety of *Acetobacter* strains. Proposal for the improvement of the taxonomy of the genus *Acetobacter* Beijerinck 1898, 215<sup>AL</sup>", *Syst. Appl. Microbiol.*, **1983**, 4, 338-368.
27. R. R. Navarro, T. Uchimura and K. Komagata, "Taxonomic heterogeneity of strains comprising *Gluconacetobacter hansenii*", *J. Gen. Appl. Microbiol.*, **1999**, 45, 295-300.
28. H. Toyosaki, Y. Kojima, T. Tsuchida, K. I. Hoshino, Y. Yamada and F. Yoshinaga, "The characterization of an acetic acid bacterium useful for producing bacterial cellulose in agitation cultures: The proposal of *Acetobacter xylinum* subsp. *sucrofermentans* subsp. nov.", *J. Gen. Appl. Microbiol.*, **1995**, 41, 307-314.
29. P. Lisdiyanti, R. R. Navarro, T. Uchimura and K. Komagata, "Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov.", *Int. J. Syst. Evol. Microbiol.*, **2006**, 56, 2101-2111.
30. I. Cleenwerck, M. De Wachter, Á. González, L. De Vuyst and P. De Vos, "Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter*

- kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*", *Int. J. Syst. Evol. Microbiol.*, **2009**, *59*, 1771-1786.
31. D. Dutta and R. Gachhui, "Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea", *Int. J. Syst. Evol. Microbiol.*, **2007**, *57*, 353-357.
  32. A. Jagannath, A. Kalaiselvan, S. S. Manjunath, P. S. Raju and A. S. Bawa, "The effect of pH, sucrose and ammonium sulphate concentrations on the production of bacterial cellulose (Nata-de-coco) by *Acetobacter xylinum*", *World J. Microbiol. Biotechnol.*, **2008**, *24*, 2593-2599.
  33. S. Keshk and K. Sameshima, "The utilization of sugar cane molasses with/without the presence of lignosulfonate for the production of bacterial cellulose", *Appl. Microbiol. Biotechnol.*, **2006**, *72*, 291-296.
  34. V. T. Nguyen, B. Flanagan, M. J. Gidley and G. A. Dykes, "Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from Kombucha", *Curr. Microbiol.*, **2008**, *57*, 449-453.
  35. P. R. Chawla, I. B. Bajaj, S. A. Survase and R. S. Singhal, "Microbial cellulose: Fermentative production and applications", *Food. Technol. Biotechnol.*, **2009**, *47*, 107-124.
  36. S. Kongruang, "Bacterial cellulose production by *Acetobacter xylinum* strains from agricultural waste products", *Appl. Biochem. Biotechnol.*, **2008**, *148*, 245-256.
  37. F. Hong and K. Qiu, "An alternative carbon source from konjac powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter acetii* subsp. *xylinus* ATCC 23770", *Carbohydr. Polym.*, **2008**, *72*, 545-549.
  38. R. Jonas and L. F. Farah, "Production and application of microbial cellulose", *Polym. Degrad. Stabil.*, **1998**, *59*, 101-106.
  39. S. M. A. S. Keshk and K. Sameshima, "Evaluation of different carbon sources for bacterial cellulose production", *Afr. J. Biotechnol.*, **2005**, *4*, 478-482.
  40. S. Y. Kim, J. N. Kim, Y. J. Wee, D. H. Park and H. W. Ryu, "Production of bacterial cellulose by *Gluconacetobacter* sp. RKY5 isolated from persimmon vinegar", *Appl. Biochem. Biotechnol.*, **2006**, *131*, 705-715.
  41. E. Trovatti, L. S. Serafim, C. S. R. Freire, A. J. D. Silvestre and C. P. Neto, "*Gluconacetobacter sacchari*. An efficient bacterial cellulose cell-factory", *Carbohydr. Polym.*, **2011**, *86*, 1417-1420.
  42. P. Lisdiyanti, K. Katsura, W. Potacharoen, R. R. Navarro, Y. Yamada, T. Uchimura and K. Komagata, "Diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines", *Microbiol. Cult. Coll.*, **2003**, *19*, 91-99.