Research Article

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First report of biocellulose production by an indigenous yeast, Pichia kudriavzevii USM-YBP2

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Abstract: Herein, we describe the first report of Pichia strain producing biocellulose (BC). This yeast was isolated from rotten pineapple and was identified as Pichia kudriavzevii USM-YBP2 using 18S rDNA analysis. The formation of pellicle was characterized using a scanning electron microscope (SEM). Based on SEM analysis, pellicles are presented as an interwoven network of fibers. Next, to produce high BC, optimization was conducted using different carbon and nitrogen sources, carbon and nitrogen concentrations, inoculum size, and cultivation condition. The optimized parameters for maximum BC production were as follows: glucose 8.0% (w/v), peptone 2.0% (w/v), yeast extract 0.5% (w/v), disodium phosphate 0.270% (w/v), citric acid 0.115% (w/v), ethanol 0.5% (v/v), and inoculum size 10% (v/v). The production yield is 12.24 ± 0.43 g/L and the productivity of 0.128 g/L/h. Then, BC was further treated using sulfuric acid to destructuring to bionanocellulose (BNC) and was characterized using the transmission electron microscope (TEM), attenuated total reflection-Fourier transform infrared spectroscopy, and X-ray diffraction (XRD) analysis. After acid hydrolysis treatment, BNC showed a spherical shape as in TEM. Interestingly, BNC characteristic band displayed high similarity with cellulose from other bacterial species. Moreover, BNC showed high crystallinity index (~82%) as estimated using XRD. Thus, our findings substantiated that strain USM-YBP2 showed remarkable BNC production with unique BNC property that can be further explored in various applications.

Keywords: yeast producing cellulose, Pichia kudriavzevii, biocellulose production optimization, two-level factorial design, bionanocellulose

1 Introduction

As one of the oldest natural polymers in the world, cellulose has been isolated from variety of cellulosic materials such as cotton wood pulp, stalks, coconut husks, tunicates, and bacteria [1]. Cellulose is the earth’s most ancient and important natural polymer, and destructuring cellulose to nanoscale dimension has attracted more attention owing to the nanocellulose (NC) unique property as a novel and advanced nanomaterial evidenced by its growing use in biotechnology area [2]. NC, which is synthesized by plants, algae, some species of bacteria, and animals and also composed of nano-sized structure materials, is a promising, attractive, unique, and sustainable material made from native cellulose [3,4]. Furthermore, NC can be divided into three groups based on their production methods, processing conditions, and properties, namely micro-fibrillated cellulose, crystalline nanocellulose (CNC), and bacterial nanocellulose [5]. NC is beneficial in food packaging, biomedicine, mechanical reinforcement of matrices, and membrane filtration [6] since it is composed of nanofiber structure or nano-sized networks of fibers [7].

Generally, plant is a massive cellulose producer, and plant-based cellulose consists of three major components known as lignin, hemicellulose, and cellulose [8]. This systematic arrangement of the cellulosic fibers is known as the greatest strength for the plants [9]. Globally, each year, around 100–150 massive amount of cellulose is synthesized naturally over the world [10]. As a result, it is expected that this naturally synthesized cellulose will be sufficient to produce eco-friendly and more sustainable biorefinery products such as bioplastics and biomaterials such as NC, and bio-ethanol, which will be able to replace petrorefinery products such as plastics and...
petroleum fuels [11]. Although cellulose is produced mostly by plants, which is abundant, why microbial cellulose is still demanded? Often the question arises and the answer lies in the peculiarity of microbial cellulose. Wang et al. [12] have provided an account of the uniqueness of microbial cellulose, namely bacterial cellulose in their work. Its high purity, as opposed to plant cellulose, and its excellent properties such as its unique nanostructure, high crystallinity, high water-holding capacity, excellent mechanical strength, and high degree of polymerization make it a dynamic material available for diverse applications ranging from membrane material in batteries to wound dressing material in the medical field. Moreover, in most common way, the extraction of plant cellulose is attained through a harsh pretreatment of the cellulosic fibers, which involves environmentally hazardous chemical reagents and achieved a relatively low plant cellulose yield [13]. Alternatively, cellulose derived from bacteria, algae, or fungi could provide a feasible solution for cellulose production in more sustainable and greener approach in which plants create 80 tons of cellulose on one hectare of land in 7 years, whereas microbial cellulose is produced in 500,000 L in only 22 days [14]. Cellulose can be divided into two native forms: pure cellulose and complex cellulose. Moreover, most of the pure cellulose is synthesized naturally from cotton, algae, tunicates, and bacteria, whereas complex cellulose is discovered from the cell wall of higher vascular plants [15].

Considering microbial cellulose might have been a good substitute for plant-based cellulose in addressing the sustainable demand for biodegradable and eco-friendly products, the demand for microbial cellulose has been expanded considerably, recently. This has put the researchers in toes to increase the microbial cellulose production. However, low yield and high production costs are the key limitations affiliated with microbial cellulose production which then impeding its industrial production and thus making it available at a relatively high cost in the market [11,16]. As a result, it is imperative that substantial research be conducted toward identifying potent microbial strains with a greater potential to produce biocellulose (BC). The ability of microbes to synthesize and characterize BC has been well documented over the last few decades. Nonetheless, low yield and high cost of BC posed a barrier to its commercial use, as the characteristics and yield of cellulose produced by microorganisms are influenced by several factors such as medium composition, the cultivation process, microorganism strain, pH, inoculum density, temperature, and oxygen [12,17,18]. The material morphology and physical properties of the resulting material are mostly influenced by the culture medium composition, which in turn affects the range of potential applications [14].

The optimal medium design is vital not only for microorganism cell growth but also for stimulating cellulose production [19].

The selection of nutritional components has a profound influence on the BC. To increase yield, it is necessary to control parameters such as nitrogen concentration, pH, temperature, and inoculum size, regardless of the substrate employed in BC cultivation. This is explained by the fact that each microorganism has an optimal conditions for the growth, which subsequently affects its productivity [20]. According to Hutchens et al. [21], micronutrients also play a key role in BC production such as ethanol, potassium salt, magnesium salts, and disodium phosphate. This claim can also be supported by similar research done by Singhania et al. [16] who had reported that the use of additives such as ethanol, sodium alginate, glycerol, xanthan gum, and carboxymethyl cellulose can enhance the production of BC. The nutrient medium used is important for enhancing biosynthesized cellulose yield with the increase of cell. In general, there are two main cultivation methods for the production of BC namely static and agitation [22,23]. A simple static culture causes the BC gelatinous membrane to accumulate at the gas–liquid interface and follow the shape of the culture vessel [22,24]. Another method is an agitated culture, in which BC is synthesized in a fermenter or shake flask under agitated and aerated conditions and dispersed in the fermentation broth as pellets, irregular masses, or fibrous suspensions [23,25,26]. Moreover, changes in its allomorph crystallinity and mass fraction can be caused by different strains and culture conditions [27,28]. Despite the fact that both types of bioprocesses have advantages and drawbacks, screening for strains with desirable physiochemical properties and high BC yield remains a great challenge in this research area.

Since its discovery, BC has demonstrated enormous potential in a variety of fields and known as a sustainable, fascinating, and critical biopolymer. Bacterial cellulose, for example, is far better in physicochemical, mechanical, and structural properties compared to plant cellulose, in terms of a high degree of polymerization, light transparency, hydrophilicity, a high crystallinity index (CrI), biocompatibility, three-dimensional network of ultrafine and nanoscale fibers [29,30], chemical purity, and high mechanical strength [4,18]. As a result, due to its versatility and advanced biomaterial properties, bacterial cellulose is used in a wide range of commercial applications, including cosmetics, food products as well as textiles, biomedical applications, and other fields. The majority of BC production research has been done by employing bacteria as a cellulose producer [31]. Currently, Komagataebacter xylinus is the most prominent and frequently used producer of
bacterial cellulose since *K. xylinus* is the most efficient bacterial strain for bacterial cellulose production [32]. *Komagataeibacter*, a former *Glucanobacter/Acetobacter* sp., has been found in various acidic fruits, as well as their residue and juice, including grapes, plums, citrus fruits, and apples [33,34].

Other bacteria such as *Achromobacter, Agrobacterium, Athrobacter, Pseudomonas, Rhizobium*, and *Sarcinar* genera have also been reported to possess the ability of producing high-purity cellulose via environmentally friendly and sustainable approaches [25]. Although numerous bacterial strains are involved in BC synthesis, *Komagataeibacter* has been extensively studied and its mechanism for BC synthesis is also well documented. This is the reason that it has been employed as a model organism for BC production and has been exploited in the industrial production of BC by various industries. Furthermore, the number of published articles referring to bacterial cellulose production via aerobic fermentation using both synthetic and nonsynthetic medium from various bacterial species including *Acetobacter* and *Glucanoacebacter, Achromobacter, Agrobacterium, Athrobacter, Pseudomonas, Sarcinar*, and *Salmonella* is also increased significantly over the years [10,23]. As shown in Figure 1, the number of BC publications has increased tremendously since 2010 from 483 to 1,450 in 2021. However, till date, there is no similar study that has been encountered in the literature, in which microbial cellulose production by using yeast species. As a consequence, it would be a good strategy and is of great importance to explore the potential of yeast species in cellulose synthesis, which can then be further implemented for BC production.

As to acquire hyperproduction of any metabolite as well as obtaining desirable properties, a potent microorganism is the most vital necessity. Hence, the main purpose of this research is to isolate potential cellulose producing microorganism from rotten fruits. Most of the research on BC production is carried out with complex media or alternative media that comprises modified carbon and nitrogen components [35] as well as cultivation conditions. In an attempt to enhance BC yield, this study therefore incorporated nonconventional components where the production of BC was optimized using different carbon and nitrogen sources, various carbon and nitrogen concentrations, inoculum size, and different cultivation conditions. In addition to that, the characterization of pellicles was carried out via a scanning electron microscope (SEM). Meanwhile, the characterization of BNC was conducted morphologically using transmission electron microscope (TEM) and structurally using attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR) and X-ray diffraction (XRD)). Ultimately, this study proposed that the *Picha kudriavzevii* USM-YBP2

![Figure 1: Number of publications on bacterial cellulose since 2010–2021. Scopus search engine system, the search term “bacterial cellulose.”](image-url)
might showed a remarkable potential for green and sustainable production of BC that can cater for biotechnology exploration, biopolymer production, and biodiversity study, in future.

2 Materials and methods

2.1 Materials

-d-Glucose (HiMedia, India), peptone (HiMedia, India), yeast extract (HiMedia, India), agar (HiMedia, India), nutrient broth (NB) (HiMedia, India), sucrose (HiMedia, India), ethanol (HmbG, Germany), glycerol (ChemAR System, Malaysia), disodium phosphate (HmbG, Germany), citric acid (HmbG, Germany), casein hydrolysate (HmbG, Germany), ammonium sulfate (HmbG, Germany), sodium hydroxide (HmbG, Germany), and sulfuric acid (Qrec, New Zealand) were purchased from Al-Ilimah Enterprise Sdn. Bhd. These materials were used for the preparation of culture media and analysis assays.

2.2 Methods

2.2.1 Preparation of culture media

Hestrin–Schramm (HS) culture medium was used for cultivation, maintenance, and screening the strains producing cellulose [36]. HS broth was prepared by adding 5.0 g peptone, 5.0 g yeast extracts, 2.70 g disodium phosphate, and 1.15 g citric acid in 800 mL double distilled water. Then, pH value for this medium was adjusted to 5.0 ± 0.2 before autoclaved at 121°C for 15 min. About 20.0 g d-glucose was autoclaved separately from other medium components before mixed with HS medium. For the preparation of HS agar, 20.0 g of agar powder was added to the broth.

2.2.2 Preparation of rotten pineapple

In this study, rotten pineapple was selected for the isolation of microbial-producing cellulose. Pineapple sample was purchased from a night market near Sungai Dua, Penang, Malaysia and was stored in a sealed glass jar for 1 week at room temperature. Then, the flesh of rotten pineapple was cut into small pieces before transferred aseptically into the autoclaved HS broth and was agitated at 180 rpm, 30°C for 48 h.

2.2.3 Isolation and screening of strain for BC production

Serial dilution and the streak plate technique were used to isolate the cellulose producing microorganisms. A volume of 100 µL of 10⁶ dilution from each sample was spread on HS agar plate. The agar plate was then incubated invertedly at 30°C for 24–48 h. A loopful of a single colony was inoculated into 9 mL of HS broth and cultured for 7 days at 30°C in a static condition. Isolates that produced white pellicles on the medium surface were selected for further analysis. The pellicles were collected by centrifugation at 6,000 rpm/4,032 g for 10 min, after which they were treated with 1N NaOH at 80°C for 15 min and washed rigorously 3–4 times with distilled water [37].

2.2.4 18S rDNA sequencing of the isolate USM-YBP2

Based on characterization analysis, a dominate isolate that showed remarkable BC production was being selected and identified based on the morphological characteristics under light microscope. The identification was also supported by a molecular tool that sequenced the 18S rDNA gene nucleotide. For DNA isolation, the yeast cells from an overnight HS culture were used. Molecular identification of the selected isolate was carried out at the Centre of Chemical Biology, Universiti Sains Malaysia, Penang, Malaysia. Using the Basic Local Alignment Search Tool (BLASTn), the nucleotide sequence was compared to those on the National Center for Biotechnology Information GenBank Database (BLAST). The identified strain was then stored at −20°C in a 40% (v/v) glycerol stock.

2.2.5 Inoculum preparation

Prior to inoculating into the production medium, the seed culture was prepared in NB. In a 50 mL NB, the glycerol stock containing seed culture was activated and incubated for 24 h at 30°C on a rotary shaker with 200 rpm agitation. A 5% (v/v) cell suspension of a 24 h culture (optical density 600 (OD₆₀₀) = 1) was used as the inoculum.

2.2.6 Production of BC using different carbon and nitrogen sources

The effects of different nutritional parameters, such as carbon and nitrogen sources, on the metabolic processes of USM-YBP2 cells in HS broth medium were investigated in order to enhance BC production. To evaluate the best
carbon source, HS broth medium was prepared by manipulating different types of carbon sources such as glucose, glycerol, and sucrose. Peptone, casein hydrolysate, and ammonium sulfate were used as nitrogen sources to enhance BC yield. For the following steps of the experiment, the carbon and nitrogen source that promoted the most BC production was used.

### 2.2.7 Production of BC under different cultivation conditions

The effects of agitation intensity on BC production were determined by changing agitation speeds, namely at 0 rpm (static), 100 rpm, and 200 rpm for 4 days at 30°C. For each agitation rate, the wall speed was calculated as follows (Eq. 1):

\[
\text{Wall speed} = \pi ND
\]

where \(\pi\) is 3.142, \(N\) represents the agitation rate (rps), and \(D\) is the diameter of the shake flask = 0.058 m.

### 2.2.8 Optimization of BC production using two-level factorial design approach

Using Design Expert 10.0.0 software, a two-level factorial design was used to screen and evaluate the significant factors of the three factors, namely nitrogen concentration, carbon concentration, and inoculum concentration, using a set of eight runs with one central point. Each factor was evaluated on two levels namely high level (+1) and low level (−1). Nitrogen concentration, carbon concentration, and inoculum concentration are indicated as independent variables, whereas BC yield is indicated as dependent variable (response). The variable values studied were determined through a literature search [21,22]. Each flask containing the modified HS broth medium was inoculated with the USM-YBP2 culture, and all flasks were incubated for 4 days in an orbital shaker under agitated conditions at 200 rpm at 30°C. Table 1 shows the two-level factorial experimental design for BC production.

### Table 1: Experimental design for BC production

| Independence variables | Coded symbol | Range and level |
|------------------------|-------------|-----------------|
| Glucose concentration  | A           | 2% (w/v) - 8% (w/v) |
| Peptone concentration  | B           | 0.5% - 2% (w/v) |
| Inoculum size          | C           | 5% - 10% (v/v)  |

### 2.2.9 Harvesting and purification of the produced BC

The pellicle of the culture medium was extracted by centrifugation for 20 min at 6,000 rpm/4,032g. To remove the excess media stick on the pellicles, distilled water was used to rinse them. The pellicles were then soaked in a 1 M NaOH solution for 15 min at 60°C to deactivate the USM-YBP2 cells that attached to the pellicles. The tube was cooled down to room temperature. The purified cellulolic pellicles were then freeze-dried for overnight. Following that, the dry weight of each pellicle or BCs obtained from both one factor at one time (OFAT) and two-level factorial experiments was weighed to determine production yield (expressed in g of dry BC/L of culture media) [19].

### 2.2.10 Determination of BC yield

The yield of BC formed was calculated as weight of BC (dry) produced divided by a volume of media used as shown in Eq. 2 below:

\[
\text{Yield of BC (g/L)} = \frac{\text{Weight of BC (g)}}{\text{Volume of media (mL)}} \times 1,000 \text{mL/L}
\]

Meanwhile, the productivity was determined by yield of BC divided by fermentation time period (h) according to Eq. 3:

\[
\text{Productivity (g/L/h)} = \frac{\text{BC yield (g/L)}}{\text{Time (h)}}
\]

### 2.2.11 Treatment of BC using acid hydrolysis technique

Strong acid hydrolysis was used to further treat the produced BC. The BC was hydrolyzed using sulfuric acid at concentration of 64% with cellulose-to-acid ratio of 1:25. The hydrolysis process was then stopped by adding 10-folds (250 mL) of excess distilled water to the reaction mixture. The colloidal suspension was generated and centrifuged for 30 min at 6,000 rpm/4,032g. To neutralize and eliminate the sulfate ions, the colloidal suspension was dialyzed for 5 days in a dialysis membrane submerged in distilled water. The samples were then homogenized, sonicated, and freeze-dried. The freeze-dried
2.2.12 Characterization of BC and BNC

2.2.12.1 SEM analysis

The white pellicle or BC was characterized using SEM at Electron Microscopy Unit, School of Biological Sciences, Universiti Sains Malaysia. The BC sample was prepared by gently fixed on an aluminum stab with two-side adhesive tape and coated with 5–10 nm thick layer of gold. The surface morphology was then examined under FE-SEM (FEI Verios 460 L) operating at 10 kV. The micrographs were taken at several magnifications.

2.2.12.2 TEM analysis

The BC and BNC samples were analyzed using TEM at Electron Microscopy Unit, School of Biological Sciences, Universiti Sains Malaysia, for the observation of BNC morphology. A drop of the diluted aqueous suspension of the treated BNC was allowed to dry on the copper-coated grid (400 mesh) and was observed at 200 kV with FEI TECNAI G² 20 S-twin TEM.

2.2.12.3 ATR-FTIR analysis

To determine the characteristic profile of BNC, the sample was analyzed using ATR-FTIR. The sample was sent to the Division of Instruments and MUPA, School of Chemical Sciences, Universiti Sains Malaysia. This analysis was performed using PerkinElmer FT-NIR spectrometer at the wavelength ranging from 400 to 4,000 with 4 cm⁻¹ resolution [2].

2.2.12.4 XRD analysis

The degree of polymerization of the crystalline region of cellulose was determined using XRD-Bruker AXS D8 Advance equipment operating at Ni-filtered Cu Kα1 radiation on the treated BNC sample. The BNC sample was scanned at intervals of 10°–70° 2θ with a step size of 0.05° at a scan time of 2 s per step. The CrI of the BNC sample was then calculated using the Segal empirical equation [39] as shown in Eq. 4 by referring to the peak intensity of diffraction angles derived from crystalline and amorphous regions.

\[
\text{CrI} \% = \left( \frac{I(200) - I_{\text{am}}}{I(200)} \right) \times 100 \tag{4}
\]

where \(I(200)\) is the intensity of the peak at \(2\theta = 22°\) and \(I_{\text{am}}\) is the background height between the peaks \(2\theta = 22°\) and \(2\theta = 16°\).

2.2.13 Statistical analysis

All experiments were performed in independent triplicates. Mean and standard deviations of the three experiments were calculated. Design-Expert® (Version 10.0.0) software was used for the experimental design and analysis of the experimental data to identify the significant factors and their corresponding coefficients. Sum of squares, \(F\)-value, and \(p\)-value were evaluated to analyze the response value of each experiment.

3 Results and discussion

3.1 Isolation, screening, and identification of cellulose producing microorganisms

The colonies formed on HS agar were randomly selected and nourished in HS broth medium. After 5 days of cultivation, the formation of pellicles at the air–liquid interface of the broth culture was observed, as shown in Figure 2a and b. Microorganisms predominantly produce cellulose by consuming dissolved oxygen during microbial cellulose production, according to Campano et al. [31], and the cellulose development is observed with the turbidity in the culture media. In addition to that, when the amount of oxygen decreases, bacteria begin to congregate at the surface and produce BC in layers [40]. The ability of microorganism to produce cellulose was primarily determined by the formation of pellicles on the surface of the HS medium, grown in static condition. This physical characteristic is an early indicator that the isolates produce cellulose [14]. HS medium is commonly used to screen and evaluate microorganism that can produce cellulose [19,41]. This medium contains only glucose as the sole carbon source and probably can promote microorganism growth and stimulate cellulose biosynthesis.

The characteristics of the formed pellicles are presented as in Table 2. The pellicle produced by isolate USM-YBP1 was observed as a floating network of cell and showed weak strength, as the pellicle was easily dissolved during the extraction process. Meanwhile, the
pellicle produced by isolate USM-YBP2 showed better rigidity and easily been extracted from the culture medium. Due to this reason, isolate USM-YBP2 was selected for further study.

As shown in Figure 3, the morphology of USM-YBP2 is in ovoid to elongate shapes. Molecular analysis was conducted to verify the isolate. By using 18S rDNA gene marker, the isolate showed 100% homology to *P. kudriavzevii* species with the accession number of MH545928.1. Therefore, it is named and coded as *P. kudriavzevii* USM-YBP2. *P. kudriavzevii*, a teleomorph of *Candida krusei*, is a yeast that is widely distributed in natural environment [42]. It is commonly found in spontaneous fermentation and involve in variety of traditional fermented food [43]. Hence, it is advantageous for cultivation in large-scale manufacturing due to its nonpathogenic characteristic. In fact, it has been used in food products for centuries, such as fermented milk in Sudan and Tibet, maize beverages in Colombia, and fermented cassava and cacao in Africa [44]. Moreover, according to the Food and Drug Administration, it is “generally recognized as safe” [42,44]. In addition, *P. kudriavzevii* is known as a remarkably stress-tolerant organism, and it plays a significant role in biotechnology area, specifically in the production of succinic acid [45], bioethanol [46,47], and glycerol [48].

### 3.2 Optimization of BC production by strain USM-YBP2

The culture media must be optimized to promote the production of BC. It is usually being influenced by the nutrients required by the microorganisms, culture type, additives, pH, and temperature [16,19]. Different compositions, morphologies,
3.2.1 Production of BC via OFAT approach

3.2.1.1 Production medium

Carbon, nitrogen, and other micro and macronutrients are required for microorganism growth in culture media, and the proportion of these components affects product formation directly or indirectly [50]. Until date, the most widely used culture medium for BC production has indeed been Hestrin and Schramm, also known as H–S medium or standard medium, which is composed of α-glucose, peptone, yeast extract, sodium phosphate dibasic, and citric acid [36]. Following this composition, some modifications have been studied that vary the carbon source, namely mannitol, glycerol, sucrose, and fructose. Ramana et al. [51] assessed the impact of a wide range of complex and defined carbon and nitrogen sources. The results revealed that glucose and sucrose were the best carbon sources for cellulose formation, with peptone, casein hydrolysate, and ammonium sulfate as nitrogen sources [51].

With the current advancement of microbial biotechnology, more attention is being placed on the expanded use of renewable resources, which is in congruence with the general concept of a circular economy and implies that waste products from one industry should serve as raw materials for another [52,53]. Numerous by-products, waste of agricultural and residues as well as biofuel or food sectors, are proposed and used for this purpose [50], which, in addition to substantial economic benefits, also create a very favorable environmental impact. Among them, glycerol, the simplest 3-C polyol, is already of substantial value and is extremely promising in this regard as a sufficiently widely available and relatively affordable compound. This is mainly because of the biodiesel production, as well as other products such as fatty ester industries, fatty acid, and soap, whose technologies involve the triglyceride transesterification reaction, that can produce large amounts of glycerol as a by-product [54]. As a result, glucose, sucrose, and glycerol were chosen as to determine the best carbon source for BC production by this strain. Furthermore, peptone, casein hydrolysate, and ammonium sulfate were selected as well to investigate the best nitrogen sources for BC production.

Based on the data presented in Figure 4, the highest BC yield (9.60 ± 0.58 g/L) was attained when glucose was employed as main carbon source, followed by glycerol (8.22 ± 0.46 g/L) and sucrose (4.39 ± 0.13 g/L). Albeit the percentage of carbon in glucose (40%) is slightly lower than sucrose (42.11%), it still can produce high BC yield. Glucose is a simple sugar monomer that can be transported directly and easily during the cellulose production process without any molecular breakdown [22]. Indeed, different from other carbon sources, glucose precursors were capable of forming cellulose polymers with a well-defined structure and it was also demonstrated that the amount of glucose consumed by the organisms during their growth was equivalent to the amount of BC synthesized as the by-product [41]. In a similar study carried out by Costa et al. [55], Komagataebacter hansenii successfully produced the highest BC yield using glucose with 9.60 g/L. It shows a similar result with this study as the maximum BC yield obtained was 9.60 g/L when glucose was used as main carbon source. Another study conducted by Chen et al. [56] had discovered that when they used conventional medium that includes glucose in their study, resulted in BC productivity of 0.43 g/L/day by Komagataebacter DHU-ATCC-1. This study has shown a higher BC yield percentage of 5.6

![Figure 4: BC yield (g/L) produced by P. kudriavzevii USM-YBP2 supplemented with various carbon sources; glucose, sucrose, and glycerol at 30°C under static culture.](image)
than that found by Chen et al. [56] in their BC production study as the productivity of BC yield by this strain; *P. kudriavzevii* USM-YBP3 was 2.4 g/L/day.

However, a study conducted by Kuo et al. [57] had reported that the major drawback of using glucose as a sole carbon source is that it is usually associated with the formation of gluconic acid, which then resulting in a decrease in the pH of the medium, subsequently preventing further fermentation. Anyhow, as the microorganism used to synthesize BC in this study is yeast, glucose can also be utilized by this strain to produce high ethanol concentration. As a result, the pH stayed at 4.5, resulting in prolonged fermentation of BC production and, as a consequence, increased pH which then can facilitate to stop the fermentation. Moreover, referring to Figure 4, sucrose was found to be the least preferred carbon source, with the lowest BC yield of 4.39 g/L in this study. This is very probable that sucrose needs to be hydrolyzed to glucose and fructose before can easily be consumed. Similarly, study by Mikkelsen et al. [62] reported that medium containing sucrose produced the lowest bacterial cellulose (3.83 g/L) since *G. xylinus* strain unable to utilize sucrose directly as it needs to be converted to glucose first before bacterial cellulose production can be initiated. Hence, it can be concluded that the production of BC may differ from strain to strain in order to utilize particular carbon source and follow a particular metabolic pathway.

Nitrogen source is also important for cellulose-producing microorganisms since it can provide not only amino acids but also vitamins and mineral salts essentially for the microorganisms. Three different types of nitrogen sources were selected, namely peptone, casein hydrolysate, and ammonium sulfate. Based on the results, shown in Figure 5, the highest BC yield (9.15 ± 0.43 g/L) was attained when peptone was used as main nitrogen source, followed by casein hydrolysate (7.63 ± 0.40 g/L) and ammonium sulfate (5.91 ± 0.10 g/L). For the production of BC, peptone had been identified as an essential nitrogen source because it contains plentiful nitrogen compounds [63]. This finding also in agreement with a study by Singhania et al. [16]. They found that *Glucanacetobacter* sp. produced high cellulose (2.15 g/L) when peptone was used in the medium. Similarly, Santos et al. [64] observed that about 3.906 g/L of BC yield was attained by *Komzatothacter intermedius* (BCRC 910677) when 38 g/L peptone used as supplement with medium.

Another study by Santos et al. [65] reported that mixed peptone and yeast extract produced the highest amount of bacterial cellulose which was around 3.0 g/L. They also highlighted the importance of yeast extract combined with other nitrogen sources. In fact, peptone and yeast extract are the most preferred nitrogen sources in the model medium developed by Hestrin and Schramm [36]. This is due to the fact that peptone is a product of protein enzymatic digestion that contains small peptides that can be utilized by microorganism [50]. As for yeast extract, it is commonly employed as a medium component for a variety of microorganisms due to its high concentration of amino acids, peptides, carbohydrates, trace elements, growth factors, and water-soluble vitamins [50]. As a result, it is possible to deduce that the

![Figure 5: BC yield (g/L) produced by *P. kudriavzevii* USM-YBP2 supplemented with different type nitrogen sources; peptone, casein hydrolysate, and ammonium sulfate at 30°C under static culture.](Image)
combination of peptone and yeast extract is important for BC production. However, with the usage of peptone as sole nitrogen source also can contribute to the reduction of other essential nutrients in the medium by the production of toxic substrates which can destructed the cells followed by deterioration of the end-products [66].

As indicated in Figure 5, the lowest BC yield (5.91 g/L) by *P. kudriavzevii* USM-YBP2 was obtained when ammonium sulfate was used as a sole nitrogen source. However, in contradict to the finding by Khan et al. [4] where they reported by employing ammonium sulfate in the culture medium gave the highest BC yield of 7.2 g/L by *Lactobacillus hilgardii* IISTRKH159. Based on this study and the previous research, it is quite difficult to precisely determine the BC yield between different strains since different parameters were employed in the experimental design. However, if conventional HS media is employed in the production process, it has been demonstrated that our strain produces the best yield of any of these strains.

### 3.2.1.2 Culture conditions

There are two cultivation techniques for BC production at the lab scale that provides cellulose with distinct properties and microstructure namely static and agitated/stirred conditions. Among other factors, carbon source concentration, surface area, and air supply have a significant impact on BC synthesis in a static state. A major drawback of aeration under static conditions is the difficulties of supplying air without disturbing the liquid. Despite the fact that the BC produced is considered superior to that produced in an agitated condition, the industrial production is recommended via agitated fermentation, which produces irregularly shaped pellets and masses known as sphere-like cellulose particle as it also can control the oxygen transfer and the cultivation time is usually shorter than in static cultivation [67]. Thus, different cultivation conditions, which are static and agitated cultivation, also were determined throughout this study as there are no report regarding the best cultivation condition for yeast, particularly for this species.

As depicted in Figure 6, the synthesis of BC under agitated culture at 200 rpm produced higher BC yield compared to the production in the static culture. Under agitated culture, about 6.38 g/L of BC yield was attained at 200 rpm (wall speed, 0.911 m/s) and about 4.25 g/L of BC yield was attained at 100 rpm (wall speed, 0.456 m/s). The yield of BC produced from *P. kudriavzevii* USM-YBP2 under static culture was 5.26 ± 0.02 g/L. In general, yield of BC is higher under agitated condition with agitation speed of 200 rpm as compared to static condition. This result was consistent with the previous reports by Toyosaki et al. [68] and Son et al. [69] which showed that the yield of BC production was higher under agitated culture condition. In contrary to study by Lu et al. [70], they found that *Komagataeibacter* sp. nov. CGMCC 17276 successfully produced the highest BC yield of 8.29 g/L under static condition. Another study demonstrated by Jahan et al. [71] also had reported that *Glucnaacetobacter* sp. F6 strain was capable of producing maximum BC yield (0.91 g/L) under static condition compared to the agitated condition with different speed of 50, 100, and 200 rpm. They had observed the production of BC declined as the shaking speed elevated. The reduction amount of BC yield from *Glucnaacetobacter* sp F6 occurred mainly due to the presence of cells with negative mutants (conversion of cellulose-producing strain to noncellulose-producing strain) followed by the mutation process in the bacterial gene that codes for cellulose synthase [71].

In agitated cultivation, oxygen is continuously mixed in the medium and sufficiently supplied to the yeast cells, resulting better production of BC. Moreover, during agitated cultivation, microorganism will be exposed to the equal conditions both in terms of nutrients and population distribution that leads to the even nutrient transfer and population distribution. As a result, the agitated cultivation system is more favorable and can be applied in wider range to produce large amount of BC. Fascinatingly, the agitated fermentation process can result in several forms of cellulose, depending on the rotational speed applied, from fibrous suspension to spheres and pellets, to different shapes and sizes [23,72]. Still, it can increase the probability of mutation from cellulose-producing cells to cellulose-negative mutants when exposed to high turbulence and shear stress [73,74].
3.2.2 Optimization of BC production using two-level factorial design experiment

Fermentation experiment was statistically designed based on several factors to obtain an optimum condition for BC production. Based on the OFAT approach, glucose (carbon source), peptone (nitrogen source), and agitation condition (200 rpm) were selected. These three main variables, glucose concentration (A), peptone concentration (B), and inoculum size (C), were tested at 200 rpm. The highest yield of BC is 12.24 ± 0.43 g/L with productivity 0.128 g/L/h as shown in Figure 7. This condition was attained from set 8 presented in Table 3.

The half-normal plot graph in Figure A1 (Appendix) illustrated the significant effects of the studied factors. It is clearly showed that (A) and (B) factors fall well-off from the straight line, which indicate statistically significant effects and suggest their importance in BC production. While (C) factor falls closer to the straight line, it is a negligible factor. Further determination using Pareto chart (Figure A2) revealed that only (B) factor falls above the t-limit. Due to this reason, the tested model is not statistically significant when tested using analysis of variance (ANOVA; p > 0.05) (Table A1 in Appendix).

In Table 3, peptone concentration showed a positive influence on BC yield. The result showed that increased production of peptone from 0.5% to 2% (w/v) significantly increased the yield of BC from 5.46 ± 0.26 to 12.24 ± 0.43 g/L. It signified the importance of high amount of nitrogen to support biomass and BC production. No doubt, carbon also showed an important role in the production of BC. A similar trend can be observed for glucose where BC yield increases as the initial glucose concentration increases. Study by Rani and Appaiah [75] described that G. hansenii UAC09 produced high amount of bacterial nanocellulose (7.40 g/L) when the glucose concentration was elevated up to 40 g/L. Nonetheless, they also highlighted that NC production was drastically

![Figure 7: The BC yield (g/L) produced by P. kudriavzevii USM-YBP2 with different carbon concentration, nitrogen concentration, and inoculum size at 30°C, agitated at 200 rpm.](image)

Table 3: The effect of different glucose and peptone concentrations, as well as different inoculum sizes, on the yield of BC produced by P. kudriavzevii USM-YBP2 using a two-level factorial experimental design

| Run | A: Glucose concentration (% w/v) | B: Peptone concentration (% w/v) | C: Inoculum size (% v/v) | BC yield (g/L) | Productivity (g/L/h) |
|-----|---------------------------------|---------------------------------|-------------------------|----------------|---------------------|
| 1   | 2                               | 0.5                             | 5                       | 6.64 ± 0.08    | 0.069               |
| 2   | 8                               | 0.5                             | 5                       | 8.41 ± 0.14    | 0.088               |
| 3   | 2                               | 2                               | 5                       | 9.93 ± 0.32    | 0.103               |
| 4   | 8                               | 2                               | 5                       | 10.60 ± 0.09   | 0.110               |
| 5   | 2                               | 0.5                             | 10                      | 5.46 ± 0.26    | 0.057               |
| 6   | 8                               | 0.5                             | 10                      | 6.48 ± 0.22    | 0.068               |
| 7   | 2                               | 2                               | 10                      | 7.63 ± 0.12    | 0.079               |
| 8   | 8                               | 2                               | 10                      | 12.24 ± 0.43   | 0.128               |
| 9   | 5                               | 1.25                            | 7.5                     | 11.75 ± 0.35   | 0.122               |
reduced when the glucose concentration exceeding 40 g/L. This is likely due to the accumulation of gluconic acid that directly affects the pH medium and causes bacterial growth inhibition. It is agreed that correlation between carbon and nitrogen substrate induced better metabolic activity. Furthermore, glucose and peptone are most commonly used carbon and nitrogen sources in many research studies due to their importance for rapid cell build-up and metabolite biosynthesis. So, an appropriate concentration and carbon-to-nitrogen ratio are required for organism survival [63].

No significant effect can be found for inoculum size factor since both highest and lowest yield of BC were observed when 10% (v/v) inoculum size was used (sets 8 and 5). Another study by Hutchens et al. [21] also reported similar finding, where the inoculum ratio of G. hansenii was proven to be an insignificant factor for cellulose optimization under fractional factorial design analysis. Therefore, it is suggested that once the cell division reached at the maximum point, competition for the limiting nutrients in the medium occurred to maintain their survival. The insufficient amount of nutrients could lead to cell reduction, which significantly impacts the cellulose production. Table 4 displays the typical optimal media required for different strains. According to Table 4, the most of strains required 7–16 days of cultivation under static culture conditions to achieve the highest BC yield. However, our newly isolated strain produced a spectacular outcome attributed to the reason that our strain required only a shorter cultivation period under agitated culture conditions to successfully produce high BC yield. As a result, it is a good strategy to further incorporate this strain for commercial application since it can produce BC with high yield within short period of time (4 days). Additionally, as indicated in Table 4, to date, there is no report is available for cellulose production by this species. This appears to be the first report of P. kudriavzevii sp. cellulose synthesis. Thus, our study proposed that P. kudriavzevii USM-YBP2 can produce BC and might exhibit an appealing alternative for BC production since it can be safely employed on a large-scale production.

3.3 Characterization

3.3.1 Morphological characteristics

SEM analysis provides the image of surface morphology of extracted membranous pellicles formed by P. kudriavzevii USM-YBP2. As shown in Figure 8, the structural morphology was observed as an interwoven network of fibers. Also, the coherent interwoven network of fibers was clearly observed within the pellicle produced by USM-YBP2. This analysis speculated the formation of BC, and further characterisation was conducted by destructuring BC to nanometer size forming BNC.

TEM analysis presented a unique spherical shape of BNC (Figure 9). As expected, BC was destructured into nanometre size after acid hydrolysis treatment. BNC was also observed to be closely attached to each other and formed several clusters of nanosphere. This analysis was conducted using freeze-dried sample where electrostatic repulsion is minimized, which might contribute to loss of colloidal stability. Therefore, aggregated with irregular spherical size of BNC was observed. Generally, CNCs with rod-like or needle-like shapes were extracted from highly crystalline cellulose sources such as cotton, wood, tunicates, and bacterial strains with highly ordered crystalline domains [84]. Uniquely, formation of spherical CNC (SCNC) is commonly observed from fruit source. Study by Trilokesh and Kiran [85] reported a novel and unique SCNCs extracted from dried jackfruit peel, which also treated using acid hydrolysis technique. CNCs extracted from grape skin and sugarcane bagasse also appeared as spherical nanoparticles [19,86]. Some study described that the formation of SCNCs might occurred during the collection and sample preparation through the accumulation with other substances and further suggesting self-assembly activity of nanocrystal with other fragments [38,84]. The characterization of the cellulose structure and crystalline nature of spherical shape BNC produced from P. kudriavzevii USM-YBP2 strain was further determined using FTIR and XRD analysis.

3.3.2 Structural analysis

In Figure 10, FTIR spectrum of BNC produced from P. kudriavzevii USM-YBP2 is presented. The infrared spectrum displayed an intense absorption peak at the wave-number 3275.32 cm⁻¹ which attributed to stretching valence vibration of intra and inter free hydroxyl (O–H) which commonly found in pure NC [87]. At around 2923.61 cm⁻¹, characteristic bond was corresponded to the C–H stretching vibrations of CH₂ symmetry and asymmetry stretching or CH₃ and CH₂ groups which commonly found in cellulose fiber components, confirming the properties of amorphous cellulose [87]. These two important peaks indicate the formation of pure and crystalline characteristics of NC [88]. Similarly, study reported by Auta et al. [89] showed strong absorption peaks at 3,000–3,700 cm⁻¹ and 2,800–2,970 cm⁻¹ which were identified as the O–H stretching vibration and
Table 4: Optimal growth medium of other strains

| Genus and species | Strain | Strain origin | Culture media | Carbon source | Culture condition | Cultivation period (days) | pH | Temperature (°C) | Max. yield (g/L) | References |
|-------------------|--------|---------------|----------------|---------------|-------------------|--------------------------|----|-----------------|----------------|------------|
| P. kudriavzevi    | USM-YBP2 Rotten pineapple | HS medium | Glucose | Agitated | 4 | 5 | 30 | 12.24 | This study |
| K. intermedius    | MO     | Rotten guava  | Seed medium | Cheese whey | Static  | 7 | 7 | 30 | 15.8 | [76] |
| K. xylinus        | SU12   | Kombucha tea | HS medium | Mannitol | Static | 14 | 7 | 30 | 15.82 | [79] |
| Gluconacetobacter intermedius | MO     | Millet vinegar biofilm | Pear residue | Glucose | Static | 7 | 7 | 30 | 10.94 | [80] |
| Komagataeibacter sp. | M12, 6–5 | Rotten apple | HS medium | Mango extract | Static | 16 | 6 | 28–30 | 11.44 | [81] |
| Malus domestica   | IITRDKH20 | Rotten apple | HS medium | Fruit peel wastes (banana, pineapple, sweet lime, and orange) | Static | 7 | 6 | 30 | 2.4 | [82] |
| K. xylinus        | SB3.1  | Gelatinous membrane of mixed of various fruits (apple, guava, pineapple) | HS medium | Mannitol | Static | 10 | 6.5 | 30 | 8.28 | [83] |
| K. rhaeticus      | TJPU03 | Rotten orange peel | HS medium | Glucose | Static | 14 | 6 | 30 | 8.85 | [70] |
| Komagataeibacter sp. nov. | CGMC 17276 | Rotten green jujube | HS medium | Glucose | Agitated | 7 | 6 | 30 | 3.22 | [70] |
C–H stretching, respectively. Concomitantly, the FTIR analysis result in Surma–Ślusarska et al. [90] was also observed in the similar region, around 3,400 cm$^{-1}$ for bacterial cellulose produced by Acetobacter xylinum.

For ester and acetyl linkages in hemicellulose and lignin, the band around 1,735 cm$^{-1}$ attributed to C–O stretching vibration [87]. Another strong peak at the wavenumber of 1,638.32 cm$^{-1}$ was determined as H–O–H bending, and the peak around 1,450 cm$^{-1}$ was denoted as CH$_2$ scissoring of the BNC. Likewise, pursuant to the study of Wang et al. [20] had also found that the peak around 1,650 cm$^{-1}$ corresponded to the H–O–H bending of absorbed water while the peak around 1,450 cm$^{-1}$ corresponded to the CH$_2$ scissoring from the cellulose compound produced by Komagataeibacter sp. strain W1. Moreover, the BNC sample revealed strong bands at 1399.91, 1307.10, and 1127.60 cm$^{-1}$ which were attributed to C–H bending, CH$_2$ wagging, and O–H in plane, respectively. Similarly, Gunduz et al. [91] reported that FTIR analysis from the bacterial nanocellulose produced by A. xylinum also generated band at around 1,375, 1,315, and 1,125 cm$^{-1}$ which determined the presence of crystalline domain within the cellulose structure.

Ultimately, the absorption band at 1047.21 cm$^{-1}$ might correspond to 1,4-β-D-glycosidic bond which is one of the main characteristic bond in cellulose structure. For NC produced by A. xylinum, FTIR band at around 1,045 cm$^{-1}$ represents C–O–C stretching vibration and strongly indicates the formation of sugar ring [91]. While for G. xylinus, the β-(1,4)-glycosidic linkages were observed at around 1015.65 cm$^{-1}$ [89]. Overall, this study proposed high structural similarity of BNC with other bacterial producing cellulose as discussed previously.

The crystalline and amorphous regions of BNC produced by P. kudriavzevii USM-YBP2 were determined using XRD analysis. As shown in Figure 11, the XRD profiles revealed four major peaks at 2θ position. Diffraction angles at 15.5°, 19.4°, and 22.3° represent highly ordered crystalline regions, while diffraction angle at 18.3° represents the amorphous region matched with other reported cellulose structure [41,84,91]. This finding confirmed the BNC crystalline nature. Interestingly, the CrI of BNC in this study is considerably high with the calculated value of 82.1% compared to A. xylinum (71%), grape

![Figure 8](image-url) **Figure 8:** SEM image of membranous pellicle produced by USM-YBP2 showing an interwoven network of fibers under 500× magnification.

![Figure 9](image-url) **Figure 9:** TEM images of unique spherical shape of BNC after acid hydrolysis treatment: (a) 40,000× magnification and (b) 20,000× magnification.
Figure 10: FTIR spectrum of extracted BNC. The highlighted peaks are the main functional groups found in the cellulose structure produced by \textit{P. kudriavzevii} USM-YBP2.

Figure 11: XRD analysis of extracted BNC produced by \textit{P. kudriavzevii} USM-YBP2. The highlighted areas are the peak intensity with diffraction angles of crystalline (15.5°, 19.4°, and 22.3°) and amorphous (18.3°) regions.
skin (54.9%), wood (73.5%), and cotton (65%) [84]. Moreover, the crystallinity of BNC reflected its properties. For instance, if crystallinity is higher than tensile strength, its dimensional stability and density are also expected to be high [92]. High in purity and crystalline nature had made BNC in a great demand in various technology applications such as utilization in biomedical devices, food and cosmetic industries, paper reinforcement, and biopackaging, where high purity and strength of the material are required.

4 Conclusion

The isolated microorganism from rotten pineapple that can produce pellicles was identified as P. kudriavzevii USM-YBP2 using 18S rDNA molecular analysis. From SEM analysis, the pellicle is presented as an interwoven network of fibers. The optimization of BC production was conducted using two-level factorial design, and the optimum medium composition was glucose 8.0% (w/v), peptone 2.0% (w/v), yeast extract 0.5% (w/v), disodium phosphate 0.270% (w/v), citric acid 0.15% (w/v), ethanol 0.5% (v/v), and inoculum size 10% (v/v). After acid hydrolysis treatment, BNC showed a spherical shape as illustrated in TEM analysis. Interestingly, BNC characteristic band displayed high similarity with cellulose from other bacterial species. Moreover, the BNC showed high CrI (~82%) as estimated using XRD. Therefore, formation of highly crystalline spherical BNC produced from P. kudriavzevii USM-YBP2 potentially applied in wide and diverse area. To the best of our knowledge, this is the first report of yeast species shows the capability of producing BC and presents a remarkable property when destructuring to nanometer size.

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Appendix

Table A1: ANOVA table of the effect of different concentrations of glucose and peptone and different inoculum sizes on BC yield (g/L) by *P. kudriavzevii* USM-YBP2

| Source                | Sum of squares | Df | Mean square | F-value | p-value | Remarks     |
|-----------------------|----------------|----|-------------|---------|---------|-------------|
| Model                 | 32.55          | 3  | 10.85       | 4.89    | 0.0798  | Not significant |
| A – glucose concentration | 10.90     | 1  | 10.90       | 4.91    | 0.0910  | Not significant |
| B – peptone concentration | 18.47     | 1  | 18.47       | 8.32    | 0.0448  | Not significant |
| C – inoculum size     | 3.18           | 1  | 3.18        | 1.43    | 0.2978  | Not significant |
| Curvature             | 11.01          | 1  | 11.01       | 4.96    | 0.0900  | Not significant |
| Residual              | 8.88           | 4  | 2.22        |         |         |             |
| Cor total             | 52.44          | 8  |             |         |         |             |

Figure A1: Half normal plot analysis of the effect of different glucose and peptone concentrations, as well as different inoculum sizes, on BC yield (g/L) produced by *P. kudriavzevii* USM-YBP2 at 30°C, agitated at 200 rpm.

Figure A2: Pareto chart analysis of the effect of different concentrations of glucose and peptone and different inoculum sizes, on BC yield (g/L) by *P. kudriavzevii* USM-YBP2 at 30°C, agitated at 200 rpm.