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A Thesis for the Degree of Master of Engineering

**Efficient Bacterial Cellulose Production
from Buffered Fruit Juices**

완충 용액을 이용한 과일즙에서의
효율적 바이오 셀룰로오스 생산

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이 논문을 석사학위논문으로 제출함

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Efficient Bacterial Cellulose Production from Buffered Fruit Juices

Abstract

Bacterial cellulose (BC) is an exopolysaccharide produced by numerous bacterial species including *Acetobacter*. BC has many advantages such as high tensile strength, water holding capacity, and biodegradability. The problems are low BC yield and high production cost. To solve the problem various carbon sources have been researched to produce BC. In Southeast Asia, coconut water is commonly used as carbon source to produce BC called “nata de coco”. However, there have been very few attempts to produce BC from juices of other fruit sources, such as pear, apple, and grape, which are major fruits produced in East Asia including South Korea. Since those fruits have relatively low pH because of organic acids, the control of pH is necessary to achieve the maximum BC production yield. In this study, optimum fermentation conditions of the strain KCCM 41431 were investigated to achieve the maximum BC production using response surface methodology (RSM). Also, the BC fermentation characteristics of various fruit juices and optimum conditions for

the BC production with buffered media were investigated. The factors for RSM were screened through literature review, and the effective factors and range of the factors were selected by one factor at a time research. Three effective factors, fructose (X_1), agar (X_2), and agitation speed (X_3), were set for the optimization. Among the variables agitation speed (X_3) showed 95% significance. The coefficient of determination (R^2) of the BC production was 0.7048, indicating that the three variable model could explain 70.48% of the total variation in response. The optimum condition for BC production was decided to be 112 g/l of fructose concentration, 0.55% (w/v) of agar concentration, and 133 rpm of agitation speed. The predicted maximum BC production was 19.55 g/l and the experimental BC production at these conditions was 17.07 g/l. Acetate buffer and citric-phosphate buffer were used to control pH of fruit juice media. *A.xylinus* KCCM 41431 was inoculated and BC fermentation was performed for 10 days. In buffered fruit juice media, BC productions were 1.67, 1.44, 2.54 fold higher than those of pure pear, apple, and grape juice, probably due to the reduction of acid stress. Even though apple juice contained high concentration of total sugar, the BC yield was low due to its high concentration of organic acids. The BC production was inhibited at high buffer concentration (>200mM). The results demonstrated that BC production from various fruit juices depended on concentration of organic

acids, concentration of buffer solution, and type of buffer solution. The maximum BC production was 17.02 g/l in 75mM acetate buffered pear juice media. The maximum BC production from buffered fruit juice was comparable to the RSM result. The result of this study suggested the feasibility of BC production from various fruit juices.

Keywords: bacterial cellulose, optimization, response surface methodology, fruit, buffer solution, fruit juice

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I . Introduction

Cellulose, β (1 \rightarrow 4)-linked D-glucoses polymer, is the most plentiful biopolymer on earth with cotton and wood being the major sources. Cellulose usually composes cell wall in plants in combination with other components such as lignin, hemicellulose, and pectin. Separating cellulose from the other components in plants requires extra processing steps, which are known to be the major technical and economical barrier to utilize pure cellulose in plants (Lynd, Weimer, Van Zyl, & Pretorius, 2002).

Bacteria cellulose (BC) that has the same molecular structure as the one in plants is highly beneficial in many aspects, such as purity, mechanical strength, crystallinity, water holding capacity, and biodegradability (Jonas & Farah, 1998; Ross, Mayer, & Benziman, 1991; Vandamme, De Baets, Vanbaelen, Joris, & De Wulf, 1998). Due to these benefits, it is widely used in biomedical applications such as a temporary substitute for human skin, tissue engineering and artificial blood vessels (Klemm, Schumann, Udhardt, & Marsch, 2001; Svensson et al., 2005). In Asian countries it is popular as a food material because of its unique soft texture (Sheu, Wang, & Shyu, 2000). Other than these, BC is even used in a wide variety of applications including paper, wound

dressing, and cosmetic (Amnuaikit, Chusuit, Raknam, & Boonme, 2011; Basta & El-Saied, 2009; Ciechańska, 2004)

While a number of bacterial species such as *Acetobacter*, *Sarcina*, *Agrobacterium*, and *Rhizobium* can produce BC, *Acetobacter xylinus* is widely used to produce BC (Hestrin & Schramm, 1954; Kongruang, 2008; Ramana, Tomar, & Singh, 2000). In the biochemical pathway to synthesize BC, first, uridine diphosphoglucose (UDP-Glc) is synthesized by UDPGlc pyrophosphorylase, and glucose molecules are then linked with β (1 \rightarrow 4) linkage (Figure1). The BC is produced as a floating pellicle or film depending on agitation as shown in Figure2 (De Wulf, Joris, & Vandamme, 1996; Lustrì et al., 2015).

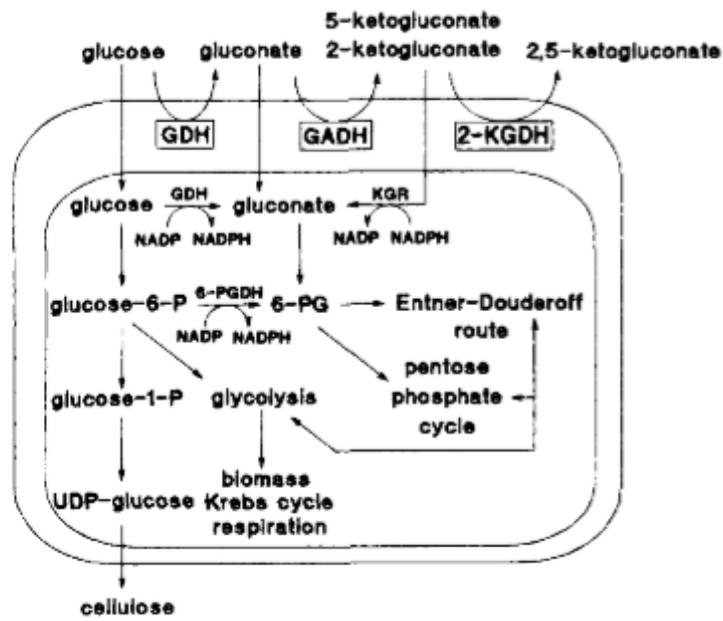


Figure 1. Biochemical pathway to produce BC (De Wulf et al., 1996)

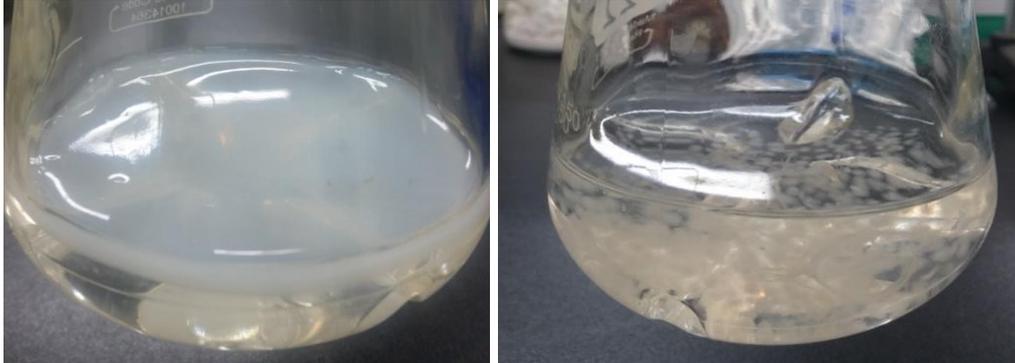


Figure 2. Film (left) and pellicle (right) shaped BC

Despite of those advantages of BC, its low production efficiency with high cost is still problematic. Statistical experimental designs have been used for optimizing medium components to increase production efficiency of BC. Among them, the Box-Behnken design, the central composite design, and Taguchi robust design are popular. Response surface methodology (RSM) is generally used to find the optimal conditions, determine significance factors, and establish relationship between response and factors (Myers, Montgomery, & Anderson-Cook, 2016). The least number of experiments is required for optimization. The optimization technique can also be used to predict the value of the response via estimate equation (Bas & Boyaci, 2007; Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). First of all factors are selected and the ranges are set by one-factor-at-a-time method to do RSM. Central composite

design (CCD) is implemented with the preliminary results to estimate a second-degree polynomial model. If the number of factor is N, the complete design consisted of 2^n factorial points, $2N$ axial points, 5 to 7 center points and α is $\sqrt[4]{2^N}$.

The first objective of this research was the optimization of physicochemical factors to improve BC production by *A.xylinus* KCCM41431. Central composite design (CCD) of three factors at five levels was adopted. Effects of these factors on the BC production were statistically analyzed with RSM.

BC is commonly synthesized from the media, where main carbon sources are glucose, sucrose, fructose, and mannitol and other co-substrates as nitrogen sources and salts are also added (Embuscado, Marks, & Bemiller, 1994; Ramana et al., 2000; Son et al., 2003). Typically, HS medium, Yamanaka, and Zhou medium are used (Hestrin & Schramm, 1954). Recently many researchers have attempted to produce BC from various carbon sources such as high sugar contents crops, residues from agriculture, and starch based materials after pretreatment and detoxification processing Recently many researchers have attempt to produce BC from various carbon sources that are cost effective and eco-friendly (Guo, Cavka, Jönsson, & Hong, 2013; Hong, Zhu, Yang, & Yang, 2011). Fruits are rich in soluble sugars such as glucose, fructose, sucrose,

and sorbitol, which can be used for synthesizing BC. Especially coconut water is commonly used as a carbon source for producing BC called “nata de coco” in Southeast Asia (Kongruang, 2008). However, there have been very few attempts to produce BC from juices of other fruit sources, such as pears, apples, and grapes, which are major fruits produced in East Asia including South Korea. Since those fruits have relatively low pH because of a large quantity of organic acids, the fruits are thought to be improper to produce BC. During the period of synthesizing BC, the pH of culture media further decreases due to the production of gluconic acid as a by-product, which inhibits the BC production (Yang, Park, Hwang, Pyun, & Kim, 1998). Therefore, the proper control of pH is necessary to achieve the maximum BC production yield.

The second objective of this study was to investigate the performance of the BC production from juices of pear, apple, and grape which are major fruits produced in South Korea. Sodium acetate and citric-phosphate buffer solutions were investigated in terms of their buffering ability and effectiveness in BC production from fruit juices.

II. Background and literature review

1. Production of bacterial cellulose with *A.xylinus* KCCM 41431(ATCC 11142)

A. J. Brown (1886) first reported that *A.xylinus* could produce BC in 1886. It has also been reported that certain bacteria belonging to the genera *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, *Rhizobium*, or *Sarcina* could synthesize BC (El-Saied, Basta, & Gobran, 2004). Among them, the *A.xylinus*, a gram-negative strain of acetic acid producing bacteria, has been frequently applied to produce BC (Hestrin & Schramm, 1954; Kongruang, 2008; Ramana et al., 2000).

This strain has been used for the optimization by Taguchi methods with six factors, *A.xylinus* strains, basic medium type, initial pH, glucose concentration, acetic acid concentration, and liquid height, at three levels. The three different types of strains, ATCC 23768, ATCC 23769, ATCC 11142, were used and the maximized production was obtained when ATCC 23768 was inoculated (Lin, Sung, Chen, Lin, & Kuo, 2012). BC film has been produced from ATCC11142 and MF03 using three different drying methods, room temperature drying, freeze drying, and supercritical CO₂ drying. The yield of BC film produced by

the former strains was 0.6–0.2 g/mL, and the produced BC film had 60~90 % porosity and their maximum water absorption capacity 66 times higher than that of dried BC (M. L. Zeng, Laromaine, & Roig, 2014). The new strain (DR-1) was isolated from the temple wash water and its BC production was compared to ATCC11142 and ATCC23747 (Raghunathan, 2013). The cellulose yields in sugarcane juice media were 1.71 ± 0.12 (g/l), 1.68 ± 0.01 (g/l), 1.65 ± 0.04 (g/l) for ATCC 23747, DR-1, ATCC 11142 respectively.

2. Factors affecting BC production

2.1 Carbon source

It was reported that BC was synthesized from various carbon source such as 5- or 6-carbon monosaccharides, oligosaccharides, starch, alcohol, and organic acid (Hestrin & Schramm, 1954). Fructose, glucose, sucrose, lactose, and its combinations were studied to find effect of sugar type. For example, fructose was the most effective sugar followed by fructose + sucrose, fructose + lactose and sucrose. The BC yields ranged from 5.25 to 7.38 g/l (Embuscado et al., 1994). Various substrates such as sorbitol, glucose, galactose, lactose, acetic acid, mannitol, maltose, starch and sucrose were also investigated (Ramana et al., 2000). Among them, glucose, sucrose and mannitol were

suitable to produce BC. Others produced BC lower than 2 g/l. The effective sugar was different depending on strains and sugar mixture had different results compared to each sugar used alone.

2.2 Nitrogen source

Organic nitrogen sources such as peptone, tryptone, yeast extract, urea and inorganic nitrogen sources such as $(\text{NH}_4)\text{SO}_4$, $(\text{NH}_4\text{H}_2)\text{PO}_4$, KNO_3 were investigated in the BC production. The highest BC production yield of 5.12 g/l was achieved when peptone was used, followed by tryptone and yeast extract. Among inorganic compounds, $(\text{NH}_4)\text{SO}_4$, $(\text{NH}_4\text{H}_2)\text{PO}_4$ gave the best BC yield. BC yield was improved when organic nitrogen sources and inorganic nitrogen sources were used together (Embuscado et al., 1994). Casein hydrolysate, ammonium sulphate, glycine, soybean meal, peptone and sodium glutamate have been evaluated as nitrogen source, and peptone, casein hydrolysate showed good BC production performance as nitrogen source with sucrose as base sugar (Ramana et al., 2000). The fruit juices with and without added nitrogen sources were also compared, and with the supplemented nitrogen sources in HS medium composition BC production improved (Kurosumi, Sasaki, Yamashita, & Nakamura, 2009).

2.3 pH of the medium

A.xylinus was grown well at the pH range 4 to 7. The optimal pH was different up to strains. At pH 5, most of the strain produced BC the most. The effect of initial pH on the cellulose production was tested in the range pH 2.5 to 7.7. The amount of cellulose increased linearly with culture time for 3 d. The optimum pH for cellulose production was 4.0 to 6.0. When the initial glucose concentration was less than 2.4 g/flask the pH value became lower during fermentation but the value was within the range of optimum pH value. It didn't reduce the BC production. However, when the initial glucose concentration was 4.8 g/flask the pH value was lower than optimum pH range. BC production was decreased (Masaoka, Ohe, & Sakota, 1993). The pH of the medium was changed on purpose during fermentation in this research. Shifting the pH from 4.0 to 5.5 during the cellulose production phase in batch cultures improved cellulose production and reduced the total fermentation time, compared to batch cultures at constant pH. In this research pH was controlled by automatic addition of 2N-HCl during fermentation (Hwang, Yang, Hwang, Pyun, & Kim, 1999). The final pH of media was different depending on which carbon sources were used. When the medium contained glucose the final pH was lower than other sugar was used. The same results had shown the glucose was combined with other sugars to produce BC (Embuscado et al., 1994).

In order to avoid the reduction of pH under 5 caused by organic acid and the accumulation of gluconic acid, buffer solution was used as medium. The buffer solution was usually used to keep pH almost steady in accordance with Le Chateilier's principle. HS medium which was common BC culture, use citrate-phosphate buffer. Acetate buffer solution was also reported that have a good effect on producing BC. BC produced from 100 mM, pH 4.75 acetate buffered medium was 3.56 g/L, much higher than that obtained from YPD medium (0.66 g/L) and HS medium (1.23 g/L), respectively (Kuo, Chen, Liou, & Lee, 2016).

2.4 Other chemicals

Inorganic salts were commonly used in medium to produce BC. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ improved the level of BC production and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, NaCl slightly improved BC production. The effect of trace elements (0.0005%); $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, H_3BO_3 were studied. Only $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and H_3BO_3 increased the level of BC production. Vitamins (0.00002%); p-Aminobenzoic acid, Biotin, Calcium pantothenate, folic acid, myo-Inositol, nicotinamide, pyridoxine-HCl, riboflavine, thiamine-HCl was added. Only nicotinamide

increased the level of BC production (Son et al., 2003). In other research revealed that, p-aminobenzoic acid (PABA) in synthetic medium increased the BC production. The type of vitamins; hesperitin, orotic acid, ascorbic acid, cobalamin, and nicotinamide, nicotinic acid, pyridoxine, thiamin, pantothenic acid, riboflavin, p-aminobenzoic acid (PABA), and biotin were investigated (Ishikawa, Matsuoka, Tsuchida, & Yoshinaga, 1995).

2.5 Optimization of the factors to improve BC production

Many researchers tried to optimize some strains with several factors. *A.xylinum* BPR 2001 strain was optimized about four factors, maple syrup, incubation time, size of inoculums, rotation speed (X. B. Zeng, Small, & Wan, 2011). There was other optimization research of this strain about fructose, corn steep liquor, dissolved oxygen, and agar concentration. In this case the predicted BC result was 14.3 g/L (Bae & Shoda, 2005). The variables for optimizing *G. hansenii* UAC09 strain were pH, Corn steep liquor, alcohol, acetic acid, and water to coffee cherry husk ratio. The amount of BC obtained was 6.24 g/l (Rani, Rastogi, & Appaiah, 2011). Also the same strain was analyzed with other factors, glucose, corn steep liquor, temperature, and pH. The maximum BC of the strain produced was 7.40 g/l (Rani & Appaiah, 2011).

3. Production of BC using various carbon sources

A variety of carbon sources have been researched to produce BC. For example, ligno-cellulosic feed stocks, the residues from agriculture, forestry, and pulp meals were researched. Also, the starch-based materials consist of carbohydrates could be used after polysaccharide converted to monosaccharide. Depends on components, detoxification treatment was needed to remove inhibitors, phenolic compounds, furan derivatives, and aliphatic acids which were formed during acid hydrolysis process (Guo et al., 2013). High sugar-content crops have also been reported which are also suitable for BC productions as cost effective method. Sugar beets molasses (Keshk, Razek, & Sameshima, 2006), corn steep liquor (El-Saied, El-Diwany, Basta, Atwa, & El-Ghwas, 2008), konjac powder (Hong & Qiu, 2008), were reported that can be converted sugar to BC.

Waste water of candied jujube-processing industry for the production of BC was investigated. Acid pretreatment was implemented to hydrolyze the waste water of candied jujube for higher glucose concentration. 2.25 g/l of BC was produced in hydrolysate medium. It was 1.5-folds higher than BC produced in waste water of candied jujube (Li et al., 2015).

Pine-apple (PA) and watermelon peels (WM) as substrates in the culture

media (containing 5 % sucrose and 0.7 % ammonium sulfate) were used to produce BC. The wet weight of cellulose obtained from PA medium was highest, i.e., 12.5 g/100 ml, as compared to WM medium, (10 g/100 ml) and HS medium (3 g/100 ml). The substrate was utilized very rapidly in the case of HS medium during the first 2 to 3 days of culture, whereas it was comparatively slower in PA and WM media. A sharp decline in pH of the HS medium from 5.0 to 3.1 was observed whereas in the case of PA and WM media, the decrease in medium pH was gradual (Kumbhar, Rajwade, & Paknikar, 2015).

Grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor were used to produce BC as a cost-effective method. The low amount of BC was produced. It was because the presence of inhibitors derived from the industrial processes inhibited bacterial growth. To minimize the effectiveness of inhibitors and to optimize carbon substrate concentration different dilutions of the residues were tested. 0.1 g/l of BC was produced with grape peel whereas 0.63 g/l of BC was synthesized with 4 fold diluted grape peel (Carreira et al., 2011).

Orange, pineapple, apple, Japanese pear and grape were investigated. The BC yield to sugars (%) was 6.9 ± 0.2 , 3.9 ± 0.3 , 3.9 ± 0.2 , 4.8 ± 0.3 , 1.4 ± 0.2 ,

respectively. The yields of the bacterial cellulose were 2.1 ± 0.2 , 0.6 ± 0.1 , 0.2 ± 0.1 , 0.6 ± 0.1 , 0.3 ± 0.1 when the nitrogen source to the fruit juices were not added (Kurosumi et al., 2009).

III. Efficient BC production from buffered fruit juices using *Acetobacter xylinus*

1. Materials and methods

1.1 Materials and microorganisms

Glucose, fructose, mannitol, sucrose, KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract was purchased from Becton, Dickinson and Company (NJ, USA). Agar was purchased from Junsei Chemical Co.,Ltd. (Tokyo, Japan). Ethanol was purchased from Merck KGaA (Darmstadt, Germany). Sodium hydroxide was purchased from Daejung Chemicals (Gyeonggi-do, Korea). Disodium hydrogen phosphate was purchased from Showa Chemical Co.,Ltd. (Tokyo, Japan).

Pear, apple, and grape were washed first with acetic acid and cut into small shape to grind by grinder. Any additional materials such as water were not put. The fruit extraction was strained through a sieve to eliminate peel, seed and other pectin components which were not grinded perfectly. The liquids were preserved in the -80°C deep freezer for future using

A.xylinus KCCM 41431 was acquired from Korean Culture Center of Microorganisms (KCCM). *A.xylinus* KCCM 40198 were provided from Nano and biomaterials lab, Seoul National University, Seoul, Korea.

1.2 Fermentation method of BC production

1.2.1 Culture media and cultural conditions for RSM

A 50 μ l of liquefied and diluted cryostock was spread on the mannitol agar plate (mannitol 10 g, peptone 1.2 g, yeast extract 2.0 g, agar 6.2 g, distilled water 400ml), followed by the incubation at 26°C for 4-5 days for the formation of bacterial colonies. One single colony was picked from the agar plate and put into 50 ml mannitol broth (mannitol 1.5 g, yeast extract 1.0 g, KH_2PO_4 1.0 g/l, $(\text{NH}_4)_2\text{SO}_4$ 3.3 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.8 g/l) and incubated in a shaking incubator at 26°C for 4-5 days. The media were filtered through an autoclaved cotton cloth to an autoclaved beaker in the clean bench. The bacterial cryostock was prepared with glycerol at 1:1 volume rate. Then the cryostocks were kept in the deep-freezer at -80°C.

The 1ml of thawed cryo-stock was inoculated to 50 mL of medium containing glucose 10 g/l, yeast extract 20 g/l, KH_2PO_4 1.0 g/l, $(\text{NH}_4)_2\text{SO}_4$ 3.3

g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.8 g/l. The culture was incubated at 26°C, 100rpm shaking incubator. After 48hour the growth of microbial approached to stationary phase and used for pre-culture. 10% of the pre-culture was inoculated to 50 mL of fruit juice medium.

1.2.2 Preparation of fruit juice media

The fruit juices have low pH under 5 because of organic compounds. After adding nitrogen sources and salts followed by HS medium, the pH of the fruit juices were slightly increased. To adjust fruit juice media pH to pH 5 which was optimum value, two types of buffer was used. First, Sodium acetate, the conjugate base, was put into the fruit juice media. To set final pH 5 with acetic acid, the weak acid, the pH of the medium should be over pH 5 after the first step. And then pH was set to 5 with glacial acetic acid and 0.1 N acetic acid. To find optimum concentration of sodium acetate, 0 mM to 250 mM sodium acetate was investigated. Same steps were done with citrate-phosphate buffer. It was composed with citric acid and disodium hydrogen phosphate. The 10% of pre-culture was inoculated to the autoclaved fruit juice media.

1.2.3 Purification of pellicle, determination of yield and cell growth

The harvested pellicle was washed with 0.1 N NaOH during 20 min at 80°C, three times to get rid of attached cells and other impurities set in the pellicle. After repeating three times, the pellicle was rinsed again with deionized water. The pellicle was put on the aluminum plate and then dried at 80°C dry oven until the weight did not change.

To investigate the microbial growth in fruit juice media, 1mL of the culture medium was measured by spectrophotometric at 660nm. The value was need to substrate the value of fruit juice measured by spectrophotometric at 660nm because the fruit juice had a color. The OD value of fruit juices was measured after centrifuging at 6000 rpm in 3 minutes to eliminate cells.

1.3 Optimization of BC production

1.3.1 One-factor-at-a-time method to select factors and set ranges for RSM

As the experimental factors glucose, fructose, sucrose, mannitol, glycerol, agar, agitation speed, and ethanol were first selected based on the preceding papers (Bae, Sugano, & Shoda, 2004; Mikkelsen, Flanagan, Dykes, & Gidley, 2009; Mohite, Salunke, & Patil, 2013; Naritomi, Kouda, Yano, & Yoshinaga,

1998). The concentration of fructose was selected because it produced high yield of BC compared to other carbon source. The concentration of agar improved BC production by increasing medium viscosity. The agitation speed supplied more oxygen which was reported to result in increase of BC productivity. Each factor was executed one factor at a time experiment to find effectiveness and set range. Three experimental factors, fructose, agar, and agitation speed were chosen for optimization of BC production.

1.3.2 A five level three factor central composite design (CCD)

CCD and RSM of three-factors, five-levels design was used as shown in Table 2. The CCD contained total of 19 experimental trials that included 8 trials for factorial runs, 6 trials for axial runs, and 5 trials for the central point. The response variable was the maximum BC productions (Y) which indicated the measured value of the results from experiments. The value of α was 1.682. All 19 runs of experiments were conducted in duplicate the average values were investigated.

1.3.3 Response surface methodology (RSM)

A second order polynomial equation (1) was then fitted to the data by using multiple regressions.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad \text{Eq.(1)}$$

Where, Y is fitted response, b_0 is intercept, b_1 , b_2 , b_3 , are coefficients of linear terms, b_{11} , b_{22} , b_{33} are coefficients of quadratic terms, b_{12} , b_{23} , b_{13} are interaction coefficients, and X_1 is fructose concentration (g/l), X_2 is the agar concentration (%), X_3 is the agitation speed (rpm).

1.3.4 Statistical Analysis

PROC RSREG of SAS 9.3 software (SAS Institute Inc.) was performed for optimization BC production. Three-dimensional surface plots and contour plots were visualized to show interaction between two factors by using 'Minitab 13

(Minitab Inc., USA). Through the results maximum point, minimum point, saddle point, and rising point were analyzed.

1.4 Analysis of sugars and organic acids in fruit juices

The components of carbon sources was analyzed by high-performance liquid chromatography using a Aminex 87P column (300mm x 7.8mm / Bio-rad, USA) with a dionex ultimate 3000 pump plus auto sampler (Dionex, USA) and Shodex RI-101(Shodex, Japan) detector. The flow rate was 0.5ml/min, and the mobile phase used was distilled water and the oven temperature was 80 °C. The injected sample volume was 20.0 µl.

The components of organic acids were analyzed by high-performance liquid chromatography using a Aminex 87H column with Dionex Ultimate3000 (USA) and RI (ERC, Refracto MAX520, Japan) detector. The flow rate was 0.5ml/min, and the mobile phase used was 0.01N H₂SO₄ and the oven temperature was 40 °C. The injected sample volume was 20.0 µl and detection time was 30 minutes.

2. Results and discussions

2.1 Optimization of BC production

2.1.1 Comparison of *A.xylinus* strains

Two types of strains, KCCM 41431 and KCCM 40198, were used to compare the ability of producing BC. 1.81 g/l BC was produced from KCCM 41431 and 1.67 g/l BC was synthesized from KCCM 40198. The former type of bacterial produced BC better than the other.

2.1.2 Comparison of sugar types

To find main substrate of the medium, glucose, fructose, sucrose, and mannitol were investigated as carbon sources. The concentration of carbon source was 30 g/l and nitrogen source and salts were put at equal concentration, respectively. The highest BC production was 6.06 g/l from fructose medium followed by mannitol 5.79 g/l, sucrose 5.10 g/l, and glucose 3.84 g/l.

2.1.3 Determination of independent variables and their levels

2.1.3.1 *The concentration of fructose*

It was turned out that the *A.xylinus* KCCM 41431 effectively used fructose to produce BC as above. To determine the effect of fructose concentration on the BC production, the microbes were grown in broth containing at a range of fructose concentration from 50 g/l to 100 g/l. As shown in Table1 the BC weight tended to increase until fructose concentration reached to 70 g/l. The BC weight decreased when the fructose concentration was over 70 g/l (Table1).

2.1.3.2 *The concentration of Agar*

It was proved that addition of agar in the medium improve BC production (Bae et al., 2004). The range of 0.2-1.0% (w/v) agar was added to medium and the highest yield of 12.8 g/l BC was obtained from strain BPR2001 at 0.4% (w/v) agar concentration. 11.6 g/l of BC was produced from mutant EP1 at 0.6% (w/v) agar concentration. However, excessive concentration of agar made medium solid which means the broth was not stirred during fermentation. For this reason, the highest concentration of agar was decided when the broth could be stirred even at the lowest agitation speed of 116rpm. The highest amount of BC was produced in medium which contain 0.8% concentration of agar (Table.1).

2.1.3.3 The speed of Agitation

In BC production using *A.xylinus* that is an aerobic bacterium, the agitation speed is important, because agitation supply oxygen into the broth. To investigate the effect of agitation speed on the production of BC agitation speeds of 116-284 rpm were used. Generally, the BC production increases when the agitation speed increased (Krystynowicz et al., 2002). The maximum BC production was at 135 rpm. However, excessive agitation speed inhibited BC production (X. B. Zeng et al., 2011).

2.1.3.4 The concentration of ethanol

Naritomi et al., 1998 reported that an increase in ethanol concentration improved the yield of BC production using *A.xylinus* BPR3001A. However, *A.xylinus* KCCM41431 used in this study did not show an improvement of BC production as presented in Table 1. The effect of ethanol on BC production might be different depending on the strain. For this reason, ethanol concentration was decided not to be considered as a variable for the RSM factors. According to the above results, three factors and five levels CCD was

decided as shown in Table 2.

Table 1. The results of BC weight by one factor at a time test

Fructose g/l	40	50	70	80	100
BC weight g/l	7.82	12.8	21.60	19.01	14.32
Ethanol g/l	0	5	10	15	20
BC weight g/l	11.68	9.53	10.60	8.02	6.75
Agar % (w/v)	0.6	0.7	0.8	1.0	
BC weight g/l	4.95	12.26	14.74	9.64	

Table 2. Factors and their levels for RSM

Factor	Unit	Symbol	- α	-1	0	1	+ α
Fructose concentration	g/l	X ₁	27.5	50	70	90	112.05
Agar concentration	% (w/v)	X ₂	0.42	0.45	0.50	0.55	0.58
Agitation speed	rpm	X ₃	116	150	200	250	284

2.2 Optimization of BC production using RSM

2.2.1 Full experimental design and regression summary

RSM was carried out to determine the optimum value of BC production. Full experimental design was implemented to find optimum condition. The results of each individual run of the experimental design were shown in table 3. The RSREG SAS procedure was used to fit the second order polynomial equation. The regression equation (2) between factor and response value was obtained. The coefficients of the variables in the models were shown in Table 4. The results obtained after full experimental design were analyzed by standard analysis of variance (ANOVA) which indicated how significant the second-order polynomial model Equation (2) was. P-value lower than 0.05 indicated the model was statistically significant at 5 % probability level. The statistical analysis showed that the linear and agitation speed variation were significant at 5% level (Table. 5). Therefore, the agitation speed was the most important factor for the BC production. The analysis of variance also showed that there was a non-significant lack of fit, which further validated the model. The coefficient of determination (R^2) of the BC production was 0.7048. It was indicated that the quadratic equations could describe approximately 70.48% of the correlation between the factors and responses.

$$Y = 15.78 + 1.76 X_1 - 1.21 X_2 - 8.23 X_3 - 2.80 X_1 * X_1 + 4.10 X_2 * X_2 - 8.83 X_3 * X_3 - 2.51 X_1 * X_2 - 6.76 X_1 * X_3 + 1.61 X_2 * X_3 \text{ _____(2)}$$

Table 3. Results of RSM using three factors and six center points

Run order	Coded levels			Bacterial Cellulose g/l(Y)
	Fructose (X ₁)	Agar (X ₂)	Agitation (X ₃)	
1	0	0	+ α	2.37
2	-1	-1	1	10.53
3	-1	1	1	6.68
4	1	-1	1	7.42
5	1	1	1	4.58
6	0	0	0	19.33
7	0	0	0	14.62
8	0	0	0	19.83
9	0	0	0	10.07
10	0	0	0	15.97
11	- α	0	0	9.66
12	+ α	0	0	12.9
13	0	- α	0	15.44
14	0	+ α	0	20.54
15	-1	-1	-1	18.98
16	-1	1	-1	17.44
17	1	-1	-1	30.10
18	1	1	-1	20.16
19	0	0	- α	8.11

2.2.2 Response Surface Plotting

The overall shape of the curve was accomplished by canonical analysis. The relationship between response and factors could be examined by these plots. Three-dimensional (3D) response surfaces and the contour plots for BC production shown in figure 3 indicated the interaction between the two factors while the other factor was set at zero level. The interaction effects of fructose and agitation speed shown in Fig. 3A and B described maximum point for response. The effects of agar and agitation speed were shown in Fig. 3C and D. The stationary point of the response surface illustrated the saddle point which indicates the possibility of the factors at either the maximum or minimum value. The effects of fructose and agar on BC production were shown in Fig. 3E and F. The type of response surfaces was also saddle behavior.

2.2.3 Optimum condition and model verification

The first aim of this study was to achieve the optimal conditions using RSM in order to accomplish maximum BC production with ATCC11142. The maximum production was obtained at the concentration of fructose 112 g/l, concentration of agar 0.55% (w/v), agitation speed 133 rpm. The predicted BC

yield at this condition was 19.55 g/l. The experimental value at these factor levels was 17.07 g/l. This value was closed with the predicted one. The weight of BC produced in the HS medium described by Hestrin and Schramm was 2.57 g/l. The experimental weight at optimum condition was 6.6 times larger than the result of HS medium.

Table 4. Coefficient of regression

Variable	Coefficient	t-value	P > t
X ₁	1.76	1.17	0.27
X ₂	-1.21	-1.01	0.34
X ₃	-8.23	0.92	0.38
X ₁ * X ₂	-2.51	-0.5	0.63
X ₁ * X ₃	-6.76	-1.28	0.23
X ₂ * X ₃	1.61	0.32	0.76
X ₁ ²	-2.80	-0.69	0.51
X ₂ ²	4.10	1.04	0.33
X ₃ ²	-8.83	-2.19	0.06

Table 5. Analysis of variance for the second order response surface model

Variation	DF	Sum of squares
Linear	3	352.35 ^a
Quadratic	3	190.65
Crossproduct	3	55.10
Total model	9	598.09
Lack of fit	5	187.66
Pure error	4	62.81
Total error	9	250.47
R ²	0.7048	
Fructose conc(X ₁)	4	80.27
Agar conc(X ₂)	4	47.40
Agitation speed(X ₃)	4	512.25 ^a

^aSignificant at 5% probability level

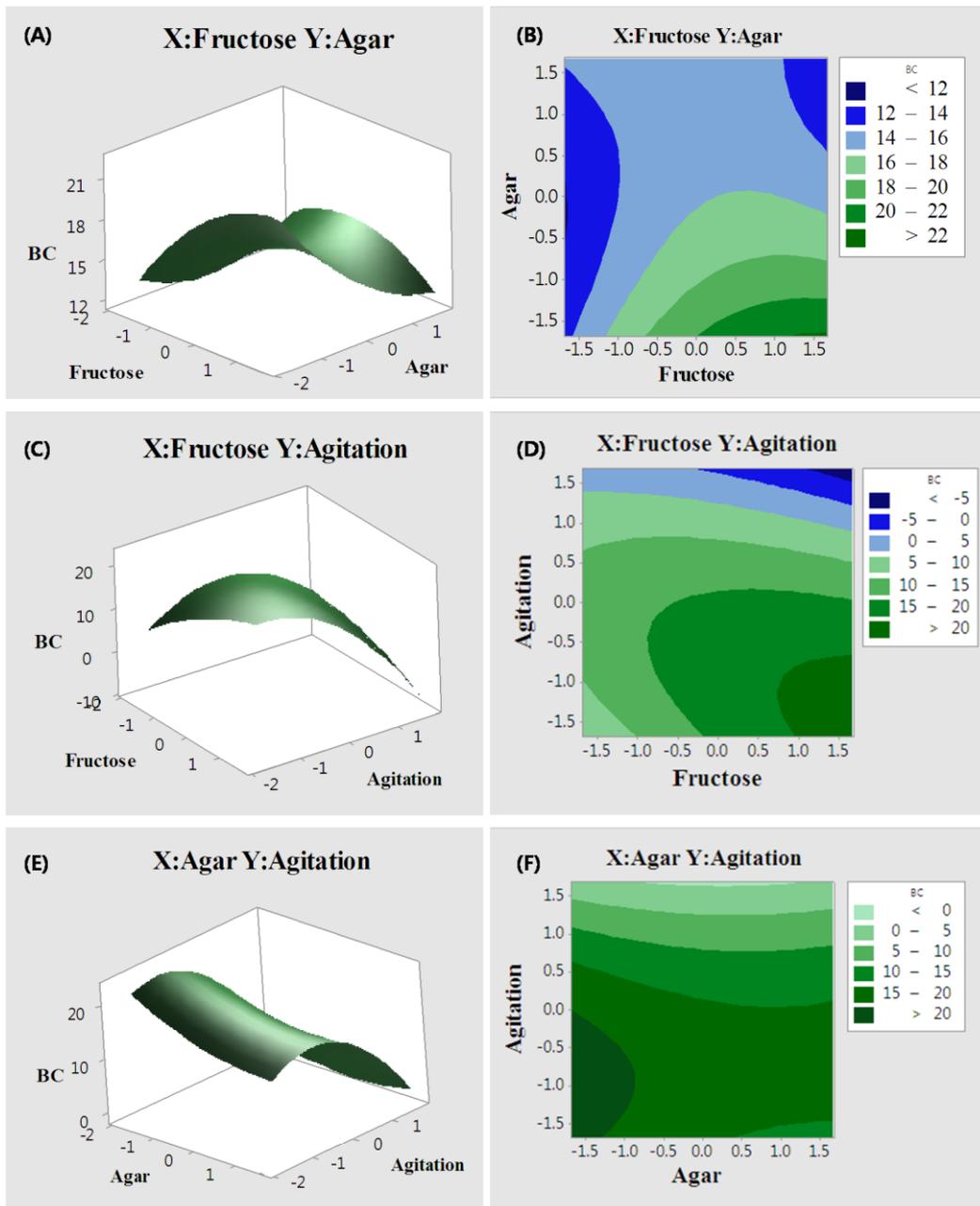


Figure 3. 3D curve and contour plot of interaction on bacterial cellulose production

2.3 Characterization of various fruit juices

The sugar levels of the fruit juices and organic acids were analyzed to evaluate the fruit juices media capacity as medium to produce BC.

2.3.1 The composition of sugars in fruit

As shown in Table 6, the amount of total sugars in fruit juices were 155.10 g/l, 131.70 g/l, 102.31 g/l for apple, grape, and pear juices, respectively. The types of sugar were also different depending on fruit juices. For all fruit juices, fructose content was the highest and glucose content was the second. Sucrose and sorbitol were not detected in grape juice.

Table 6. Types of sugars and their concentrations in fruit juices

g/l	Sucrose	Glucose	Fructose	Sorbitol	Total
Pear	7.92	27.49	48.86	18.04	102.31
Apple	24.19	34.15	91.26	5.50	155.10
Grape	0.000	55.88	75.83	0.00	131.70

2.3.2 The composition of organic acids and pH in fruit juices

The pH of the fruit juices was different because concentration of organic

acids was varied. All the fruit juices had citric and malic acids (Table.7). Pear juice and apple juice mainly had malic acid and only grape juice had tartaric acid. The amount of total organic acids was highest for apple followed by grape and pear highest. Grape juice showed the lowest pH and pear had the highest (Table.8). The initial pH values of the fruit juice were not appropriate for *A.xylinus* which had optimum pH at 5 in common. The final pH was near 3 after 10 days of fermentation (Table.8). It became lower than initial pH mainly because of an acid byproduct, gluconic acid, formed during fermentation. The appearance of end products with BC was shown in figure 4 and 5. When BC was formed at agitation condition, showed a in pellicle shape (Fig.4). When the BC was formed in static condition, film shape resulted (Fig.5). The BC had a color of each fruit.

Table 7. Types of organic acids and their concentrations in fruit juices

g/l	Citric acid	Malic acid	Tartaric acid	Total
Pear	0.50	3.16	-	3.67
Apple	0.03	9.82	-	9.85
Grape	0.11	1.35	3.57	5.03

Table 8. Initial pH of fruit juices and final pH after 10days of fermentation

	Initial pH	Final pH
Pear	4.72	3.05±0.16
Apple	4.04	2.98±0.21
Grape	3.08	2.83±0.08

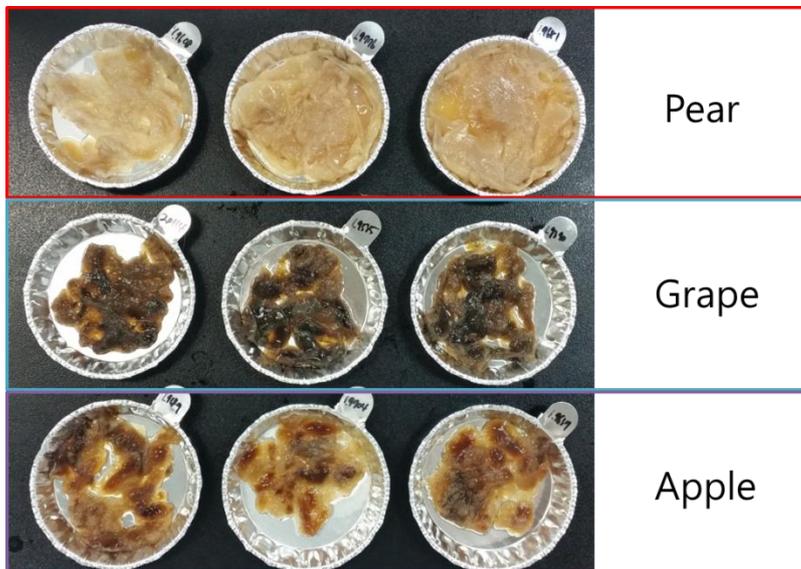


Figure 4. The BC produced from various fruit juices at agitation condition



Figure 5. The BC produced from various fruit juices at static condition. (Apple, pear, and grape juices from left to right)

2.4 Effect of buffered juice medium on BC production

2.4.1 Production of BC using fruit juices without pH adjustment

BC was produced from pure fruit juices with supplemented nitrogen sources and salts. BC production yields were 8.60 ± 0.96 , 6.15 ± 0.44 , 4.83 ± 0.01 g/l for pear, apple, and grape juices, respectively. The consumed sugar level had no relation with BC production (Table 9). The results of BC weight were relatively low even though the fruit juices had high total sugar concentration.

In order to consider different total sugar concentration in fruit juices, BC conversion yield was calculated using equation (3). The BC conversion yields were 8.41, 3.97, and 3.67 for pear, apple, and grape juices, respectively. Pear juice was the most efficient medium among three when buffer solution was not used. It has been reported that the type and level of organic acids could affect BC production either positively or negatively (Jung et al. 2010a; Dudman, 1959). When appropriate amount of organic acids were used as co-substrate the BC production improved except for fumaric acid, with 0.35% (w/v) succinic acid showing the greatest effect (Jung et al. 2010a). The optimum acetic acid concentration for BC production was reported as 0.1–0.2% (v/v) and at higher concentration up to 1% (v/v) BC production was

decreased (Jung et al. 2010b). Also, 0.1% of malic acid improved production of BC (Jung et al. 2010a). High concentration of citric acid inhibited cell growth and high concentration of acetic acid resulted low BC production (Dudman, 1959). It was postulated that the high concentration of organic acids 9.85 g/l in apple juice inhibited BC production (Table 7).

BC conversion yield

$$= \frac{BC\ Production(g/l)}{Total\ Sugar\ Concentration\ of\ Fruit\ Juice(g/l)} \times 100(\%) \quad eq(3)$$

Table 9. Variation of sugar level during fermentation and BC yield

	Final Total Sugar Level (g/l)	Consumed Sugar Level (g/l)	BC production (g/l)	Conversion Yield (%)
Pear	30.44 ±0.25	71.88	8.60±0.96	8.41
Apple	66.28 ±0.14	88.83	6.15±0.44	3.97
Grape	59.33 ±1.42	72.37	4.83±0.01	3.67

2.4.2 Effect of acetate buffer on BC production

The different concentrations of sodium acetate were in fruit juices and then acetic acid was used to set pH at 5. It was found that sodium acetate

concentration higher than 300 mM severely inhibit BC production (data not shown). Therefore, the highest limit concentration of sodium acetate was set to 200 mM. The pH of sodium acetate and fruit juice mixtures should be higher than 5 before adding acetic acid. However, 200 mM of sodium acetate was not enough to increase the pH of 100% apple and grape juices to higher than 5. In order to solve this problem, fruit juice concentration was adjusted in the case of apple and grape juices, so that their pH could be higher than 5 with the addition of sodium acetate at the level of 200 mM or less. Since, the pH of pear juice after adding nitrogen sources and salts was already over 5, 100% pear juice could be used (Table.10-12).

In the case of pear juice, the BC production increased as sodium acetate concentration decreased. However, with apple juice, 200 mM buffer concentration showed better performance to produce BC than 150 mM buffer concentration. As a result, the optimum BC production was achieved in 100% pear juice with 50 mM buffer, 90% of apple juice with 200 mM buffer, and 60% of grape juice with 200 mM buffer. BC productions in buffered pear, apple, and grape juice media were 1.67, 1.44, and 2.54 fold higher than those in non-buffered juice medium, respectively (Fig.6).

Table 10. BC production and initial pH in pear juice media with sodium acetate buffer

Pear Juice (%)	Concentration of Sodium Acetate (mM)	Initial pH	BC weight (g/l)
100	200	5.7	4.30±0.91
	150	5.6	5.05±0.76
	100	5.46	9.38±3.62
	50	5.15	14.35±2.24

Table 11. BC production and initial pH in apple juice media with sodium acetate buffer

Apple Juice (%)	Concentration of Sodium Acetate (mM)	Initial pH	BC weight (g/l)
90	200	5.21	10.03±0.83
	150	5.13	6.84±0.90
	<150	<5	-
80	200	5.30	9.95±0.85
	150	5.22	7.54±0.26
	<150	<5	-

Table 12. BC production and initial pH in grape juice media with sodium acetate buffer

Grape Juice (%)	Concentration of Sodium Acetate (mM)	Initial pH	BC weight (g/l)
70~90	<200	<5.0	-
60	200	5.16	7.73±1.18
	<200	<5.0	-
50	200	5.47	7.14±2.02
	150	5.39	3.66±0.62
	100	<5.0	-
40	200	5.40	4.97±0.79
	150	5.29	4.76±0.09
	100	5.2	5.78±0.24
	50	<5.0	-

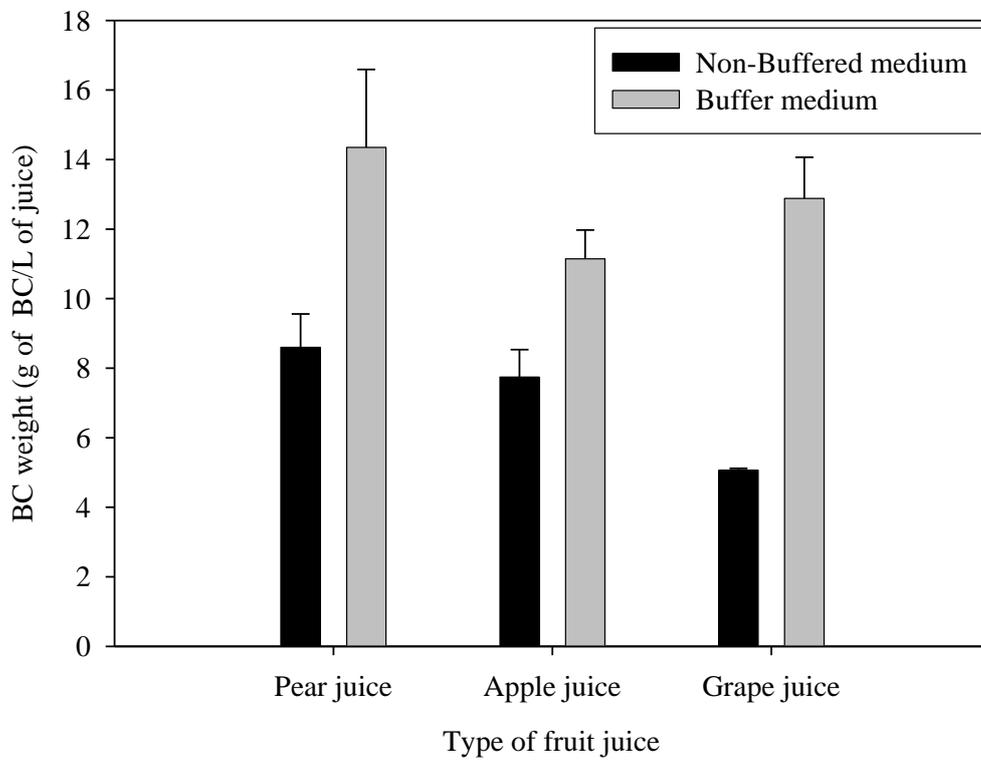


Figure 6. The produced BC weight in buffered and non-buffered fruit juices

2.4.3 Fermentation profiles of buffered fruit juices

Sucrose, glucose, fructose, and sorbitol were utilized to produce BC in pear juice and apple juice (Fig.7-8). All the sugars except fructose were almost completely used. The glucose was used more than fructose in the early stage of fermentation and after 7 days all glucose was depleted. After the glucose was depleted, the fructose was more rapidly utilized compared to early stage of fermentation (Fig.9). This result agreed with previous work on mixed sugar fermentation done by Sangok Bae & Shoda (2004). They reported that glucose was consumed preferentially and after exhaustion of glucose, fructose was consumed. It was revealed that cell growth measured from OD value occurred rapidly from the beginning of the fermentation, and BC production started to be active after day 2 in average.

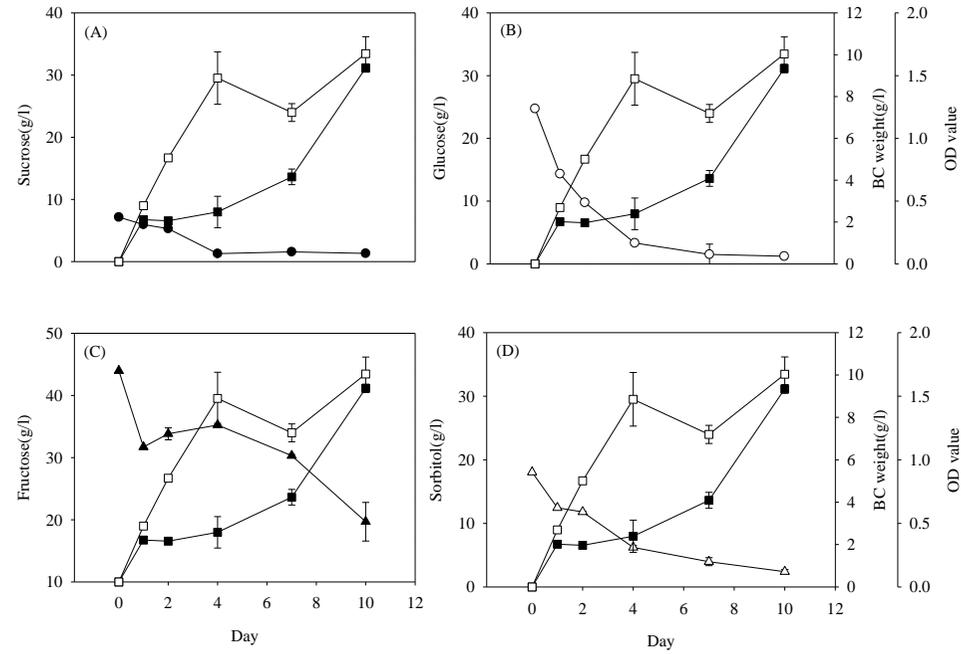


Figure 7. Fermentation profiles of buffered pear juice media during 10days. Filled circle: sucrose, empty circle Glucose, filled upright triangle: fructose, empty upright triangle: sorbitol, empty square: OD value, filled square: BC production.

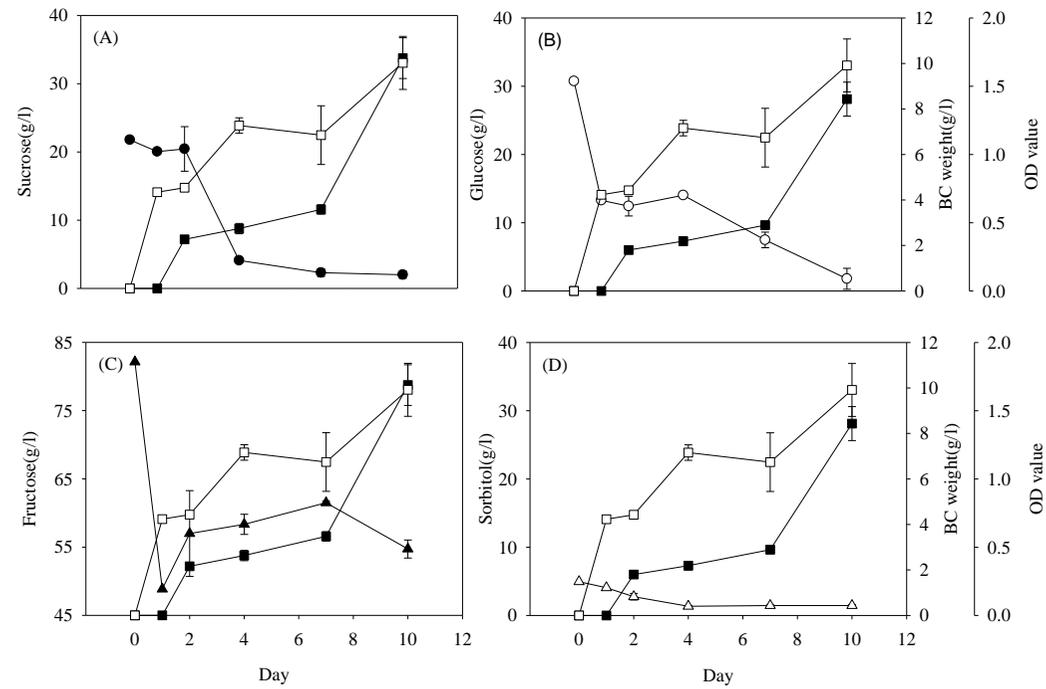


Figure 8. Fermentation profiles of buffered apple juice media during 10days. Filled circle: sucrose, empty circle: glucose, filled upright triangle: fructose, empty upright triangle: sorbitol, empty square: OD value, filled square: BC production.

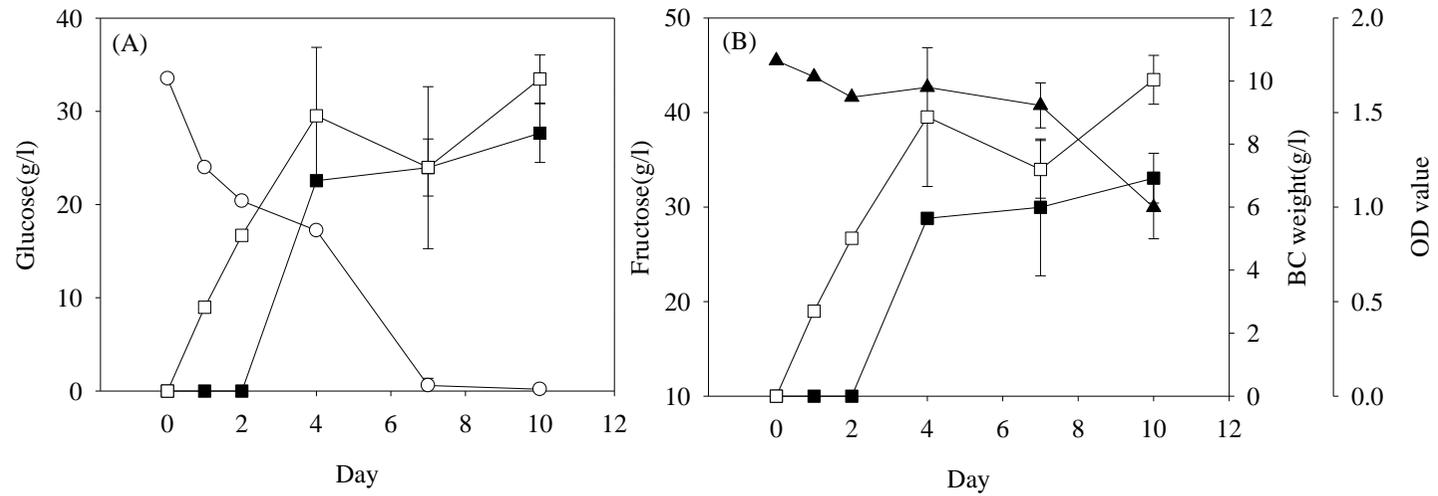


Figure 9. Fermentation profiles of buffered grape juice media during 10days. *Empty circle: glucose, filled upright triangle: fructose, empty square: OD value, filled square: BC production.*

2.4.4 The effect of buffer type on BC production in pear juice media

Sodium acetate buffer and McIlvaine buffer, also called citrate-phosphate buffer, was compared in BC production using pear juice that was confirmed to be the most efficient for BC production (Fig.6). The citrate-phosphate buffer consisted of disodium hydrogen phosphate as a conjugate base and citric acid as a weak acid. HS medium, one of the popular media to produce BC, contains these components.

As presented in Figure 10, BC productions in both buffered media were higher than those in non-buffered media at low buffer concentrations. With sodium acetate buffer, BC production was increased until 75 mM and then decreased. At buffer concentration, higher than 150 mM, BC production was severely inhibited. As for citrate-phosphate buffer, BC production was generally improved until the buffer concentration reached to 150mM, and then abruptly decreased at higher concentration. The highest BC production was achieved when sodium acetated buffer was used at 75mM.

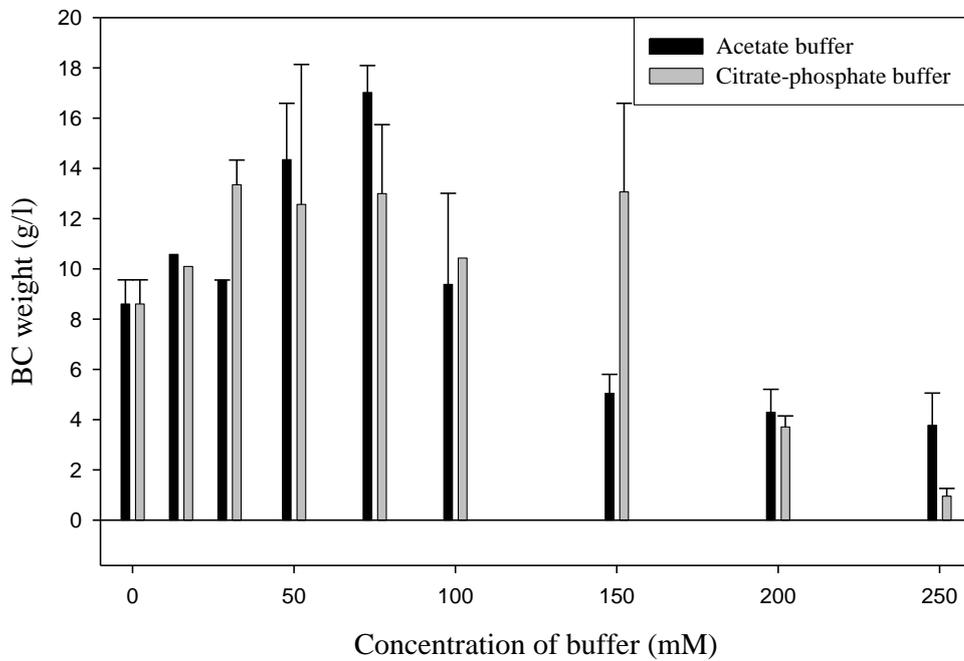


Figure 10. The effect of type and concentration of buffers on BC production

2.4.5 Buffering performance of different buffered media

The initial pH of citrate-phosphate buffered media was higher than that of sodium acetate buffered one because of higher conjugate base strength of the former. The final pH after fermentation increased as the concentration of conjugate base increased (Table.13). The final pH of sodium acetate buffered media was maintained between 4 and 5 at buffer concentration higher than 75

mM, whereas citrate-phosphate buffer could not maintain the pH higher than 4 even at 200 mM. This might partly explain the reason why the highest BC production occurred in sodium acetate buffered media. However, regardless of buffer type, finding optimum buffer concentration was more important than buffering capacity, because high concentration of buffer dramatically inhibited BC production.

Table 13. Final pH of the pear juice media after fermentation depending on concentration of the different buffer solutions

Concentration of the buffers (mM)	0	15	30	50	75	100	150	200	250
Final pH of acetate buffered medium	3.30± 0.01	3.50± 0.02	3.5± 0.01	3.7± 0.01	4.3± 0.01	4.45± 0.01	5.16± 0.04	4.8± 0	4.86± 0.01
Final pH of citrate-phosphate buffered medium	3.30± 0.01	3.32± 0.02	3.41± 0.01	3.48± 0.01	3.57± 0	3.6± 0	3.78± 0	3.95± 0.01	4.73± 0.02

IV. Conclusions

In this study, RSM was successfully implemented to optimize factors that could maximize BC production. The fructose concentration, agar concentration, and agitation speed were the main factors of fermentation process. The optimized conditions for *A.xylinus* KCCM 41431 as determined using RSM were X_1 (fructose concentration) = 112 g/l, X_2 (agar concentration) = 0.55% (w/v), and X_3 (agitation speed) = 133 rpm. The 19.55 g/l of BC was produced at the optimum condition, which was approximately 6.6-fold higher than the yield in common media (2.57 g/l). The validated BC production at optimum fermentation conditions was 17.07 g/l. The results obtained from this study provided a better strategy to produce BC with the strain.

The suitability of fruit juices as carbon sources to produce BC and efficient BC production method with fruit juices were also investigated. Even though the fruit juices had high total concentration of sugar, relatively low amount of BC was produced. Low initial pH and excess amount of organic acids in fruit juices could be the possible reason for low BC production. After adjusting pH with acetate buffer, BC production was significantly improved in all fruit juices by preventing pH decrease. Also, the optimum concentration of buffer solution existed. Comparing sodium acetate and citrate-phosphate buffers, the former

provided higher buffering capacity and the maximum BC production yield, whereas the inhibition effect of buffer at high concentration was less pronounced for the latter. However, the excessive concentration of both buffer severely inhibited the BC production.

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국문 초록

셀룰로오스는 자연에서 가장 많이 존재하는 생물고분자이다. 박테리아 셀룰로오스는 초산균의 일종인 *A.xylinus* 등에 의해 합성되는 천연 바이오 소재로 바이오 셀룰로오스로 주로 불린다. 식물 속 셀룰로오스는 다른 헤미셀룰로오스, 펙틴, 리그닌 등과 함께 존재하지만 이와 달리 바이오 셀룰로오스는 순수한 셀룰로오스로 구성되어 있다. 바이오 셀룰로오스의 특성으로는 높은 인장강도, 보수력, 생분해성 등이 있다. 하지만 낮은 수율로 인해 높은 생산 비용이 든다는 단점이 있다. 따라서 본 연구의 첫 번째 목적은 실험계획법을 이용한 최적화를 통해 생산량을 높이는 것이다. *A.xylinus* KCCM 41431(ATCC 11142) 균주를 사용하였고 반응표면분석법(RSM)을 통해 해당 균주의 최적 공정조건을 구명하였다. 논문 조사 및 예비 실험을 통해 바이오 셀룰로오스 생산에 큰 영향을 미치는 실험인자로는 과당 농도, 한천 농도, 교반 속도 3가지의 조합으로 결정되었다. 가장 많은 양의 바이오 셀룰로오스가 생산되는 공정 조건은 과당 농도 112 g/l, 한천 농도 0.55% (w/v), 교반속도 133 rpm 인 것으로 조사되었다. 이 때의 예상되는 바이오 셀룰로오스 생산량 예상치는 19.55 g/l 이며 실제 실험 결과 17.07 g/l 와 유사하였다. 목질 자원, 당 함량이 높은 작물, 과일 등 과 같은 다양한 농업 물질들을 이용하여 효과적으로 바이오 셀룰로오스를 생산하는 배양방법에 대한 연구가 이루어 지고 있다. 동남아시아에서는 흔히 코코넛 워터를 이용하여

"nata de coco"라고 불리는 바이오 셀룰로오스를 생산한다. 그러나 한국을 포함한 동아시아 지역에서 많이 생산되는 과일인 배, 사과, 포도와 같은 과일 주스를 이용한 바이오 셀룰로오스 생산 연구는 미비한 실정이다. 이러한 과일들은 유기산의 농도가 높아서 낮은 pH를 지니기 때문에 많은 양의 바이오 셀룰로오스를 생산하려면 pH 통제가 필요하다. 따라서 완충용액을 통한 pH 조절로 다양한 과일즙에서 바이오 셀룰로오스를 효율적으로 생산에 대해 연구하였다. 완충용액으로는 아세트산 버퍼와 시트르산-인산염 버퍼를 사용했다. *A.xylinus* KCCM 41431 균주를 접종 후 10일간 발효시켰다. 완충 효과를 지닌 과일주스 배지에서 바이오 셀룰로오스 생산량은 순수한 과일주스에서 키웠을 때 보다 1.98, 1.44, 2.55배 증가했다. 이는 산에 의한 스트레스가 감소하였기 때문으로 판단된다. 아세트산의 농도가 75 mM일 때 생산량이 최대였고 200 mM 이상의 고농도 버퍼가 첨가되었을 경우는 바이오 셀룰로오스의 생산이 저해되었다. 시트르산 인산염 버퍼는 생산량이 증가하긴 하나 농도별 유효적 차이는 보이지 않았고 아세트산 버퍼와 마찬가지로 고농도에서는 바이오 셀룰로오스 생산이 저해되었다. 즉, 최적 acetate 버퍼 사용 농도 조건에서 RSM 최적 조건의 BC 생산량에 준하는 생산 수율을 획득하였으며, 다양한 과일 주스에서의 BC 생산 수율은 과일의 종류에 따른 유기산 량, 산도를 조절하기 위한 완충용액의 종류와 농도에 의존적임을 구명하였다