Enhancement and Optimization of cellulose production by *Gluconacetobacter xylinus* N2

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Abstract:

To enhance the ability of *G. xylinus* N2 for cellulose production, this isolate was subjected to random mutagenesis using UV radiation, two enhanced producer mutants (*G. xylinus* N2_87 and *G. xylinus* N2_90) were obtained and the productivity of cellulose in their culture filtrate was 6.3 and 6.9 g/L (increased by about 26% and 38%) respectively. Optimum conditions for cellulose production by *G. xylinus* N2_90 were growing this bacterium in production medium (HS medium) containing date syrup (2%) as a sole source of carbon and energy, yeast extract (2%) as a nitrogen source, in an initial pH 6.5 and incubation at 30°C for one week. Under these conditions, cellulose dry weight in culture filtrate of *G. xylinus* N2_90 was 8.5 g/L.

Key Words: Cellulose, *Gluconacetobacter xylinus*, Mutagenesis, Optimum conditions.

Introduction

Gluconacetobacter xylinus (formerly *Acetobacter xylinum)* is a gram-negative, rod-shaped, aerobic, non-pathogenic bacterium that secretes bacterial cellulose in the form of a pellicle on the surface of liquid culture (Ross *et al.*, 1991; McKenna *et al*., 2009). Bacterial cellulose (BC) is synthesized by several bacterial genera, *Gluconacetobacter xylinus*, which is the most efficient producer of cellulose. The production of this BC, is receiving great attention because of its unique properties such as lacking impurities, like lignin, hemicellulose or pectin, and its crystallinity and degree of polymerization are higher than those of plant cellulose (Ross *et al*., 1991). Because of its high tensile strength and water-holding capacity, bacterial cellulose has been used in various fields such as healthcare, cosmetics and beauty, clothing and shoes, baby care products, and audio products. It is also being used in paper industry to enhance paper strength and for making electronic paper. In pharmaceutical industry as wound dressing and gelling agents and in medicines in artificial skin, duraplasty, nerve anastomosis, artificial blood vessels or barrier to bone defects. It was used in preparation of nanocomposites for biomedical purposes and proton-

conducting membranes of fuel cells (Panesar *et al*., 2009; Lavoine *et al*., 2012; Cavka *et al*., 2013). The wide application of bacterial cellulose brings big interests towards the bacterial cellulose production for large commercial scale. Some attempts have been made in the area of optimization of culture conditions (Kouda *et al*., 1997), medium composition (Matsuoka *et al*., 1996) and strain improvement (Vandamme *et al*., 1998). Strain improvement is one approach to combat bacterial cellulose productivity, which included the isolation of mutants that effect cellulose production. A variety of mutagens have been used in an effort to induce mutation in *G. xylinus* i.e., N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), nitrous acid, ethyl methanesulfunate (EMS) and ultraviolet light are very effective. Ishikawa *et al*., (1995) obtained mutants of *A. xylinum* BPR 2001 by NTG (MNNG) treatment. They reported increased cell growth and cellulose production up to 40% higher than that of the parent strain. While Hungund and Gupta, (2010) reported that cellulose production for EMS and UV mutants of *G. xylinus* were 50% and 30% higher than the parent strain respectively.

In view of the facts mentioned above, aim of the present investigation was to increase cellulose productivity of *G. xylinus* N2 by ultraviolet (UV) radiation and study the optimum conditions for cellulose production.

Materials and Methods

Microorganism

Gluconacetobacter xylinus N2 was isolated in a previous study (Hameed *et al.*, 2012). The isolate was maintained at 4°C on modified Carr's agar medium [v/w: 3% yeast extract, 2% ethanol and 2% agar] (Sowden and Colvin, 1978). Hestrin-Schram broth medium (HS-medium) [v/w: 2% glucose, 0.5% yeast extract, 0.5% peptone, 0.27% Na_2HPO_4 and 0.115% citric acid] (Hwan *et al*., 2004) was used for the activation of this bacterium.

Mutagenesis of *G. xylinus* N2

Mutagenesis was induced according to Siripong *et al*., (2012) in an attempt to improve the ability of this isolate for production of cellulose by subjecting to UV radiation using the UV- transiluminator. The tray for irradiation was 15X25 cm, which exposed sample in glass petri dish, to direct irradiation from four bulbs of 15 watts, 254 nm and the distance between the UV source, and irradiated suspension was 11 cm. The dose rate of UV irradiation was 2.5 $J/m^2/s$. *G. xylinus* N2 was first grown in modified Carr's agar medium at 30°C for 48 hr, and then bacteria were harvested in

sterile phosphate buffer (pH 7) and mixed with a vortex mixer, followed by centrifugation (10 ml) at 4000 rpm for 10 min. The cell pellet was suspended in phosphate buffer and the suspension was poured in sterilized Petri dishes and exposed to UV radiation for 0, 20, 40, 60, 70, 80 seconds under sterile conditions. Then 0.1 ml of cell suspension was taken after each treatment, diluted to appropriate dilution and plated on modified Carr's agar medium. Plates were incubated at 30°C for 48 hr. to determine the viable count and survivals of bacterial cells. According to the survival curve, the treatment that led to a survival percentage of approximately 10% as compared with the control was considered to have a higher mutation rate. From this treatment, a number of colonies were picked up randomly and tested for cellulose production. One hundred colonies of suspected mutants were selected to examine cellulose production using HS broth medium (100 ml dispense in 500 ml Erlenmeyer flasks) and compared with wild type population.

Determination of optimal conditions for cellulose production by selected mutant of *G. xylinus* N2

Modification in HS medium (100 ml dispense in 500 ml Erlenmeyer flasks) components were tested to determine optimum culture conditions, like effect of carbon and nitrogen sources, and their concentrations, and effect of pH and temperature.

For these tests, modified HS media were inoculated with 1% (v/v) of fresh culture of mutant and incubated statically at 30°C for one week, after which, cellulose dry weight in culture filtrate was measured.

Cellulose production

Ability of bacterial isolate for cellulose production was determined by inoculating cellulose production medium (HS-medium) with 1 % (v/v) of fresh culture of bacterial isolate, and incubated statically at 30 °C for one week. Cellulose production was verified by appearance of white pellicle of cellulose on the surface of culture medium. Cellulose was then extracted from the production medium according to Son *et al*., (2001) by harvesting cellulose pellicles by filtration through pre-weighed filter paper No.1. It was washed with distilled water, heated in water bath with 0.5% NaOH at 80°C for 15 minute to remove microbial cells and other medium constituents, then washed with distilled water, placed in glass Petri dish and dried in oven at 105°C for 1-2 hrs. to determine cellulose dry weight (g/l) by difference.

Results And Discussion

Mutagenesis of *G. xylinus* N2

Results in figure 1 indicate that the survival percentage of *G. xylinus* N2 was 9.6% (killing 90.4%) after subjecting to UV irradiation for 70 seconds. So this treatment was selected for mutants' isolation because many studies mentioned that treatment allowing survival of approximately 10% of cell population generate more mutants (Queener and lively, 1986; Siripong *et al*., 2012).

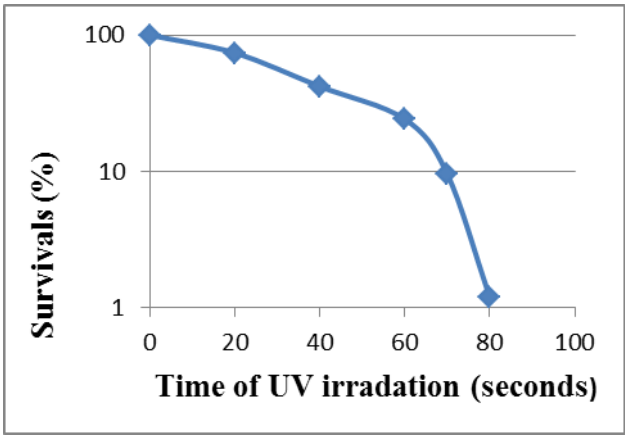

Figure 1: Effect of UV irradiation on *G. xylinus* N2 for different periods.

It is known that agent such as UV irradiation whose mutagenesis is affected via missrepair of damaged DNA by photoreactivation and SOS repair system can induce mutation in bacteria. These agents have been termed indirect mutagens (Al- Bakri and Umran, 1994).

In order to get cellulose enhanced hyper producer mutants of *G. xylinus* N2, one hundred colonies were selected from mutagenized culture. Results showed that ability of cellulose production varied among these mutants from 0 g/L to 6.9 g/L. Two of these mutants, N2_87 and N2_90 showed enhanced cellulose production of 6.3 and 6.9 g/L respectively, which was 26% and 38% higher in comparison with the productivity of wild type cells (5 g/L). 38% of these mutants lost their ability of cellulose production, while 13% of these mutants maintained their ability of cellulose production, and 47% of these mutants showed a decrease in their ability of cellulose production.

UV radiation was used successfully to induce random mutations in *G. xylinus* for different purposes. It was reported that UV mutants of *G. xylinus*

showed higher cellulose yield (30% more than that of the wild strain) (Hungund and Gupta, 2010). Also Siripong *et al*., (2012) reported that cellulose production by UV mutant of *G. xylinus* was 39.60% higher than that of the parent strain. However, some mutation may occur in cellulose production genes or in regulation genes also causing loss or decrease in cellulose production.

Optimum conditions for cellulose production by the mutant (*G. xylinus* N2_90)

1. Effect of carbon source: In order to examine the effect of carbon source on the ability of this mutant for cellulose production, HS medium was supplemented with one of the five different carbon sources (glucose, fructose, maltose, date syrup and ethanol) at a final concentration of 2%. Results (figure 2) showed that the maximum production of cellulose was achieved when date syrup was used as a sole source of carbon and energy. Cellulose dry weight in culture filtrate of this mutant was 7.8 g/L.

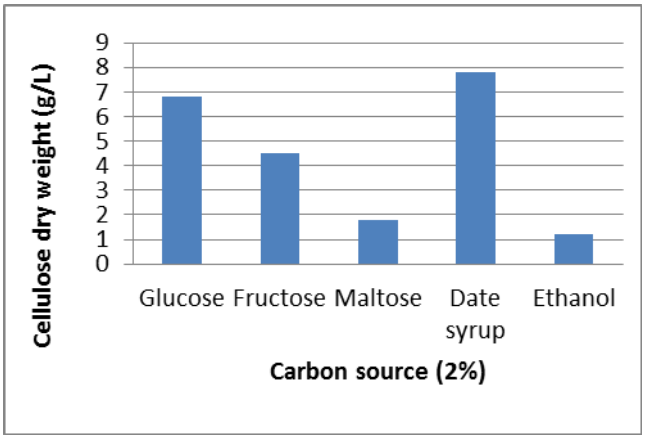

Figure 2: Effect of carbon source on cellulose production by *G. xylinus* N2_90 grown in modified HS media (pH 7) and incubated statically at 30°C for one week.

As mentioned above, the optimal carbon source for cellulose production was date syrup: this may be because it is rich in nutrients, which promote cell growth, and leads to increase cellulose production. It was found that yields of cellulose by *G. xylinus* in medium containing date syrup was approximately two times more than that in the medium containing sucrose (Marzieh and Alireza, 2011). Sang *et al*., (2010) also referred that cell growth and cellulose yield was higher in the date syrup medium than in the glucose medium.

2. Effect of date syrup concentration: Different concentrations (1, 2, 3, 4 and 5%) of the optimal carbon source (date syrup) were used to determine the optimum for cellulose production by this mutant. Results presented in figure 3 show that maximum cellulose production was obtained when date syrup was added to the HS production medium in a concentration of 2%. At this concentration cellulose dry weight was about 7.8 g/L. As a consequence of these results, date syrup (2%) was subsequently used.

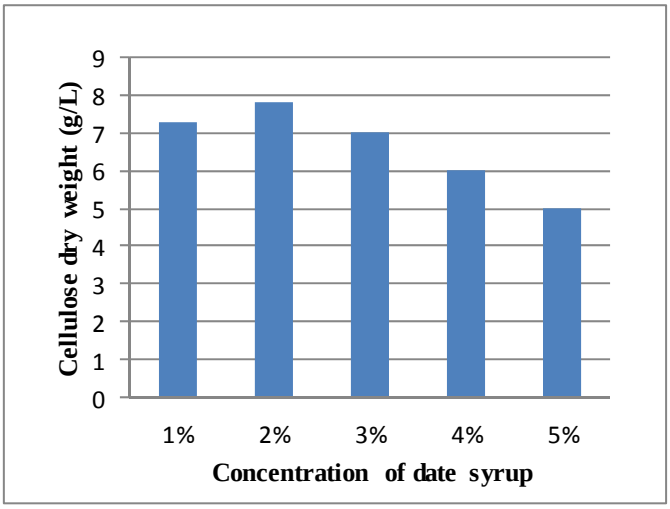

Figure 3: Effect of date syrup concentration on cellulose production by G. xylinus N2_90 grown in modified HS media (pH 7) and incubated statically at 30°C for one week.

Addition of date syrup in high concentration (3-5%) decrease cellulose production by the *G. xylinus* N2_90 which may be due to the increase in gluconic acid and acetic acid production that decrease medium pH leading to decrease cellulose production (Prashant *et al*., 2009).

Bae and Shoda, (2005) studied the production of cellulose by *G. xylinus* BPR 2001 using date syrup medium and they concluded that maintaining a lower concentration of the date syrup is essential for efficient cellulose production in jar fermentors, the effect being attributed mainly to the complex nature of date syrup.

3. Effect of nitrogen source: In order to examine the effect of nitrogen source on the ability of this mutant for production of cellulose, different nitrogen sources were added to the HS medium to determine the optimum for cellulose production. These nitrogen sources included organic sources (Tryptone, peptone, yeast extract and malt extract) and inorganic sources (urea and sodium nitrate) at a final concentration of 1%.

Results presented in figure 4 show that production of cellulose by *G. xylinus* N2_90 reached the maximum when production medium was supplemented with yeast extract and production of cellulose reached to 8.2 g/L. Peptone was also efficient in supplementing production medium because it induce cellulose production of 8 g/L.

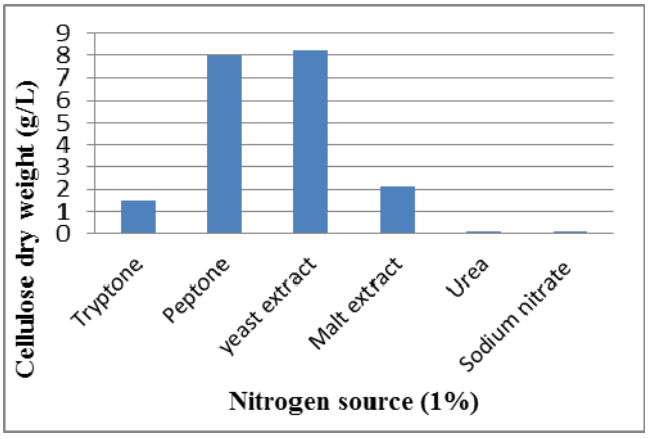

Figure 4: Effect of nitrogen source on cellulose production by *G. xylinus* N2_90 grown in modified HS media (pH 7) and incubated statically at 30°C for one week.

According to these results, yeast extract was the best among other nitrogen sources, this may be because yeast extract is an excellent stimulator of bacterial growth and nutrition providing nitrogen, amino acid, carbon and vitamins, especially vitamin B complex, that gives the requirements for microorganism for growth and cellulose production (Atlas, 2005).

This result was in agreement with Son *et al*. (2001) who reported that best nitrogen source for cellulose production by *G. xylinus* was yeast extract followed by peptone. Similar observation was reported by Al –Shmary (2007) who noticed that best nitrogen source for cellulose production by *G. xylinus* was yeast extract and next was peptone. However Panesar *et al*., (2009) found that peptone was most effective nitrogen source for cellulose production by *G. xylinus*.

4. Effect of nitrogen source concentration: Different concentrations (0.5, 1, 1.5, 2, and 2.5%) of yeast extract were used to determine the optimum for cellulose production by the mutant *G. xylinus* N2_90. Results presented in figure 5 show that maximum cellulose production (8.5 g/L) was obtained when yeast extract was added to the production medium (HS medium) at a concentration of 2% w/v. According to these results, 2% of yeast extract was used in the next experiments. However, Al –Shmary (2007) reported that optimal yeast extract concentration for cellulose production by *G. xylinus* was 0.8%.

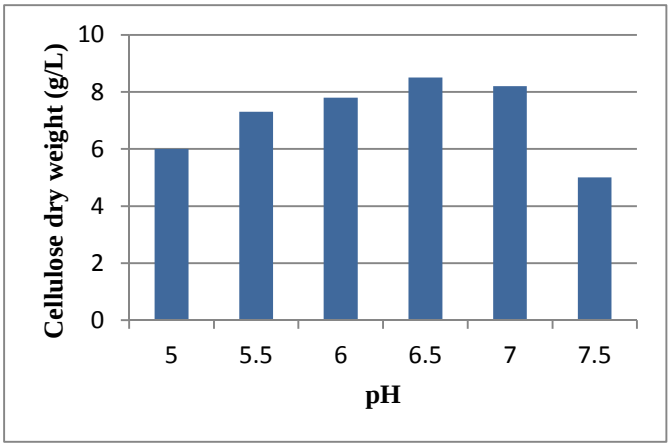

Figure 5: Effect of yeast extract concentration on cellulose production by *G. xylinus* N2_90 grown in modified HS media (pH 7) and incubated statically at 30°C for one week.

5. Effect of pH: To investigate the effect of initial medium pH on cellulose production by the mutant (*G. xylinus* N2_90), production medium (HS medium) was adjusted to different pH values (5, 5.5, 6, 6.5, 7 and 7.5).

Results presented in figure 6 show that maximum cellulose production was obtained when the medium pH value was adjusted to 6.5, at this pH cellulose dry weight in culture medium reached 8.5 g/L. pH 7 was also efficient for cellulose productivity in which cellulose dry weight reached to 8.2 g/L; it decreased above and below the optimum pH value. Because of these results, subsequent experiments were carried out at pH6.5.

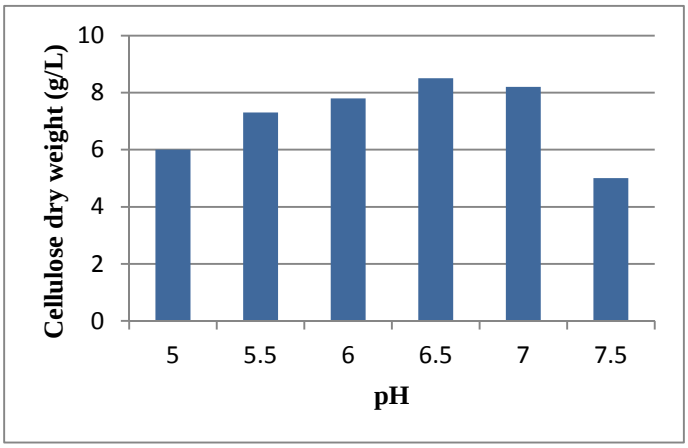

Figure 6: Effect of pH on cellulose production by mutant *G. xylinus* N2_90 grown in modified HS media and incubated statically at 30°C for one week.

The pH decreases during fermentative production because of the accumulation of gluconic, acetic or lactic acids in the culture broth, which bring down pH value of the production medium leading to inhibited cell growth.

This result was in agreement with Al –Shmary (2007) and Panesar *et al*., (2009) who found that optimal pH value for cellulose production by *G. xylinus* was 6.5-7.0.

6. Effect of temperature: Different incubation temperatures (20, 25, 30, 35, 40 and 45°C) were used to determine the optimum for cellulose production by the mutant (*G. xylinus* N2_90). Results depicted in figure 7 show that the maximum cellulose production was obtained when the culture medium was incubated at 30 °C for one week. At this temperature, the cellulose production reached its maximum of 8.5 g/L.

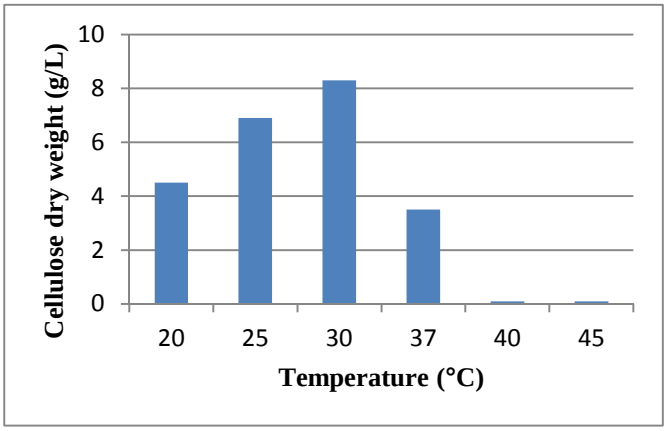

Figure 7: Effect of incubation temperature on cellulose production by *G. xylinus* N2_90 grown in modified HS media (pH 6.5) and incubated statically at 30°C.

This result is similar to that reported by Al –Shmary (2007). He found that optimal temperature for cellulose production by *G. xylinus* was at 28°C and 30°C. Meanwhile, Son *et al*., (2001) reported that there was no significant difference in the amount of cellulose production by *G. xylinus* when incubated at 25°C and 30°C with preference of the second on the first and a decrease in the productivity of cellulose when raising the temperature to 35°C.

Conclusions

- Mutagenesis of *G. xylinus* using UV ray was efficient in obtaining cellulose hyper producer mutants.
- The optimum conditions for cellulose production by *G. xylinus* N2_90 were growing this mutant in date syrup and yeast extract as a carbon and nitrogen sources respectively with concentration of 2% for both, at an initial pH of 6.5 and incubation at 30°C for one week.

Abbreviations:

BC: Bacterial cellulose; *G. xylinus*: *Gluconacetobacter xylinus*; *G. xylinus* N2_87 and *G. xylinus* N2_90: mutants of *G. xylinus* N2; UV: Ultraviolet; MNNG or NTG: N-methyl-N'-nitro-N-nitrosoguanidine; EMS: ethyl methanesulfunate; HS-medium: Hestrin-Schram broth medium.

References:

Al-Bakri, G. H. and Umran, M. A. 1994. Mutagenesis of novel halotolerant bacteria (*Micrococcus* sp.) using ultraviolet light and N-methyl-N-nitro-N-nitrosoguanidine. Iraq. J. Microbial. 6: 55-65.

Al-Shmary, E. I. 2007. Production of cellulose from Localy Isolated *Acetobacter xylinum* and assessing some of its properties. PhD thesis. University of Baghdad, Iraq.

Atlas, R. M. 2005. Handbook of Media for Environmental Microbiology. (2 nd Ed), Taylor and Francis Group, USA.

Bae, S. and Shoda, M. 2005. Statistical optimization of culture conditions for bacterial cellulose production using Box-Behnken esign. Biotechnol. Bioeng. 90: 20-28.

Cavka, A., Guo1, X., Tang, S-J., Winestrand, S., Jönsson, L. and Hong, F. 2013. Production of bacterial cellulose and enzyme from waste fiber sludge. Biotechnology for Biofuels. 6:25.

Hameed, Noor, D., Al-Jailawi, M., H. and Jassim, H. M. 2012. The Role of Plasmid in Cellulose Producing *Gluconacetobacter xylinus.* J. Plant Mol. Biol. Biotechnol. 3: 16-20.

Hungund, B. S. and Gupta, S. G. 2010. Strain improvement of *Gluconacetobacter xylinus* NCIM 2526 for bacterial cellulose production. African Journal of Biotechnology. 9: 5170-5172.

Hwan, Y. S., Lee, O. S., Lee, I. S., Kim, H. S., Yu, T. S. and Jeong, Y. J. 2004. *Gluconacetobacter persimmonis* sp. nov., isolated from Korean traditional persimmon vinegar. Journal of Microbiology and Biotechnology. 14: 276-283.

Ishikawa, A., Masuoka, M., Tsuchida, T. and Yoshinaga, F. 1995. Increase in cellulose production by sulfa guanidine-resistant mutants derived from *Acetobacter xylinum* subsp. *sucrofermentans*. Biosci. Biotechnol. Biochem. 59: 2259-2262.

Joris, K. and Vandamme, E. J. 1993. Novel production and applications of bacterial cellulose. Eur. Microbiol. 1: 27-29.

Kouda, T., Naritomi, T., Yano, H. and Yoshinaga, F. 1997. Effect of oxygen and carbon dioxide pressures on bacterial cellulose production by Acetobacter in aerated and agitated culture. J. Ferment Bioeng. 84: 124 -127.

Lavoine, N., Desloges, I., Dufresne, A., and Bras, J. 2012. Microfibrillated cellulose - its barrier properties and applications in cellulosic materials: a review. Carbohydr. Polym. 1: 90(2): 735-64.

Marzieh, M. N. and Alireza, Y. 2011. Biotechnological production of cellulose by

Gluconacetobacter xylinus from agricultural waste. Iranian journal of biotechnology. 9: 94-101.

Matsuoka M., Tsuchida, T., Matsushita, K., Adachi, O., and Yoshinaga, F. 1996. Asynthetic medium for bacterial cellulose production by *Acteobacter xylinum* subsp. *Sucrofermentant*, *Biosci*. Biotech. Biochem. 60: 575-579.

McKenna, B., Mikkelsen, D., Wehr, J., Gidley, M. and Menzies, N. 2009. Mechanical and structural properties of native and alkali-treated bacterial cellulose produced by *Gluconacetobacter xylinus* strain ATCC 53524. Cellul. 16: 1047-1055.

Panesar, P. S., Chavan, Y. V., Bera, M. B., Chand, O. and Kumar, H. 2009. Evaluation of *Acetobacter* Strain for the Production of Microbial Cellulose. Asian Journal of Chemistry. 21: 99-102.

Prashant, R. C., Ishwar, B. B., Shrikant, A. S. and Rekha, S. S. 2009. Microbial Cellulose: Fermentative production and applications. Food Technol. Biotechnol. 47: 107–124.

Queener, S. W. and Lively, D. H. 1986. Screening and selection for strain improvement. In: Manual of Industrial Microbiology and Biotechnology, pp. 155-169. American Society for Microbiology, Washington, D. C.

Ross, P., Mayer, R. and Benzimann, M. 1991. Cellulose biosynthesis and functions in bacteria. Res. Microbiol. 153: 205-212.

Sang, M. P., Sang, J. Y., Hong, J. S., Chung, Y, L. and Hong, S. K. 2010. Properties of bacterial cellulose cultured in different carbon sources. Polymer. 34: 522-526.

Siripong, P., Chuleekorn, S., and Duangporn, P. 2012. Enhanced cellulose production by ultraviolet (UV) irradiation and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis of an *Acetobacter* species isolate. African Journal of Biotechnology. 11: 1433-1442.

Son, H. J., Heo, M. S., Kim, Y. G. and Lee, S. J. 2001. Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter sp*. A9 in shaking cultures. Biotechnol Appl Biochem. 33: 1–5.

Sowden, L. C. and Colvin, J. R. 1978. Morphology, microstructure and development of colonies of *Acetobacter xylinum*. Can, J. Microbiol, 24: 772-779.

Vandamme, E. J., De Beets, S., Vanbaelen, A., Joris, K. and De Wulf, P. 1998. Improved production of bacterial cellulose and its application potential. Polymer. Degr. Stabil. 59: 93-99.

التحسين وأفضل الظروف لإنتاج السيليلوز بفعل بكتريا *Gluconacetobacter xylinus* N2

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الملخص:

لزيادة قابلية بكتريا *Gluconacetobacter xylinus* N2 على إنتاج السيليلوز، عرضت هذه البكتريا للتطفير العشوائي باستخدام الأشعة فوق البنفسجية، وتم الحصول على طافرتين (*G. xylinus* N2_87, *G. xylinus* N2_90) امتازت بزيادة قابليتها على إنتاج السيليلوز، إذ كان الوزن الصافي للسيليلوز المنتج 6.3 و 6.9 غم/ لتر وبزيادة بلغت حوالي 26% و 38% على التوالي.

استخدمت العزلة الطافرة ذات الإنتاجية الأعلى (*G. xylinus* N2_90) لدراسة الظروف المثلى لإنتاج السيليلوز، أظهرت النتائج أن الظروف المثلى لإنتاج السيليلوز بفعل هذه الطافرة هي بتنميتها في وسط الإنتاج الحاوي دبس التمر بتركيز (2%) مصدرا وحيدا للكاربون والطاقة، وخلاصة الخميرة بتركيز (2%) مصدرا نيتروجينيا وبرقم هيدروجيني ابتدائي 6.5 والحضن بدرجة 30°م مدة أسبوع. تحت هذه الظروف بلغ الوزن الصافي للسيليلوز المنتج بفعل هذه الطافرة (*G. xylinus* N2_90) 8.5 غم/لتر.

الكلمات المفتاحية: بكتريا إنتاج السليلوز، تطفير، سيليلوز، الظروف المثلى.