STUDY OF SPECIFIC METABOLIC PATTERN OF *ACETOBACTER XYLINUM* NUST4.2 AND BACTERIAL CELLULOSE PRODUCTION IMPROVEMENT

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Gluconic acid, as a by-product of bacterial cellulose (BC) fermentation, can be used as the sole carbon source for BC production by *Acetobacter xylinum*. In this study, a special metabolic pattern was found in *Acetobacter xylinum* NUST 4.2. Most of the glucose was converted to gluconic acid by NUST 4.2 in the early stage of fermentation, and then gluconic acid was used to synthesize bacterial cellulose. In order to improve the utilization rate of gluconic acid so as to improve the production of BC, we extended the fermentation period accordingly on the basis of our experience guiding us with regard to the end point of the fermentation. During this period, we paid more attention to the regulation of dissolved oxygen in the fermentation tank because of the low oxygen transfer efficiency of the system caused by a large amount of BC. The production of BC was increased exponentially after the extension of the fermentation period.

Keywords: *Acetobacter xylinum* NUST4.2, gluconic acid, metabolic pattern, bacterial cellulose

INTRODUCTION

Bacterial cellulose (BC), a biopolymer consisting of repeating units of D-glucose linked by β-1,4 glycosidic bonds, is attracting more and more research attention owing to its unique properties. Unlike plant cellulose, BC produced by certain bacteria (such as *Acetobacter xylinum*, *Agrobacterium*, *Rhizobium*, *Aerobacter*) possesses a superfine structure, superior purity and crystallinity, better morphological controllability and extremely hydrophilic surface,[1,2] which recommends BC as a multifunctional nano-biomaterial to be used in various fields, such as biomedicine, functional devices and soft tissue engineering.[3-6] However, the large-scale production and application of BC are limited because of its high production cost and low yield. The search for cheap carbon sources or high yield strains and improving the fermentation process parameters are the main ways to solve the problems mentioned above. Up to now, a large amount of work has been devoted to improving the production of BC by optimizing the composition of the broth,[7] the mutation of the bacteria,[8] looking for cheap carbon source substitutes[9,10] and improving the fermentation parameters,[11] such as pH and dissolved oxygen (DO). No matter which method is used, an obvious increase is achieved in bacterial cellulose production.

In the long-term pilot study of BC fermentation, an interesting phenomenon was observed from the patterns of fermentation. In the process of fermentation, the pH rebound phenomenon repeatedly occurred, especially when the pH of the fermentation broth was reduced to about 4.46, the pH of the broth increased rapidly. From the metabolic pathway of *Acetobacter xylinum*, a hypothesis is proposed for the phenomena mentioned above,[12] which suggests the existence of a tendency in *Acetobacter xylinum* NUST4.2 to convert most of the glucose into gluconic acid and acetic acid in the early stage of fermentation, and then a fraction (12.36%) of the glucose acid is used for the synthesis of BC.[13] Therefore, it is more practical to seek the optimal conditions for the consumption of gluconic acid to increase BC production.

Hence, a preliminary small-scale exploration was performed to find the optimal fermentation conditions of gluconic acid as the sole carbon source, which would offer a reference for the process parameters adjustment of our future industrial production of BC, especially for medium and longer periods of fermentation. During this period, we paid more attention to the regulation of DO by adjusting the stirring speed, ventilation volume, gas-liquid ratio of the fermentation tank because of

the low oxygen transfer efficiency of the system caused by a large amount of BC. Finally, we extended the fermentation period on the basis of our experience guiding us with regard to the end of the fermentation.

EXPERIMENTAL

Materials and culture conditions

The strains, *Acetobacter xylinum* NUST4.2 isolated from nature sources,[14] are stocked below -80 °C. Before fermentation, a strain activation process must be performed by culturing the *Acetobacter xylinum* NUST4.2 at 30 °C on agar slants. The strains were then inoculated into the seed medium for further culture at 30 °C for 2 days to form a certain concentration of cell suspension. Finally, the as-prepared cell suspension was inoculated into the fermentation broth designed according to our needs at an inoculum concentration of 10% (v/v).

Submerged fermentation for bacterial cellulose production

In this study, three different types of fermentation culture medium were prepared for different purposes and named as E_{11}, E_{12} and E_{21}, respectively. Gluconic acid or acetic acid were used as a sole carbon source in E_{11} and E_{12}, respectively. The purpose of preparing E_{11} and E_{12} was to study whether glucose acid and acetic acid can be used to produce bacterial cellulose. For E_{21}, a 100 L mechanical stirring fermentation tank and a 250 mL shake flask were selected. The chemical compositions of those media are described in Table 1.

Before the fermentation experiment, all the culture media were sterilized in an autoclave or in the fermentation tank at 121 °C for 15 min. A volume (50 mL) of E_{11} or E_{12}, in a 250 mL conical flask, with an initial pH of 4.6, was inoculated and incubated at 30 °C for 5 days in a Shaking incubator. A volume (75 L) of E_{21}, in a 100 L mechanical stirring fermentation tank, and a volume (50 mL) of E_{21}, in a 250 mL conical flask, were incubated at 30 °C with an initial pH of 5.4. The microbial seed was inoculated into the fermentation broth at an inoculum concentration of 10% (v/v).

Monitoring process parameters

Glucose concentration, the concentration of gluconic acid, BC production, viscosity and bacterial concentration were determined and recorded by sampling at time intervals of 2-4 hours. The DO and pH of the whole process of fermentation were continuously monitored by a pH electrode (InPro 3030, Mettler Toledo, Giessen, Germany), and a DO electrode (InPro 6800 O_2 sensor, Mettler Toledo, Giessen, Germany), respectively. The whole process was supported by the fermentation process analysis software system (FZ-D20L, Jiangsu Fengze Biological Engineering Equipment Manufacturing Co., Ltd., Nanjing, China). Fehling's method was used to determine the glucose concentration in the cultures.[15] The concentration of gluconic acid for the whole process was detected on an Agilent 1200 Series High Performance Liquid Chromatography System (Agilent Technologies Inc., Santa Clara, CA, USA).[13,16] The HPLC system was run on an SMA-H column (300×4.6 mm, SMA, USA) with a UV detector at 210 nm, and 5 mM H2SO4 was chosen as the mobile phase with a flow rate of 0.6 mL/min.

To determine the dry weight of BC, all the samples collected at different sampling time points were filtered to obtain suspended solids in the fermentation broth, and then the as-obtained suspended solids were treated with sodium hydroxide (3%, m/v) aqueous solution at 85 °C for 2 hours in order to lyse cells, followed by soaking it in 3‰ H_2O_2 solution and washing several times with deionized water until the water from the suspension showed neutral pH. The flocculated BC was oven dried at 80 °C until it reached constant weight. Viscosity was detected by a rotary viscometer (SNB-1, Shanghai Pingxuan Scientific Instrument Co., Ltd., Shanghai, China) at 30 r/min and 30 °C.

Table 1
Media and composition (g/L)

	G	G-ac	Suc	Ams	Pot	Mas	Cal	Csl	Aca	Cia	Cmc
E_{11}	-	46	-	14.5	5	0.7	0.2	-	-	-	-
E_{12}	-	-	-	14.5	5	0.7	0.2	-	49.5	-	-
E_{21}	22.5	-	27.5	1	5	0.7	0.2	20	1.5	0.6	0.4

G: glucose; G-ac: gluconic acid; Suc: sucrose; Ams: ammonium sulfate; Pot: potassium sulphate; Mas: magnesium sulfate; Cal: calcium lactate; Csl: corn steep liquor; Aca: acetic acid; Cia: citric acid; Cmc: sodium carboxymethyl cellulose; - absent

RESULTS AND DISSCUSSION

Gluconic acid or acetic acid were used as a sole carbon source in E_{11} and E_{12}, respectively. The purpose of preparing E_{11} and E_{12} was to study whether gluconic acid and acetic acid can be used to produce bacterial cellulose. In order to investigate which of the two acids could be used for the synthesis of BC, E_{11} and E_{12} were used as fermentation media, in which, gluconic acid and acetic acid were used as a sole carbon source, respectively. Corn steep liquor was replaced by inorganic nitrogen source $(NH_4)_2SO_4$ to eliminate the interference of complex components. E_{11} and E_{12} were inoculated using NUST4.2. After five days of shake flask culture, 10.42 g/L of BC was obtained from E_{11}. However, no BC was found in E_{12}. This demonstrates that the gluconic acid produced by NUST4.2 can be used as a sole carbon source by NUST4.2 for the synthesis of BC.

Gluconic acid and acetic acid, as by-products produced by *Acetobacter xylinum* in the fermentation process, reduced the pH of the fermentation broth, which was not conducive to the growth of *Acetobacter xylinum* and the accumulation of BC.[11] However, gluconic acid and acetic acid could be involved in some of the metabolic pathways of *Acetobacter xylinum*, and eventually transformed into BC, which can be reflected in the metabolic network constructed by Zhong *et al*.[12] For *Acetobacter xylinum* (CGMCCNO.29 55), Yassine *et al.*[17] believed that acetic acid could be used for cell growth by taking part in the Krebs cycle pathway that ended with CO_2 generation, or could be used as a precursor for the production of cellulose. They also found the existence of a competitive nutritional profile between glucose and acetic acid. Therefore, a hypothesis was proposed for the phenomena mentioned above. If this assumption was established, that would mean the existence of a special metabolic pattern in *Acetobacter xylinum* NUST4.2. We believed that most of the glucose was converted into gluconic acid and acetic acid in the early stage of fermentation by NUST4.2, and after that, most of the gluconic acid or acetic acid was used for the synthesis of the BC.

Analysis of fermentation profile (fermentation tank)

The fermentation profiles of the whole fermentation period for the 100 L mechanical stirring fermentation tank are given in Figure 1a and b. A lag phase of about 32.5 hours was observed from the curve of BC production shown in Figure 1a. During this phase, there is only an abysmally slow growth in the cellulose content, because of the low concentration of bacteria (about 0.21×10^8 cfu/mL), as shown in Figure 1b. In the later period of the lag phase, the growth of pH (from 5.2 to 5.7) and the decrease of DO (from 64% to 0%) were observed with a reduction in glucose concentration from 2% to 1.3%, which was caused by bacterial growth. Noro *et al*. held that the increase in pH at the early stage of fermentation was mainly due to the consumption of lactic acid, as the energy material in corn steep liquor, which can provide energy for the growth of *A. xylinum* through the TCA cycle.[18,19]

After the 32.5 h induction phase, a dramatic decline in pH from 5.7 (32.5 h) to 4.47 (37 h), followed by a sharp increase, was observed until 6.85 at about 51 h. During this period, dramatic changes occurred in the concentration of glucose (1.29~0.37%), production of BC (0.09~3.87 g/L), germ concentration (3.69~36.93 10^8) and viscosity (3.84~19.72 mPa.s). This period is usually called cellulose accumulation phase. It is worth noting that when the fermentation was carried out up to 39.8 h, the glucose concentration was almost exhausted (0.33%), a threshold value of glucose could be utilized in the mechanical tank. However, at this time, the BC production was only 1.28 g/L, and then the production of BC was greatly increased, with a rapid rise of pH, while the glucose concentration was still maintained at a constant low level. This implies the fact that the accumulation of a large amount of cellulose was based on the consumption of some acid produced by NUST 4.2 rather than of glucose.

In order to explain the accumulation and consumption of glucose acid in the fermentation broth, the concentration of glucose acid for the whole process was detected by the high performance liquid chromatography method.[16] The results are shown in Figure 1a and c. As we assumed previously, a large amount of gluconic acid accumulated in the early fermentation stage and reached a maximum value of 1.72% at 37 h, which means that 75% of the glucose was converted to gluconic acid. After that, a sharp decrease, from 1.72% to 0.6077% (52.8 h), in the concentration of glucose acid was observed with a rapid accumulation in the production of BC (1.08~3.86 g/L) and sharp increase in pH (4.46~6.8). Glucose dehydrogenase is located in the cell membrane of *A. xylinus*,[20] therefore, under certain conditions, it is possible that glucose was first oxidized to gluconic acid when it entered into the cells for the metabolism. A similar fermentation curve was obtained by using the shake flask

fermentation and the corresponding results are shown in Figure 1b and d, respectively.

However, it is worth noting that a more lengthy process of gluconic acid accumulation and consumption was found in the shake flask fermentation curve. A 90 hours gluconic acid accumulation and depletion process was observed from 20 h to 110 h, which is shown in Figure 1c. We attribute this long process to the difference of DO between the fermentation tank and shake flask. In the mechanical stirring tank, BC clumps formed more easily, while in the shake flasks, the BC was crushed into small pieces of flocculent cellulose under the action of the shear caused by the blade stirring, which made more bacteria previously wrapped in BC clumps to be released and effectively increased the contact chance between the bacteria and oxygen.

As shown in Figure 1a and c, for the whole fermentation process, whether in the fermentation tank or in the shake flask, the utilization of gluconic acid for the synthesis of BC only occurred after glucose was depleted. This shows that the competitive nutritional profile between glucose and acetic acid mentioned earlier is found between glucose and gluconic acid as well. We believe that this competitive nutritional profile originates from the degree different carbon sources are metabolized by NUST4.2. In other words, the difference in the energy demand between different metabolic patterns determines which carbon source is used first. This competitive nutritional profile represents the degree of demand for oxygen in the fermentation process.

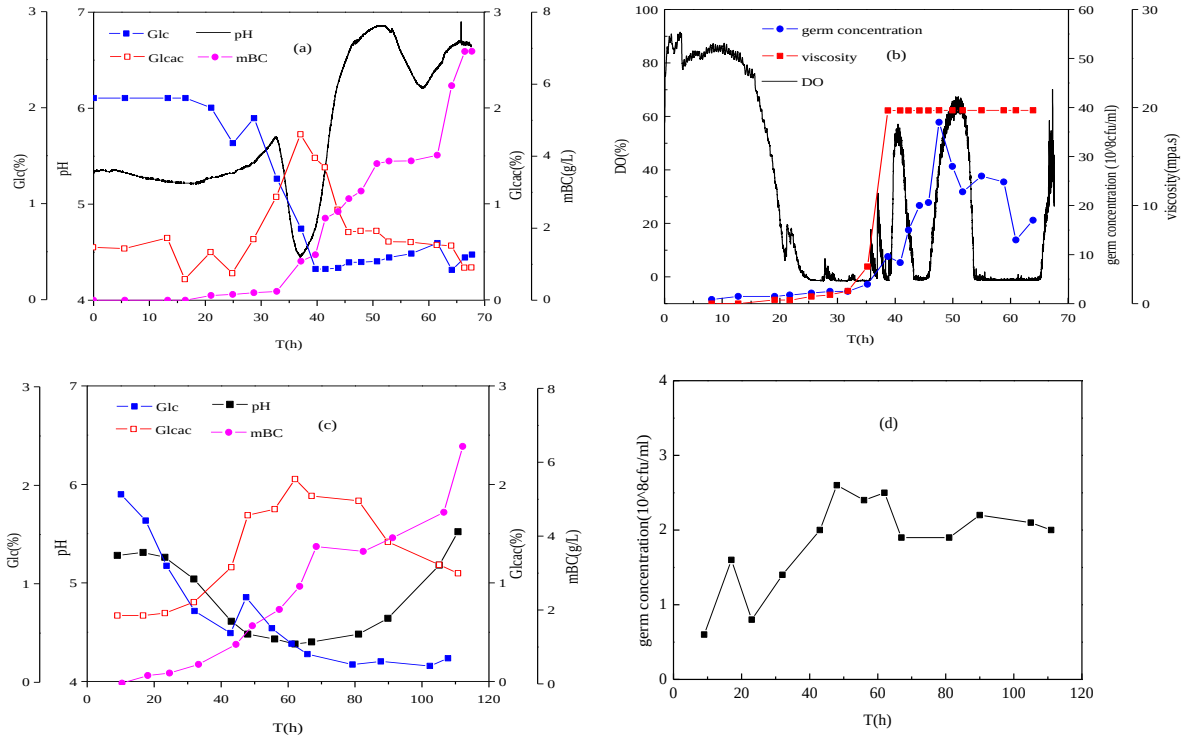

Figure 1: Process parameters curves for (a, b) 100 L fermentation tank and (c, d) 250 mL shake flask

As *Acetobacter xylinum* are aerobic bacteria, therefore, compared with the fermentation process in the shake flask, the glucose acid accumulated in the early days could be metabolized by *Acetobacter xylinum* for the synthesis of BC, due to the good conditions of DO in the fermentation tank. However, most of the glucose acid was used for the growth of bacteria rather than for the accumulation of cellulose, because of the higher bacterial concentration. This can be remarked in Figure 1b and d. Furthermore, the degradation of the production capacity of *Acetobacter xylinum* caused by the shearing action from the stirring paddle also results in the reduction of BC production. This is the reason why the dynamic fermentation has the characteristics of a short fermentation period: high production efficiency, but low yield. It is inevitable that glucose would convert into gluconic acid at the beginning of fermentation, due to the existence of glucose dehydrogenase in the cell membrane. The accumulation of BC was based on the consumption a large amount of gluconic acid. As a matter of fact, a similar metabolic pattern was found in *Acetobacter xylinum* BRC5.[21] The researchers found that the strain BRCS transmuted almost all of the glucose into gluconic acid in the early stage of

fermentation, and after that, it utilized the gluconic acid accumulated in the broth for the synthesis of BC.

In the initial stage of fermentation, it was inevitable for the glucose to be oxidized to glucose acid by the enzyme glucose dehydrogenase. Moreover, the enzymes had higher enzyme activity under acidic conditions.[22] Therefore, they found that acidic conditions could shorten the long process of glucose oxidation and the whole fermentation period. The researchers believe that the sharp decline in pH caused by large accumulation of gluconic acid is not conducive to bacterial growth and BC accumulation.

In fact, the opposite conclusion was drawn in this study. We found that acidic conditions are more suitable for the synthesis of BC when gluconic acid is used as sole carbon source. Similar to glucose, gluconic acid has a threshold value that can be used by NUST 4.2. The threshold value is reflected by the residual amount of gluconic acid at the fermentation end point. A detailed study is necessary by the experimental design to find the optimum condition of gluconic acid used for the synthesis of cellulose, which will be of great significance in regulating the process parameters in the process of cellulose accumulation.

Analysis of the control process for DO

During the later stage of fermentation, the oxygen transfer efficiency was reduced because of the increase in the yield of cellulose and the formation of other viscous by-products, such as exopolysaccharides. The growth of aerobic *Acetobacter xylinum* bacteria relies to a great extent on the conditions of DO in the fermentation system. The curves of DO, viscosity and bacterial concentration in the 100 Liter fermentor are shown in Figure 1b. In 15.8 hours of cellulose accumulation, the accumulation of a large amount of cellulose did not cause significant changes in the viscosity of the fermentation system. However, in this period, the viscosity of the system maintained the maximum value at about 19.72, which made a great impact on the oxygen transfer efficiency.

A very obvious trend can be noted in Figure 1b, namely, as the DO rose, bacterial concentration increased and then decreased. Since the conversion of glucose to gluconic acid was inevitable, in order to improve the yield of bacterial cellulose, compared with the method that reduces the production of gluconic acid by means of mutagenesis, it is more practical to seek the optimal conditions for the consumption of gluconic acid to improve the utilization of gluconic acid.

Although a large amount of gluconic acid was used for the synthesis of cellulose and the growth of bacteria, a large amount of gluconic acid still remained in the fermentation broth at the end of fermentation. Considering the culture in the shaker,[13] we can improve the utilization degree and reduce the remains of gluconic acid at the end of fermentation by extending the fermentation time and controlling the DO in the mechanical stirring fermentation tank. In this study, in order to improve the utilization rate of gluconic acid, so as to reduce the residue of gluconic acid at the end of fermentation, we extended the fermentation period correspondingly, from 56.8 h, based on our experience regarding the end point of the fermentation, as reflected in Figure 1a. During this period, 10 L of liquid material was released and we paid more attention to the regulation of DO by adjusting the stirring speed, ventilation volume, gas-liquid ratio and tank pressure of the fermentation tank because of the low oxygen transfer efficiency of the system caused by the large amount of BC and some of the by-products. Moreover, a large amount of oxygen was needed to maintain a high concentration of bacteria. The adjustment process of DO for the whole process, by adjusting the agitation speed and tank pressure, is displayed in Figure 2.

The production of BC increased exponentially after the extension of the fermentation period. This can be observed from the BC yield curve shown in Figure 1a. After prolonging the fermentation with 10.8 h, with a decrease in the concentration of gluconic acid from 0.6025% to 0.3363%, the final yield of 6.9 g/L was achieved. When the fermentation period was extended from 56.85 h to 64 h, although in the meantime the stirring speed and tank pressure were maintained at a higher level, and an increase in ventilation was performed from 5.4 Nm^3/h to 7.3 Nm^3/h, a poor DO was observed. The lower DO led to a decrease in bacterial concentration from 2.6×10^9 to 1.3×10^9.

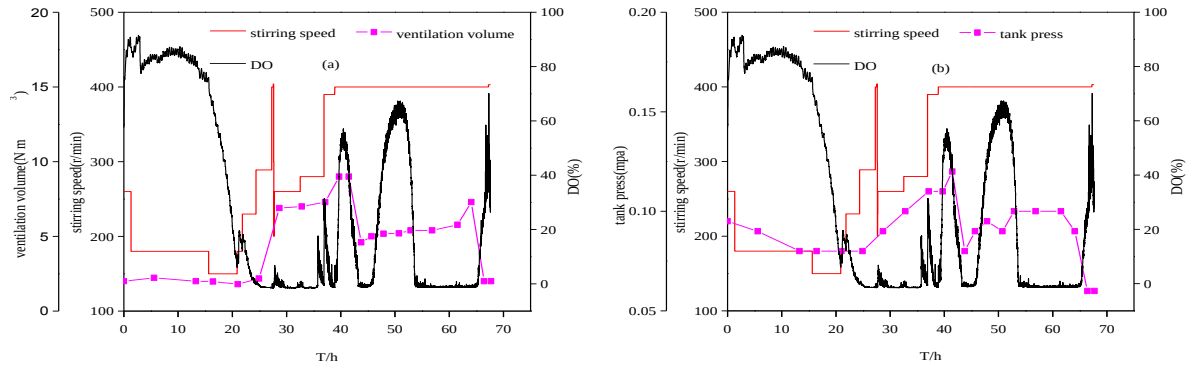

Figure 2: Control of DO by (a) stirring speed and ventilation volume; (b) tank pressure and stirring speed

After that, it is remarkable that although the tank pressure and ventilation volume were reduced to 2 Nm^3/h and 0.06 Mpa, respectively, the DO increased dramatically and was maintained at 35.73% until 67.7 h. This phenomenon cannot be attributed solely to the fact that the decrease in bacterial concentration reduced the demand for oxygen because the oxygen transfer mode in this period had changed.

At the end of the fermentation process, due to the increase of the BC floc, the fermentation broth was held by the hydrophilic cellulose; almost no liquid remained in the tank, which enabled direct contact of the bacteria on the surface of BC with the air and was more conducive to further bacterial growth. In the last part of fermentation, although the speed of 400 r/min was maintained until the end of fermentation, the high shear stress from the high speed was suitable for the bacteria at this time, because the bacteria were wrapped by flocculent cellulose. Jung *et al*.[23] believed that the BC could protect the bacteria, to a certain extent, from the effect of shear stress because the bacteria are wrapped in BC.

CONCLUSION

A unique metabolic pattern was found in *Acetobacter xylinum* NUST4.2. Comparing the fermentation curve for the fermentation tank with that for the shake flask, we believe that faster accumulation and consumption of gluconic acid in the fermentation tank was the reason why the fermentation tank was characterized by higher efficiency. The higher efficiency occurred by virtue of the good DO condition in the fermentation tank.

More favorable conditions for BC production need to be researched, for instance, the acidic condition has been noted to be more favorable for the conversion of gluconic acid to BC. Similar to glucose, gluconic acid has a threshold value that can be used by NUST 4.2. This threshold value has an important guiding role in deciding upon the new fermentation terminating point. However, in the selection of the fermentation period, we must consider both the production cost and the additional cost incurred by the extended period, which will be discussed in detail in our next paper.

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REFERENCES

1 R. Jonas and F. Farah, *Polym. Degrad. Stabil.*, **59**, 101 (1998).
2 Y. Huang, C. L. Zhu, J. Z. Yang, Y. Nie, C. T. Chen *et al.*, *Cellulose*, **21**, 1 (2014).
3 W. K. Zhu, W. Li, Y. He and T. Duan, *Appl. Surf. Sci.*, **338**, 22 (2015).
4 S. Y. Chen, S. Wei, F. Yu, W. L. Hu and H. P. Wang, *J. Appl. Polym. Sci.*, **117**, 8 (2010).
5 M. Esguerra, H. Fink, M. W. Laschke, A. Jeppsson, D. Delbro *et al.*, *J. Biomed. Mater. Res. A*, **93**, 140 (2010).
6 N. Petersen and P. Gatenholm, *Appl. Microbiol. Biotechnol.*, **91**, 1277 (2011).
7 S. Hegde, G. Bhadri, K. Narsapur, S. Koppal, P. Oswal *et al.*, *J. Bioprocess. Biotech.*, **4**, 142 (2013).
8 R. Q. Wu, Z. X. Li, J. P. Yang, X. H. Xing, D. Y. Shao *et al.*, *Cellulose*, **17**, 399 (2010).
9 A. Vazquez, M. L. Foresti, P. Cerrutti and M. Galvagno, *J. Polym. Environ.*, **21**, 545 (2013).

[10] X. Y. Yang, C. Huang, H. J. Guo, L. Xiong, Y. Y. Li *et al*., *J*. *Appl*. *Microbiol*., **115**, 995 (2013).
[11] C. H. Kuo, J. H. Chen, B. K. Liou and C. K. Lee, *Food Hydrocoll*., **53**, 98 (2015).
[12] C. Zhong, G. C. Zhang, M. Liu, X. T. Zheng, P. P. Han *et al*., *Appl*. *Microbiol*. *Biotechnol*., **97**, 6189 (2013).
[13] H. Zhang, C. T. Chen, C. L. Zhu and D. P. Sun, *Cellulose Chem*. *Technol*., **50**, 997 (2016).
[14] L. L. Zhou, D. P. Sun, L. Y. Hu, Y. W. Li and J. Z. Yang, *J*. *Ind*. *Microbiol*. *Biotechnol*., **34**, 483 (2007).
[15] J. G. Shi, *Food and Fermentation Industries*, **32**, 165 (2006).
[16] C. Zhong, F. Li, M. Liu, X. N. Yang, H. X. Zhu *et al*., *PLoS One*, **9**, e98772 (2014).
[17] F. Yassine, N. Bassil, R. Flouty, A. Chokr, A. E. Samrani *et al*., *Carbohyd*. *Polym*., **146**, 282 (2016).
[18] N. Noro, Y. Sugano and M. Shoda, *Appl*. *Microbiol*. *Biotechnol*., **64**, 199 (2004).
[19] M. Matsuoka, T. Tsuchida, K. Matsushita, O. Adachi and F. Yoshinaga, *Biosci*. *Biotechnol*. *Biochem*., **60**, 575 (1996).
[20] T. Shigematsu, K. Takamine, M. Kitazato, T. Morita, T. Naritomi *et al*., *J*. *Biosci*. *Bioeng*., **99**, 415 (2005).
[21] J. W. Hwang, Y. K. Yang, J. K. Hwang, Y. R. Pyun and Y. S. Kim, *J*. *Biosci*. *Bioeng*., **88**, 183 (1999).
[22] K. Matsushita, H. Toyama and O. Adachi, *Adv*. *Microb*. *Physiol*., **36**, 247 (1994).
[23] J. Y. Jung, J. K. Park and H. N. Chang, *Enzyme Microb*. *Technol*., **37**, 347 (2005).