Video Article

Production of Chemicals by *Klebsiella pneumoniae* Using Bamboo Hydrolysate as Feedstock

Dong Wei^{1,2}, Jinjie Gu¹, Zhongxi Zhang¹, Chenhong Wang¹, Dexin Wang¹, Chul Ho Kim³, Biao Jiang¹, Jiping Shi^{1,4}, Jian Hao¹

¹Lab of Biorefinery, Shanghai Advanced Research Institute, Chinese Academy of Sciences

²University of Chinese Academy of Sciences

³Biorefinery Research Center, Jeonbuk Branch Institute, Korea Research Institute of Bioscience & Biotechnology (KRIBB)

⁴School of Life Science and Technology, ShanghaiTech University

Correspondence to: Jiping Shi at shijp@sari.ac.cn, Jian Hao at haoj@sari.ac.cn

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Abstract

Bamboo is an important biomass, and bamboo hydrolysate is used by *Klebsiella pneumoniae* as a feedstock for chemical production. Here, bamboo powder was pretreated with NaOH and washed to a neutral pH. Cellulase was added to the pretreated bamboo powder to generate the hydrolysate, which contained 30 g/L glucose and 15 g/L xylose and was used as the carbon source to prepare a medium for chemical production. When cultured in microaerobic conditions, 12.7 g/L 2,3-butanediol was produced by wildtype *K. pneumoniae*. In aerobic conditions, 13.0 g/L *R*-acetoin was produced by the *budC* mutant of *K. pneumoniae*. A mixture of 25.5 g/L 2-ketogluconic acid and 13.6 g/L xylonic acid was produced by the *budA* mutant of *K. pneumoniae* in a two-stage, pH-controlled fermentation with high air supplementation. In the first stage of fermentation, the culture was maintained at a neutral pH; after cell growth, the fermentation proceeded to the second stage, during which the culture was allowed to become acidic.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55828/>

Introduction

Klebsiella pneumoniae is a bacterium that grows well under both aerobic and anaerobic conditions. *K. pneumoniae* is an important industrial microorganism used to produce many chemicals. 1,3-propanediol is a valuable chemical that is mainly used as a monomer to synthesize polytrimethylene terephthalate. Polytrimethylene terephthalate is a biodegradable polyester that exhibits better properties than those of 1,2-propanediol, butanediol, or ethylene glycol¹. 1,3-propanediol is produced by *K. pneumoniae* using glycerol as a substrate in oxygen-limited conditions². 2,3-butanediol and its derivatives have applications in the field of plastics, solvent production, and synthetic rubber and have the potential to be used as biofuel³. With glucose as the substrate, 2,3-butanediol is the main metabolite of the wildtype strain⁴. 2,3-butanediol is synthesized from pyruvate. First, two molecules of pyruvate condense to yield α -acetylactate; this reaction is catalyzed by α -acetylactate synthase. α -acetylactate is then converted to acetoin by α -acetylactate decarboxylase. *R*-acetoin can be further reduced to 2,3-butanediol when catalyzed by butanediol dehydrogenase. An efficient gene replacement method suitable for *K. pneumoniae* has been explored, and many mutants have been constructed^{5,6,7}. A *budC* mutant, which lost its 2,3-butanediol dehydrogenase activity, accumulates high levels of acetoin in culture broth. Acetoin is used as an additive to enhance the flavor of food⁸. When *budA*, which encodes α -acetylactate decarboxylase, is mutated, 2-ketogluconic acid accumulates in the broth. 2-ketogluconic acid is used for the synthesis of erythorbic acid (isoascorbic acid), an antioxidant used in the food industry⁹. 2-ketogluconic acid is an intermediate of the glucose oxidation pathway; in this pathway, located in the periplasmic space, glucose is oxidized to gluconic acid and then further oxidized to 2-ketogluconic acid. Gluconic acid and 2-ketogluconic acid produced in the periplasm can be transported to the cytoplasm for further metabolism. The accumulation of 2-ketogluconic acid is dependent upon acidic conditions, and higher air supplementation favors 2-ketogluconic acid production¹⁰. Gluconate dehydrogenase, encoded by *gad*, catalyzes the conversion of gluconic acid to 2-ketogluconic acid. The *gad* mutant of *K. pneumoniae* produced high levels of gluconic acid instead of 2-ketogluconic acid, and this process is also dependent upon acidic conditions. Gluconic acid is a bulk organic acid and is used as an additive to increase the properties of cement¹¹. Glucose oxidation to gluconic acid is catalyzed by glucose dehydrogenase. Xylose is also a suitable substrate of glucose dehydrogenase. When xylose is used as a substrate, *K. pneumoniae* produces xylonic acid¹².

Chemical production using biomass as a feedstock is a hot topic in biotechnology¹³. The main components of biomass are cellulose, hemicellulose, and lignin. However, these macromolecular compounds cannot be directly catabolized by most microorganisms (including *K. pneumoniae*). Cellulose and hemicelluloses in the biomass must be hydrolyzed to glucose and xylose and can then be used by microorganisms. The presence of lignin in lignocelluloses creates a protective barrier that prevents biomass hydrolyzation by enzymes. Thus, a pretreatment

process that removes lignin and hemicelluloses and reduces the crystallinity of cellulose is always performed during biomass utilization by microorganisms. Many pretreatment methods have been developed: acid, alkaline, ammonia, and steam pretreatments are common.

Bamboo is abundant in tropical and subtropical regions and is an important biomass resource. Here, the preparation of bamboo hydrolysate and chemical production using bamboo hydrolysate are presented

Protocol

1. Preparation of Bamboo Hydrolysate

- Add 5 g of bamboo powder to 40 mL of an NaOH solution to achieve a 10% (g/g) final concentration in a 250 mL flask. Use a series of NaOH solutions ranging from 0.05 M to 0.50 M in increments of 0.05 M.**
 - Incubate the mixture for 60 min at 60 °C or 100 °C in a water bath. Incubate at 121 °C in an autoclave.
- After incubation, leave the mixture to stand for 4 h at room temperature. Then, remove the supernatant. Add 100 mL of fresh water and mix.**
 - Repeat this washing 5-6 times, until the pH of the supernatant reaches 6.8. Use the obtained solid for the next step of enzymatic hydrolysis.
- For hydrolysis, adjust 50 mL of the above mixture to pH 5.0 using 98% H₂SO₄. Then, add 0.5 mL of 200 filter paper activity (FPU)/mL cellulase.
NOTE: The final ratio of cellulase to bamboo should be 20 FPU per 1 g of bamboo.
- Incubate the mixture at 50 °C for 36 h in a shaker.
NOTE: After hydrolysis, some solid will remain in the mixture.
- Obtain a clear hydrolysate by centrifuging at 7,690 x g for 5 min.
- Quantify the glucose, xylose, and other chemicals with a high-pressure liquid chromatograph system equipped with a refractive index detector and a photodiode array detector. Use an HPX-87H column (300 mm x 7.8 mm) and a mobile phase of 5 mM H₂SO₄ solution at a 0.8 mL/min flow rate¹².
- For a large volume of hydrolysate preparation, mix 2 kg of bamboo powder and 18 L of 0.25 M NaOH in a 30-L stainless-steel tank.**
 - Incubate the tank at 121 °C in an autoclave for 60 min. After incubation, wash the mixture with 40 L of water in a 60 L plastic tank. Repeat this washing 5 times.
 - Use water to adjust the total washed bamboo to a volume of 20 L. Adjust the pH of the mixture to 5.0 using 98% H₂SO₄.
 - Enzymatically hydrolyze the mixture in a modified shaking water bath, equipped with a mechanical agitator to ensure that the mixture is well distributed. Add 200 mL of 200 FPU/mL cellulase and incubate the mixture at 50 °C for 36 h.
 - After hydrolysis, centrifuge the mixture at 4,700 x g for 10 min.

2. 2,3-Butanediol Production by Wildtype *K. pneumoniae*

- Use 3 L of bamboo hydrolysate as the solvent for medium preparation.
- Prepare a fermentation medium containing 4 g/L corn steep liquor, 2 g/L (NH₄)₂SO₄, 6 g/L K₂HPO₄, 3 g/L KH₂PO₄, and 0.2 g/L MgSO₄. Adjust the pH of the medium to neutral using 2.5 M NaOH. Use a 5-L bioreactor containing 3 L of autoclaved fermentation medium for fermentation.
- Use *K. pneumoniae* cells stored in a -80 °C low-temperature refrigerator.
- For the seed culture, incubate a 250 mL flask containing 50 mL of Luria-Bertani (LB) medium overnight at 37 °C, with shaking at 200 rpm. Use LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl.
NOTE: The cell density of the seed culture should reach OD 2 (optical density at 600 nm).
- Inoculate one flask of 50 mL of the seed culture in the bioreactor. Maintain the culture at pH 6.0 and 37 °C. Use an air supplementation rate of 2 L/min and an agitation rate of 250 rpm, creating a microaerobic condition.
- Collect 5 mL samples every 2 h during the fermentation and analyze them with high-pressure liquid chromatography to determine the chemical concentrations in the broth⁴.**
 - Monitor the alkali added to the bioreactor online using MFCS/DA.
NOTE: Organic acids are produced in the fermentation process, and NaOH is added to keep the culture pH stable. The volume of alkali added represents the amount of acid produced. When the alkali-added line plateaus, the process is finished, either because of carbon source exhaustion or cell death.

3. R-acetoin Production by the *budC* Mutant of *K. pneumoniae*

- Prepare a medium for acetoin production containing 4 g/L corn steep liquor, 2 g/L (NH₄)₂SO₄, 3 g/L sodium acetate, 0.4 g/L KCl, and 0.1 g/L MgSO₄.
- Use *K. pneumoniae-ΔbudC* for R-acetoin production. Repeat steps 2.4-2.5.
NOTE: *budC* encodes 2,3-butanediol dehydrogenase. *K. pneumoniae-ΔbudC* loses the 2,3-butanediol dehydrogenase activity and produces R-acetoin instead of 2,3-butanediol.
- Maintain the culture at pH 6.0 and 37 °C. Use an air supplementation rate of 4 L/min and an agitation rate of 450 rpm, an aerobic condition.
NOTE: The oxygen supplementation is higher than that of 2,3-butanediol production.
- Assay the samples as in step 2.6.

4. 2-Ketogluconic Acid Production by the *budA* Mutant of *K. pneumoniae*

1. For 2-ketogluconic acid production, use the same medium as for *R*-acetoin production.
2. Use *K. pneumoniae*- Δ *budA* for 2-ketogluconic acid production. Repeat steps 2.4-2.5.
NOTE: *budA* encodes α -acetolactate decarboxylase. *K. pneumoniae*- Δ *budA* loses the α -acetolactate decarboxylase activity and produces 2-ketogluconic acid, using glucose as a substrate.
NOTE: 2-ketogluconic acid synthesis is an acidic condition-dependent process. A two-stage fermentation has been developed for 2-ketogluconic acid production¹⁰; in the first stage, the seed culture is inoculated in the bioreactor.
3. Maintain the culture at pH 7.0 and 37 °C. Use an air supplementation rate of 4 L/min and an agitation rate of 500 rpm.
NOTE: In these conditions, cells grow very fast (the cell density reaches OD 7 in about 4 h). Then, the fermentation progresses to the second stage, during which the culture pH decreases to 5.0. No acid is added; the organic acids produced in the culture naturally lead to the pH decrease. In these acidic conditions, cell growth stops, but 2-ketogluconic acid is synthesized and accumulates in the broth.
4. Assay the samples as in step 2.6. Using the hydrolysate of bamboo as the carbon source.
NOTE: The glucose in the medium is converted to 2-ketogluconic acid, and the xylose is converted to xylonic acid. Thus, a mixture of 2-ketogluconic acid and xylonic acid is obtained.

Representative Results

In this protocol, the bamboo was pretreated using alkali. The optimal incubation parameters—a temperature of 121 °C and 0.25 M NaOH—were determined in **Figures 1** and **2**. The pretreated bamboo was enzymatically hydrolyzed, and the glucose and xylose concentrations obtained in the hydrolysate were measured. Higher temperatures favored sugar production, so 121 °C was selected as the optimal temperature. The glucose and xylose produced in the hydrolysate increased with NaOH concentration over the range 0.05-0.25 M. Further increases in the NaOH concentration had no positive effect on sugar production. Thus, NaOH at 0.25 M was selected as the optimal concentration.

Figure 1: The effect of pretreatment temperature on glucose and xylose concentration in bamboo hydrolysate. Red column: glucose; blue column: xylose. Higher temperatures favored sugar production, and 121 °C was selected for the large-volume hydrolysate preparation. [Please click here to view a larger version of this figure.](#)

Figure 2: The effect of the NaOH concentration used during the pretreatment on glucose and xylose concentration in the bamboo hydrolysate. Red line: glucose; blue line: xylose. The glucose and xylose produced in the hydrolysate increased with NaOH concentration over the range of 0.05–0.50 M, and 0.25 M was selected for the large-volume hydrolysate preparation. [Please click here to view a larger version of this figure.](#)

About 20 g/L glucose and 10 g/L xylose were produced during the hydrolysate in the flask-scale enzymatic hydrolysis; 30 g/L glucose and 15 g/L xylose were obtained from the large-volume hydrolysate preparation. This was because the large-volume preparation was performed in an open water bath shaker, and some water evaporated in the process, leading to the concentration of the hydrolysate. The glucose and xylose in the bamboo hydrolysate were used by *K. pneumoniae* as the carbon sources for chemical production. Other compounds in the hydrolysate were: cellobiose (1.4 g/L), arabinose (8.9 g/L), acetic acid (1.9 g/L), and formic acid (0.2 g/L).

2,3-butanediol was produced by *K. pneumoniae* in microaerobic conditions (**Figure 3**). The process was divided into two periods. In the first, glucose was used by the cells to produce 7.6 g/L 2,3-butanediol, while the xylose level in the broth remained unchanged. The glucose was exhausted at 8 h, and this time marked the shift to the next period. In the second period, the xylose in the broth was used by the cells, and an additional 5.1 g/L 2,3-butanediol was produced. The production of 2,3-butanediol was slower in the second period. At the end of the process, a total of 12.7 g/L 2,3-butanediol had been produced. Byproducts of this process were lactic acid, acetic acid, and ethanol. Lactic acid and ethanol were mainly synthesized in the first period (when glucose was used as the carbon source), and acetic acid was synthesized continuously.

Figure 3: 2,3-butanediol production using bamboo hydrolysate as the feedstock. Red line: glucose; blue line: xylose; magenta line: 2,3-butanediol; orange line: lactic acid; black line: acetic acid; green line: ethanol. The glucose and xylose in the hydrolysate were both used for 2,3-butanediol synthesis, but glucose was used first, followed by xylose. [Please click here to view a larger version of this figure.](#)

R-acetoin was produced by the *budC* mutant of *K. pneumoniae* under aerobic conditions (**Figure 4**). As in 2,3-butanediol production by the wildtype strain, glucose and then xylose were used in sequence by *K. pneumoniae*- Δ *budC*. Xylose was exhausted at 16 h, and the consumption rate was faster than that in 2,3-butanediol production by the wildtype. The production of *R*-acetoin was 13 g/L at the end of the process, and the byproducts were 2,3-butanediol, acetic acid, and ethanol.

Figure 4: *R*-acetoin production using bamboo hydrolysate as the feedstock. Red line: glucose; blue line: xylose; violet line: acetoin; magenta line: 2,3-butanediol; black line: acetic acid; green line: ethanol. The glucose and xylose in the hydrolysate were both used for *R*-acetoin synthesis, and their usage was in sequence. [Please click here to view a larger version of this figure.](#)

2-ketogluconic acid and xylonic acid were produced by the *budA* mutant of *K. pneumoniae* (**Figure 5**). This process required a high air supplement. The glucose in the medium was first converted to gluconic acid, which accumulated in the broth. The gluconic acid reached a maximum level of 15 g/L at 8 h of culture, and after that, its concentration decreased. No 2-ketogluconic acid was produced until 6 h into the culture. It was then synthesized at a high rate, and 25 g/L 2-ketogluconic acid was produced by the end of the process. The xylose in the medium was converted to xylonic acid; this reaction began later than the glucose conversion to gluconic acid. Some acetic acid was produced as a byproduct during the process.

Figure 5: 2-ketogluconic acid and xylonic acid production using bamboo hydrolysate as the feedstock. Red line: glucose; orange line: gluconic acid; magenta line: 2-ketogluconic acid; blue line: xylose; black line: acetic acid; green line: ethanol. The glucose in the hydrolysate was converted to gluconic acid and was further converted to 2-ketogluconic acid. The xylose in the hydrolysate was converted to xylonic acid. [Please click here to view a larger version of this figure.](#)

Discussion

K. pneumoniae belongs to the genus *Klebsiella* in the family *Enterobacteriaceae*. *K. pneumoniae* is distributed widely in natural environments such as soil, vegetation, and water¹⁴. The wildtype *K. pneumoniae* strain used in this research was isolated from soil and is used for 1,3-propanediol production¹⁵. *K. pneumoniae* and mutants of this species produce many chemicals under different conditions.

Culture pH and air supplementation are key factors that affect chemical production by *K. pneumoniae*. Wildtype *K. pneumoniae* produces 2,3-butanediol as the main catabolite at pH 6. However, 2-ketogluconic acid changes to the main catabolite when cultured at pH 5 or lower⁹. Increasing the air supplement decreases 2,3-butanediol synthesis, while *R*-acetoin synthesis is improved⁸. Bioreactors that facilitate a stable culture pH and precise air supplementation are used instead of flasks for cell culture. Thus, liters of medium are required. The pretreatment method used in this research followed the method presented by Hong *et al.*¹⁶, which is easy to perform in the laboratory, especially on the liter-scale.

Higher pretreatment temperatures favor sugar production during enzymatic hydrolysis. Treatment at ≤ 100 °C was performed in a water bath and at 121 °C in an autoclave. These two pieces of equipment are common in labs, and several liters of liquid were processed in each batch. Higher temperatures require high-pressure reactors. For example, our lab has 50-mL and 1-L high-pressure reactors, but the volumes of these reactors limit their use during biomass pretreatments.

The bamboo hydrolysate preparation shown in this research was easy to perform in flasks. However, when done in large volume, lower levels of glucose and xylose were sometimes obtained. The hydrolysis mixture must be well distributed. Precipitation of the biomass would lead to lower levels of hydrolysis.

Unlike pure glucose and xylose, biomass hydrolysate is contaminated by many toxic chemicals that might inhibit the growth of microorganisms. Many methods have been developed to remove such inhibitors, but they add to process costs and tend to reduce sugar yields¹⁷. In this research, no special work was done to remove inhibitors. Using the bamboo hydrolysate generated in this work as the feedstock, the productivity (0.95 g/Lh) and the conversion ratio (0.25 g/g) of glucose to 2,3-butanediol during the first stage, when glucose was consumed, were lower than when purified glucose was used as the carbon source, reported previously (1.4 g/Lh and 0.3 g/gm, respectively)⁴. The productivity of 2,3-butanediol during the second stage, when xylose was consumed, was slower than in the first stage. However, the conversion ratio of xylose to 2,3-butanediol reached 0.34 g/g, which was higher than when purified glucose was used as the carbon source⁴. *R*-acetoin production using the bamboo hydrolysate as feedstock had productivity and substrate conversion ratios of 0.92 g/Lh and 0.29 g/g, respectively. Both of these ratios were lower than when purified glucose was used as the substrate (1.7 g/Lh and 0.34 g/g, respectively)⁸. The productivity and the conversion ratio of 2-ketogluconic acid in this study were 2.3 g/Lh and 0.91 g/g, respectively, lower than when purified glucose was used as the carbon source (4.2 g/Lh and 1 g/g, respectively)¹⁰.

Xylose is the second most abundant sugar in nature after glucose. Unlike glucose, xylose is not easily utilized by most microorganisms. In this study, xylose was totally consumed by *K. pneumoniae* for chemical production. 2,3-butanediol, *R*-acetoin, 2-ketogluconic acid, and xylonic acid are all valuable chemicals, and their production processes vary from microaerobic to highly aerobic conditions. The fermentation results here indicate that sugars in bamboo hydrolysate are suitable carbon sources for *K. pneumoniae* growth and chemical production under different conditions.

Using biomass hydrolysate as feedstock is a promising method for chemical production. However, there are still many shortcomings that must be overcome, such as the large amount of water needed to wash the pretreated biomass and the lengthy amount of time needed for enzyme hydrolysis.

Disclosures

The authors have nothing to disclose.

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References

1. Zeng, A. P.; Biebl, H., Bulk chemicals from biotechnology: the case of 1, 3-propanediol production and the new trends. *Adv Biochem Eng Biotechnol.* **74.**, 239-259 (2002).
2. Wei, D.; Wang, M.; Jiang, B.; Shi, J.; Hao, J., Role of dihydroxyacetone kinases I and II in the dha regulon of *Klebsiella pneumoniae*. *J Biotechnol.* **177**, 13-19 (2014).
3. Celińska, E.; Grajek, W., Biotechnological production of 2,3-butanediol-current state and prospects. *Biotechnol Adv.* **27** (6), 715-25 (2009).
4. Chen, C.; Wei, D.; Shi, J.; Wang, M.; Hao, J., Mechanism of 2, 3-butanediol stereoisomer formation in *Klebsiella pneumoniae*. *Appl Microbiol Biotechnol.* **98** (10), 4603-4613 (2014).
5. Wei, D.; Wang, M.; Shi, J.; Hao, J., Red recombinase assisted gene replacement in *Klebsiella pneumoniae*. *J Ind Microbiol Biotechnol.* **39** (8), 1219-1226 (2012).
6. Wei, D.; Sun, J.; Shi, J.; Liu, P.; Hao, J., New strategy to improve efficiency for gene replacement in *Klebsiella pneumoniae*. *J Ind Microbiol Biotechnol.* **40** (5), 523-527 (2013).
7. Chen, C. et al. Inhibition of RecBCD in *Klebsiella pneumoniae* by Gam and its effect on the efficiency of gene replacement. *J Basic Microbiol.* **56** (2), 120-126 (2016).
8. Wang, D., et al. R-acetoin accumulation and dissimilation in *Klebsiella pneumoniae*. *J Ind Microbiol Biotechnol.* **42** (8), 1105-1115 (2015).
9. Wei, D.; Xu, J.; Sun, J.; Shi, J.; Hao, J., 2-Ketogluconic acid production by *Klebsiella pneumoniae* CGMCC 1.6366. *J Ind Microbiol Biotechnol.* **40** (6), 561-570 (2013).
10. Sun, Y. et al. Two-stage fermentation for 2-ketogluconic acid production by *Klebsiella pneumoniae*. *J Microbiol Biotechnol.* **24** (6), 781-787 (2014).
11. Wang, D., et al. Gluconic acid production by gad mutant of *Klebsiella pneumoniae*. *World J Microbiol Biotechnol.* **32** (8), 1-11 (2016).
12. Wang, C., et al. Production of xylonic acid by *Klebsiella pneumoniae*. *Appl Microbiol Biotechnol.* **100** (23), 10055-10063 (2016).
13. Kumar, P.; Barrett, D. M.; Delwiche, M. J.; Stroeve, P., Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind Eng Chem Res.* **48** (8), 3713-3729 (2009).
14. Brisse, S.; Grimont, F.; Grimont, P. A., The genus *Klebsiella*. In *The Prokaryotes.*, Springer: 159-196 (2006).
15. Hao, J.; Lin, R.; Zheng, Z.; Liu, H.; Liu, D., Isolation and characterization of microorganisms able to produce 1, 3-propanediol under aerobic conditions. *World J Microbiol Biotechnol.* **24** (9), 1731-1740 (2008).
16. Hong, E., et al. Optimization of alkaline pretreatment on corn stover for enhanced production of 1,3-propanediol and 2, 3-butanediol by *Klebsiella pneumoniae* AJ4. *Biomass Bioenerg.* **77**, 177-185 (2015).
17. Pienkos, P. T.; Zhang, M., Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. *Cellulose.* **16** (4), 743-762 (2009).