Glucose Tolerance of *Clostridium acetobutylicum* Fermentation in the Anaerobic System

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Solvent-producing *Clostridium acetobutylicum* was purified and used in an acetone-butanol-ethanol (ABE) fermentation process. The objective of this study is to design a fermentation medium for the synthesis of butanol and determine the ideal glucose concentration for appropriate microbe ingestion. The fermentation medium was incubated at 37 °C for up to 90 h before inoculation while being sparged with nitrogen gas under anaerobic conditions. Based on the optical density of fermentation media, the growth rate was also monitored. At 60 g/L of glucose, which was the optimum condition for fermentation, the process followed a log phase pattern until the death phase, with the largest growth taking place between 10 h and 50 h after incubation. The *C. acetobutylicum* steadily consumed the glucose content, reaching its maximal consumption with only around 12 g/L remaining. In contrast to acetone and ethanol, which produced the highest concentrations at 6.4 g/L and 5.2 g/L, respectively, butanol productions were seen appropriately, with the greatest concentration yielding 11.2 g/L of butanol. This shows that *C. acetobutylicum* expressed its active metabolism for up to 60 g/L and further increase of glucose content will deteriorate the performance of butanol production.

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INTRODUCTION

Biobutanol is an alternative type of biofuel that can be produced mainly by the fermentation of sugars in organic feedstocks. Butanol has a low vapor pressure. It is also less corrosive than bioethanol and can be blended with gasoline or used directly (Qureshi *et al*. 2008). Unlike bioethanol, biobutanol has higher energy density, lower volatility, higher boiling point, and is less hygroscopic. Compared to other fuel alternatives, biobutanol has a relatively high energy content (Patakova *et al*. 2012). The energy density of biobutanol is 10 to 20% lower than gasoline. Low volatility means that the substance is more of a liquid or solid, which means that biobutanol does not evaporate easily at certain temperatures. The volatility of the substances and the boiling point have been related (Cheng *et al.* 2012). Biobutanol will become an attractive, cost-effective, and long-term fuel when petroleum becomes more expensive due to dwindling oil sources and increased greenhouse gas emissions in the environment (Jönsson *et al*. 2013). To improve butanol production, metabolic networks are being manipulated through genetic and metabolic

engineering. Fermentation methods for butanol production have made significant progress but are still inefficient (Koutinas *et al*. 2014). One of the methods carried out is the use of *Clostridium acetobutylicum*, which allows the production of biobutanol during the fermentation process (Mesfun *et al.* 2014). This bacterium uses sugar as a substrate and produces a mixture of solvents.

Clostridium acetobutylicum is a well-studied species that has been used in the development of an industrial fermentation process for the production of acetone, butanol, and ethanol (ABE). This species can be manipulated to achieve direct metabolism toward butanol synthesis by manipulating the redox balance (Zhang *et al.* 2014). During the fermentation process, *C. acetobutylicum* exhibits a biphasic metabolism, producing acetic and butyric acids during exponential growth and ABE during the stationary growth phase (Wietzke and Bahl 2012). However, in the presence of its products and byproducts, the fermentation process of *C. acetobutylicum* may be inefficient, resulting in low butanol production and conversion rate (Gao *et al.* 2020). Therefore, in this study, the fermentation process of *C. acetobutylicum* was carried out to evaluate its tolerance to glucose and determine the optimal conditions for biobutanol production.

EXPERIMENTAL

Fermentation Media

The pre-culture media (PCM) were prepared in 1.0 L of solution containing 10 g/L of glucose, 5 g/L of yeast extract, 0.01 g/L *p*-aminobenzoic acid, 0.01 g/L biotin, 0.80 g/L K_2HPO_4, 0.80 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 1.0 g/L NaCl, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}H_2O$, and 0.01 g/L $FeSO_4{\cdot}7H_2O$. The material was thoroughly mixed *via* agitation for an hour. To complete a further fermentation process, *Clostridium* media were also prepared using 20 to 120 g/L of glucose, whereas other constituents were kept constant at 5 g/L of yeast extract, 0.20 g/L Na_2SO_4, 0.8 g/L KH_2PO_4, 1.0 g/L tryptone, and with mineral salts added that consisted of 0.25 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.25 g/L $CoCl_2{\cdot}6H_2O$, 1.5 g/L $CaCl_2{\cdot}2H_2O$, 0.15 g/L $CuSO_4$, 0.52 g/L $ZnSO_4{\cdot}7H_2O$, 2.0 g/L $MnSO_4{\cdot}H_2O$, and 25 g/L $MgSO_4{\cdot}7H_2O$. All the initial media were maintained at pH 6.9 ± 0.2 to ensure an optimal growth condition for the microbes (Khamaiseh *et al.* 2014). The homogenized media were then autoclaved at 121 °C for 15 min to undergo sterilization.

Inoculum Preparation

Clostridium acetobutylicum ATCC 824 was purchased from the American Type Culture Collection (ATCC, Japan). The inoculum was cultured in PCM and sparged with nitrogen gas for 15 min prior to inoculation. It was then incubated for 24 h at 37 °C. All fermentation medium experiments were conducted in a self-designed 0.25-L conical flask with tubing stopper-based reactor containing 200 mL of fresh medium and 10% PCM (Khamaiseh *et al.* 2014). The fermentation was conducted under anaerobic conditions at 37 °C. Nitrogen gas was used to purge the air out through the head tubing of the fermenter and maintain the oxygen-free environment. The analysis was performed using UV-Visible spectroscopy (U-3900H; Hitachi, Ltd., Tokyo, Japan) for evaluation of optical density, whereas high performance liquid chromatography (HPLC) (Thermo Scientific Vanquish HPLC; Thermo Fisher Scientific, Waltham, MA, USA) and gas chromatography (GC) (GC-2010; Shimadzu, Kyoto, Japan) were used to assess the glucose content and

fermentation products, respectively. Each of the analyses was conducted in triplicate samples with the assistance of a calibration curve.

RESULTS AND DISCUSSION

Glucose Consumption

Glucose is the preferred carbon source for *Clostridium* species, and because all major carbon metabolic pathways are constitutively expressed, glucose is effectively utilized. Furthermore, glucose predominates among all commercially available polymer-based carbohydrates (Jaros *et al*. 2012). In this study, glucose served as the carbon source for batch fermentation of *C. acetobutylicum*. The glucose consumption by the microbes was evaluated based on the concentration of 20 to 120 g/L across the experimental periods of 10 to 90 h. The assessment was conducted to evaluate the optimum glucose concentration that should be used in fermentation.

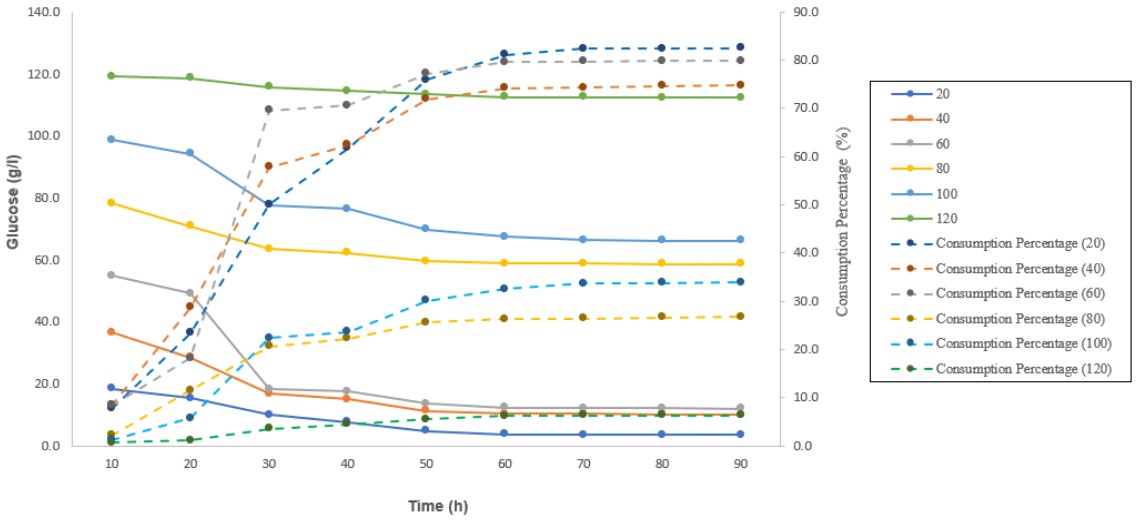

Fig. 1. Glucose concentration and percentage *vs.* time

Figure 1 provides a clear representation of the change in glucose concentration over time, indicating that the glucose concentration had a remarkable impact on the percentage of glucose consumption by *C. acetobutylicum*. Consumption patterns between 20 and 60 g/L showed that fermentation was active during that time. Because the intake over a period of 90 h demonstrated the efficient utilization of glucose, the concentration that offered the best fermentation was at 60 g/L. This concentration may be consumed for maximum to 12 g/L left, as opposed to 20 g/L and 40 g/L. This indicates a large consumption by *C. acetobutylicum*, which also concludes that the product can be achieved at maximum level. A further increase in the glucose loadings may result in a decrease in the amount of substrate that the bacteria consume. This can be attributed to suppression of solvent formation whereby *C. acetobutylicum* produces solvents including acetone, ethanol, and butanol during the later stages of fermentation, a process known as solventogenesis. Excessive glucose levels can promote acid production at the expense of solvent production. This can result in reduced yields of the desired products (Dürre 2007). The effective utilization of glucose had only occurred up to 60 g/L. The 120 g/L does not indicate a

considerable condition for fermentation process, according to the testing. The amount of glucose consumed is minimal, with a total consumption of about 8 g/L over the period of 90 h.

Optical Density

The optical density for *C. acetobutylicum* was estimated using a UV-Visible spectrophotometer. A spectrophotometer measures the quantity of light that passes straight through a sample and calculates the turbidity as a result. The quantity of transmitted light decreases as the number of cells rises. An increasing in turbidity in a culture indicates the growth and biomass of the bacterial population because turbidity is directly related to cell count. It is also referred to as optical density or absorbance, and indirectly indicates the number of bacteria (Vijayanand *et al*. 2017). Using absorbance, which is expressed as logarithmic value, it is possible to graph the bacterial growth. The sensitivity of this approach is restricted to bacterial suspensions, which limits its usefulness while being faster than a traditional plate count (Jones and Woods 1986). Figure 2 portrays the absorbance curve for the prepared inoculum of *C. acetobutylicum*. The absorbance peak appears at 600 nm of wavelength. This indicates the amount of light absorbed by the sample, which contains *C. acetobutylicum.* The absorbance value is used to analyse the presence and concentration of any dissolved compounds when light passes through the sample (Martin *et al*. 1983). A range of glucose concentrations between 20 and 120 g/L were evaluated across six consecutive differences.

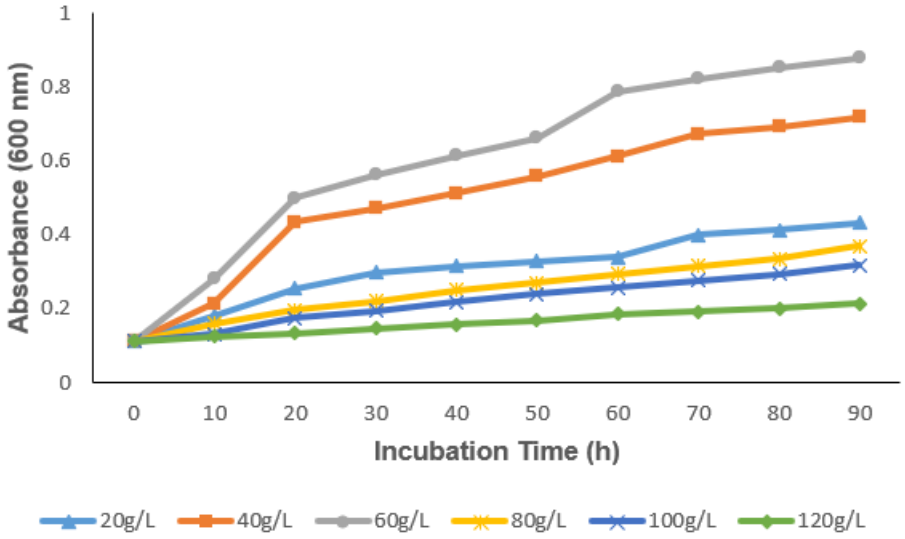

Fig. 2. Optical density of *C. acetobutylicum*

The data shown for those six different concentrations indicate that 60 g/L of glucose was the best concentration to prepare the medium. An insufficient substrate level that results from a gradual drop in glucose will hinder the bacteria's ability to grow effectively. The fermentation process will also be harmed by subsequent increases in the amount of glucose present in the medium. This happened because some strains of *C. acetobutylicum* may exhibit catabolite repression, a regulatory mechanism that prioritizes the utilization of glucose over other carbon sources. In the presence of excess glucose, the bacterium may not efficiently utilize alternative carbon sources, which can limit its overall growth and

metabolic activity (Al-Shorgani *et al*. 2019). This requirement, that the glucose concentration should be maintained at an appropriate level to reduce the substrate inhibition that may impair the functional ability of 6-phosphofructokinase and pyruvate kinase, has been confirmed by an earlier investigation (Liu *et al*. 2016). When there is a surplus of substrate present, substrate inhibition can occasionally occur. Every reaction activity has a maximal state that is specific to the rate of reaction. Thus, further increasing the substrate concentration could result in failure.

Technically, the bacteria should go through a normal growth process, whereby it needs to undergo an initial stage, which is the lag phase. Occasionally, the log phase is entered, whereby a rapid cell division and an increase in population occur. The log phase can be said to end as the rate of cell division equals the rate of cell death, resulting in a stable population size. This stage is called as the stationary phase, and its onset may be attributed to nutrient depletion or the accumulation of metabolic waste products. The growth of bacteria will come to an end when the bacterial population starts to decline. This can occur due to a lack of nutrients, accumulation of toxic metabolites, or other adverse conditions. In terms of growth rate period, it depends on the bacteria species as well as the conditions of its environment (Madigan *et al.* 2017).

In this research, a normal growth rate can be seen when the glucose concentration was optimal (60 g/L), whereby a lag phase would start at 0 to 10 h and would therefore shift to a log phase until 50 h passed after incubation. The microorganisms entered a stationary phase after a few hours and then gradually transitioned into their death phase. This can be explained by the apparent low optical density increment that begins at 50 h and continues until the end. In light of this, it may be said that *C. acetobutylicum* may only be able to ferment vigorously during 10 to 50 h of incubation.

Fermentation Products

The ABE fermentation products were analysed using gas chromatography. The results from the experimental investigation are presented in Fig. 3. These values were determined using calibration curves that were plotted for each of the main fermentation products. The evaluation was conducted to express the capability of *C. acetobutylicum* to produce butanol and made comparisons to acetone and ethanol productions.

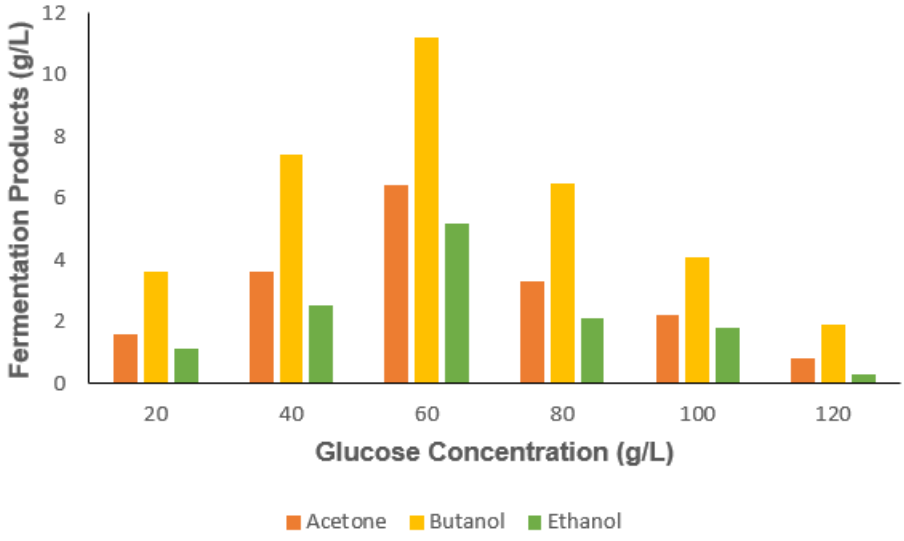

Fig. 3. Fermentation products

Analysis showed that employing 60 g/L of glucose resulted in the maximum value of butanol production. At this substrate concentration, acetone and ethanol also displayed a strong relationship with the fermentation processes. Based on these three products, ethanol and acetone are in order of decreasing peak concentration, and butanol is at the top. This has been demonstrated by Janssen *et al*. (2014), who note that the typical acetone, butanol, and ethanol ratios are 3:6:1, respectively, but that these values can vary depending on the circumstances. A lower solvent concentration occurs due to the fermentation gases, which consisted of about 60% CO_2 and 40% H_2, accounting for 50% of the glucose fermented (Janssen *et al*. 2014). In addition, this happened due to the condition where there are two unique phases in the ABE fermentation. These included an acidogenic phase where a surge in bacterial population and growth tends to promote the production of butyric acid, acetic acid, and fermentation gases (CO_2 and H_2). Glycolysis, which resulted in the formation of pyruvate from the sugars, also involved two distinct fermentation gases (CO_2 and H_2). The bacterial metabolism changed at the end of the exponential growth phase, leading to the formation of acetone, butanol, and ethanol from the acids (Jiang *et al*. 2014). Because there was less substrate present when the glucose level was below 60 g/L, fewer products were produced. Nevertheless, when the glucose level rose over the optimal level, the efficiency of the fermentation process deteriorated. These findings support the phenomenon that led to the correlation of the optical density and glucose consumption. Hence, it can be said that 60 g/L is the ideal concentration to be used for *C. acetobutylicum*'s fermentation process.

CONCLUSIONS

1. Based on the findings from the analyses of glucose consumption, growth rate evaluation, and fermentation product assessment, 60 g/L is the optimum concentration for fermentation by *Clostridium acetobutylicum*.
2. The relationship between glucose consumption and fermentation products indicates active fermentation from 20 to 60 g/L. Further increasing the concentration to 120 g/L may cause a decrease in reaction rate due to catabolite repression and suppression of solvents resulting from the toxicity of acids produced during the fermentation process.
3. This research study provides valuable insights into the optimal glucose concentration for butanol production. It highlights the limitations when working with microorganisms such as *C. acetobutylicum* to achieve desired products. Maintaining optimum conditions is crucial to maximize output.

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REFERENCES CITED

Al-Shorgani, N. K. N., Al-Tabib, A. I., Kadier, A., Zanil, M. F., Lee, K. M., and Kalil, M. S. (2019). "Continuous butanol fermentation of dilute acid-pretreated de-oiled rice bran by *Clostridium acetobutylicum* YM1," *Scientific Reports* 9(1). DOI: 10.1038/s41598-019-40840-y

Cheng, C. L., Che, P. Y., Chen, B. Y., Lee, W. J., Lin, C. Y., and Chang, J. S. (2012). "Biobutanol production from agricultural waste by an acclimated mixed bacterial microflora," *Appl. Energ.* 100, 3-9. DOI: 10.1016/j.apenergy.2012.05.042

Dürre, P. (2007). "Biobutanol: An attractive biofuel," *Biotechnology Journal* 12(2), 1525-1534. DOI: 10.1002/biot.200700168

Gao, Y., Zhang, M., Zhou, X., Guo, X., Lei, C., Li, W., and Lu, D. (2020). "Effects of carbon ion beam irradiation on butanol tolerance and production of *Clostridium acetobutylicum*," *Frontiers in Microbiology* 11. DOI: 10.3389/fmicb.2020.602774

Janssen, H., Wang, Y., and Blaschek, H. P. (2014). *Clostridium: Clostridium acetobutylicum*, in: *Encyclopedia of Food Microbiology: Second Edition*, Elsevier Inc., Amsterdam, Netherlands, 449-457. DOI: 10.1016/B978-0-12-384730-0.00070-7

Jaros, A., Rova, U., and Berglund, K. A. (2012). "Effect of acetate on fermentation production of butyrate," *Cell. Chem. Technol.* 46(5), 341-347.

Jiang, W., Wen, Z., Wu, M., Li, H., Yang, J., Lin, J., Yijun, L., Yang, L., and Cen, P. (2014). "The effect of pH control on acetone–butanol–ethanol fermentation by *Clostridium acetobutylicum* ATCC 824 with xylose and d-glucose and d-xylose mixture," *Chinese Journal of Chemical Engineering* 22, 937-942. DOI: 10.1016/j.cjche.2014.06.003

Jones, D. T., and Woods, D. R. (1986). "Acetone–butanol fermentation revisited," *Microbiol. Rev*. 50(4), 484-524. DOI: 10.1128/mr.50.4.484-524.1986

Jönsson, L. J., Alriksson, B., and Nilvebrant, N. O. (2013). "Bioconversion of lignocellulose: Inhibitors and detoxification," *Biotech. Biofuel.* 6(1), 1-10. DOI: 10.1186/1754-6834-6-16

Khamaiseh, E. I., Abdul Hamid, A., Abdeshahian, P., Wan Yusoff, W. M., and Kalil, M. S. (2014). "Enhanced butanol production by *Clostridium acetobutylicum* NCIMB 13357 grown on date fruit as carbon source in P2 medium," *The Scientific World Journal*, 2014. DOI: 10.1155/2014/395754

Koutinas, A. A., Vlysidis, A., Pleissner, D., Kopsahelis, N., Garcia, I. L., Kookos, I. K., Papanikolaou, S., Kwan, T. H., and Ki Lin, C. S. (2014). "Valorization of industrial waste and by-product streams *via* fermentation for the production of chemicals and biopolymers," *Chem. Soc. Rev.* 43(8), 2587-2627. DOI: 10.1039/c3cs60293a

Liu, H., Huang, D., and Wen, J. (2016). "Integrated intracellular metabolic profiling and pathway analysis approaches reveal complex metabolic regulation by *Clostridium acetobutylicum*," *Microb. Cell Fact.* 15(1), article 36. DOI: 10.1186/s12934-016-0436-4

Madigan, M. T., Martinko, J. M., Bender, K. S., Buckley, D. H., and Stahl, D. A. (2017). *Brock Biology of Microorganisms*, 15th Global Edition," *Pearson*.

Martin, J. R., Petitdemange, H., Ballongue, J., and Gay, R. (1983). "Effects of acetic and butyric acids on solvents production by *Clostridium acetobutylicum*," *Biotechnol. Lett.* 5, 89-94. DOI: 10.1007/BF00132165

Mesfun, S., Lundgren, J., Grip, C. E., Toffolo, A., Nilsson, R. L. K., and Rova, U. (2014). "Black liquor fractionation for biofuels production: A techno-economic assessment," *Bioresource Technol.* 166, 508-517. DOI: 10.1016/j.biortech.2014.05.062

Patakova, P., Linhova, M., Rychtera, M., Paulovab, L., and Melzoch, K. (2012). "Novel and neglected issues of acetone–butanol–ethanol (ABE) fermentation by clostridia: *Clostridium* metabolic diversity, tools for 32 process mapping and continuous fermentation systems," *Biotechnol. Adv.* 31(1), 58-67. DOI: 10.1016/j.biotechadv.2012.01.010

Qureshi, N., Ezeji, T. C., Ebener, J., Dien, B. S., Cotta, M. A., and Blaschek, H. P. (2008). "Butanol production by *Clostridium beijerinckii*. Part I: Use of acid and enzyme hydrolyzed corn fiber," *Bioresource Technol.* 99(13) 5915-5922. DOI: 10.1016/j.biortech.2007.09.087

Vijayanand, C., Kamaraj, S., Sriramajayam, S., Karthikeyan, S., Surendrakumar, A., and Ramesh, D. (2017). "A probe into the biphasic nature of the acetone butanol ethanol (ABE) fermentation by *Clostridium acetobutylicum* ATCC 824," *International Journal of Current Microbiology and Applied Sciences* 6(4), 2179-2186. DOI: 10.20546/ijcmas.2017.604.255

Wietzke, M., and Bahl, H. (2012). "The redox-sensing protein Rex, a transcriptional regulator of solventogenesis in *Clostridium acetobutylicum*," *Applied Microbiology and Biotechnology* 96(3), 749-761. DOI: 10.1007/s00253-012-4112-2

Zhang, L., Nie, X., Ravcheev, D. A., Rodionov, D. A., Sheng, J., Gu, Y., Yang, S., Jiang, W., and Yang, C. (2014). "Redox-responsive repressor rex modulates alcohol production and oxidative stress tolerance in *Clostridium acetobutylicum*," *Journal of Bacteriology* 196(22), 3949-3963. DOI: 10.1128/JB.02037-14

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