I-BIC-O-200-03

Development of *Clostridium cochlearium* by UV- mutation for bio-butanol production

Eakkachai Klaithin[1], Kompon Glaopo[2], Yanisa Laoong-u-thai[1,3*]
1 Department of Chemical Engineering, Faculty of Engineering, Burapha University, Thailand.
2 Environmental Engineering program, Faculty of Engineering, Burapha University, Thailand.
3 Bioengineering program, Faculty of Engineering, Burapha University, Thailand.
*E-mail: yanisa@buu.ac.th

Abstract

This research intended to develop *Clostridium cochlearium* by UV-Mutation for bio-butanol production, wild type strain identification was performed by PCR technique using *16s rRNA* gene. The wild type mutation was done by UV-exposure on cooked meat broth and agar for 2, 4 and 6 minutes. The appropriate conditions for mutation was shown at 6 minutes UV-exposure in cooked meat broth. The selected 3 colonies of mutant L6-01, L6-02 and L6-03 were transferred into phosphate carbonate medium and controlled temperature at 30°C. After the fermentation finished, remaining glucose was measured by DNS method. It showed that the lowest remaining glucose was found in L6-01 clone which presented 12.9266 g/L followed by L6-02, L6-03 and wild type at 15.0039 g/L, 15.5900 g/L and 16.2035 g/L, respectively. The GC-FID analysis for butanol concentration measurement revealed that the L6-01 mutant showed the highest production of butanol at 0.02106 g/L followed by wild type and L6-03 at 0.01782 g/L and 0.01701 g/L, respectively. Meanwhile, L6-02 mutant strain did not show butanol production. The highest productivity of butanol was presented from L6-01 at 1.755×10^{-4} g/L/h, followed by wild type and L6-03 at 1.485×10^{-4} g/L and 1.418×10^{-4} g/L /h, respectively.

Key words: Bio-butanol, *Clostridium cochlearium*, UV-mutation, ABE fermentation

Introduction

Butanol is an alcohol solution applied in a variety of applications such as coatings, cleaning industry and solvent in lacquers and cosmetics industries. In addition, butanol can be used as a fuel and fuel additives as well. Butanol is important to use as alternative energy apart from fossil fuels which is important for sustainability energy. Butanol produced by chemical and biological processes using agricultural waste as raw materials. Agricultural waste has relatively low values it is easily to digested in both chemical and enzymatic digestion. Fermented alcohol productions including butanol are less complicated than chemical synthesis but it takes longer period than chemical methods as well as low alcohol concentration. Meanwhile, bio-butanol production from agriculture waste is considered promising and economically feasible and environmental friendly. Therefore, the biological process need to be developed for higher productivity. The important factors for butanol production include bacterial strain, type and concentration of medium, temperature, pH and fermentation period. However, The key factor of butanol production is type of microorganisms used in fermentation. [1,2,5,6].

Microorganisms using in bio-butanol production are *Clostridium sp*.[9,10], Cyanobacteria [11], *L. brevis* [12] etc. The most common species applied in commercial butanol production is *Clostridium acetobutylicum* along with *C. saccharobutylicum*, *C. beijerinckii* and *C. saccharoperbutylacetonicum*[13]. Interestingly, alternative species of *C.cochlearium* has been found butanol producibility[2]. This strain has been characterized in biogas fermenter of shrimp pond sediment [14]. To develop microorganism strain, the mutagenesis is the promising method along with the positive clone selection based on the expected product. One of the interesting method for mutagenesis is UV mutation method that can cause genetic change resulting to mutation. It is cheap, easy to perform and less toxic compare to other methods. Along with the compromise selective method, the expected positive clones are highly discovered [1,2,5,6]. Thus, *C.cochlearium* has been shown butanol productivity in previous study. To improve this bacterial strain for butanol production, it becomes challenging for industry purpose. Thus, the objective of this research is to develop the bacterial strain using UV mutation method and selected strains will be analyzed for butanol productivity.

Materials and methods

Materials and bacterial culture

Bacterial strain used in this research was *Clostridium cochlearium* which cultured in BCP Agar (1 L cooked meat broth, 0.4 g Bromcresol purple, 0.2 mM Allyl alcohol) [1] , Phosphate carbonate medium (20 g/L Glucose solution, 10 g/L Trypticase peptone, 6 g/L Yeast extract, 3.38 g/L Potassium dihydrogen phosphate, 4 g/L Sodium hydrogen carbonate) [14] and Cooked meat medium (for agar medium, add the agar powder 15 g/L).

Primers for PCR amplification were 16S1 forward primer (5'-GAGTTTGATCCTGGCTCA-3') and 16S2 reverse primer (5'CGGCTACCT TGTT ACGACTT-3').

Methods

The morphology study of wild type and mutant was performed using gram stain technique then visualized by light microscope and genetic identification of wild type and mutant by using molecular genetic techniques as following DNA extraction; used the GF-1 DNA extraction kit, 16s rRNA PCR amplification; for identification bacteria strain [7,8]. Setting of PCR condition was 30 second at 94 ^{o}C for denature step then 30 second at 55 ^{o}C for annealing step then 60 second at 72 ^{o}C for extension step for 35 cycles. PCR products were stored at -20 ^{o}C. Gel electrophoresis was used for PCR product detection. Then the amplicon was sequenced and blasted to database for species characterization. The mutation of wild type by UV irradiation were set in 2 forms of cultures including broth and agar cultures then exposed of UV light in difference time series of 2, 4 and 6 minutes. The single colony was pick up and transferred into cooked meat medium broth then incubated at 35 ^{o}C in anaerobic condition for 18 hours. Selected clones then were streaked onto BCP agar and incubated at 35 ^{o}C for 24 hours. The yellow colonies were selected then subjected on cooked meat agar then spray with iodine vapor for 3-5 minutes in room temperature. The dark yellow colonies were chosen and again incubated into cooked meat medium at 35 ^{o}C for 48 hours [1,4]. The fermentation for butanol production of selected clones were performed in 505 ml working volume (**Fig. 1**) comparing with wild type. For fermentation, phosphate carbonate medium (glucose 20 g/L (6.06 g)) was used at 30 ^{o}C for 5 days with initial pH of 6.5. To keep anaerobic condition, N_2 surface flushing was applied in the reactors at all time [2].

Fig. 1 Butanol fermentation

DNS method for glucose analysis was applied to measure remaining glucose quantity in phosphate carbonate medium after fermentation. Calculation of butanol Productivity (g/L/h) was used equation (1) as follow.

$$\text{Productivity} = \frac{\text{Concentration of butanol production (g/L)}}{\text{Time of fermentation (h)}} \quad (1)$$

Result and discussion

Result

The shape of colony is showed circular smooth surface colony and white opaque color and from gram staining visualized by microscope showed the purple stained of gram-positive and rod-shape cell. From PCR technique using 16s rRNA gene and BLAST search showed *C.cochlearium* strain. Selected condition of the UV mutation was 6 minutes exposure to broth culture and selected 3 colonies were named L6-01, L6-02 and L6-03 and used in ABE fermentation. The remaining glucose was measured by DNS method. It showed that the lowest remaining glucose found in L6-01 clone with the amount of 12.9266 g/L followed by L6-02, L6-03 and wild type at

15.0039 g/L, 15.5900 g/L and 16.2035 g/L, respectively, The highest productivity of butanol was presented by L6-01 at $1.755x10^{-4}$ g/L/h, followed by wild type and L6-03 at $1.485x10^{-4}$ g/L and $1.418x10^{-4}$ g/L /h, respectively. The comparison of butanol production between wild type and mutants showed that L6-01 had highest concentration of butanol at 0.02106 g/L followed by wild type and L6-03 at 0.01782 and 0.01701 g/L respectively (**Table 1.**). However, ethanol and acetone productions were also analyzed along with butanol. It found that highest ethanol and acetone production were observed in L6-02.

Table 1 The amount of organic solvents produced

culture	Concentration of organic solvents (g/L)			
	Ethanol	Acetone	Butanol	Total organic solvents
Wild type	0.09705	0.01740	0.01782	0.13227
L6-01	0.13334	0.02373	**0.02106**	0.17813
L6-02	**0.20909**	**0.04271**	0	**0.25180**
L6-03	0.10888	0.01898	0.01701	0.14487

Discussion

From morphological study of *Clostridium cochlearium* wild type and mutants showed circular smooth surface colony and white opaque colour, displayed purple colour when stained with crystal violet and rod shaped cell when observing under light microscope (Fig 2 (a)). It revealed that the mutants still classified as *Clostridium sp.* and along with molecular genetic determination of 16s rRNA gene which showed the same size of PCR product(Fig. 2 (b)). As well as the conformation of 16s rRNA gene sequencing, BLAST search was matched to *Clostridium cochlearium* species. From butanol production, L6-01 showed highest concentration of butanol followed by wild type and L6-03, respectively. For substrate consumption, lowest remaining glucose was shown from L6-01, L6-02, L6-03 and wild type, respectively and analysis with total organic solvents did not show significant difference in each sample. However, L6-01 demonstrated higher butanol production than wild type. These may result of the mutation in butanol regulatory genes such as butanol dehydrogenase gene (BDH) which can confirm by PCR amplification and sequencing in future study.

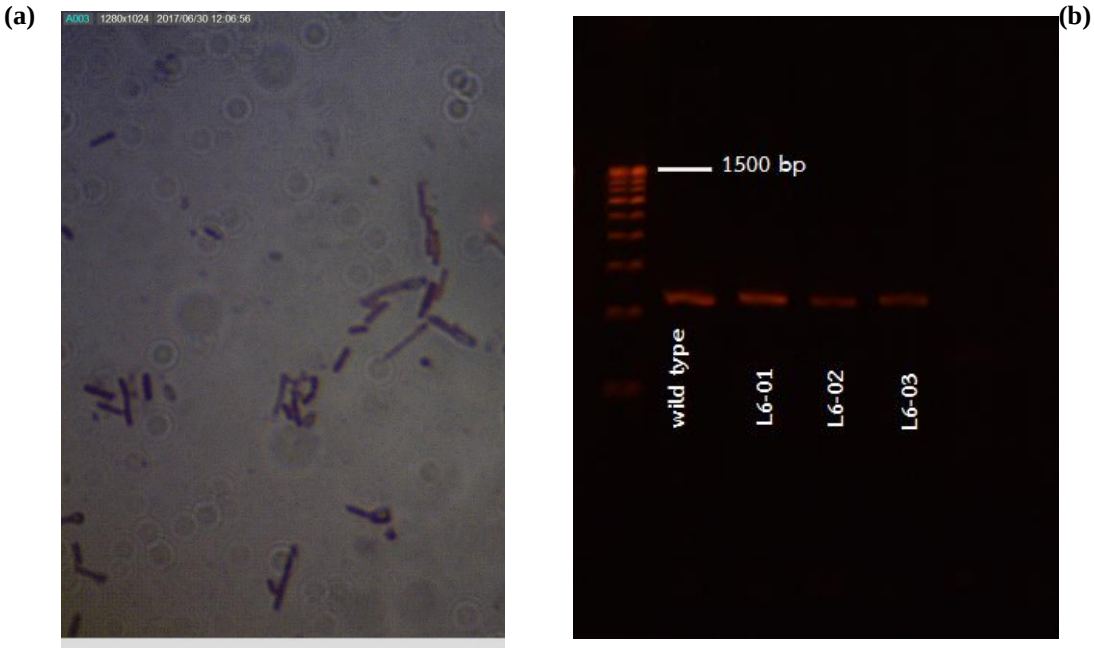

Fig.2 Bacterial gram stain (a), 16s rRNA PCR for identification bacterial strain (b)

In addition, the butanol production has been reviewed. Butanol production in this research used *C. cochlearium* wild type and mutants in Phosphate-carbonate medium, always N2 fusing and no pH control on this experiment. The butanol production was similar to Matthias et al. (1984) result (see Table 2) but difference process of pH

control, N_2 fusing at beginning of fermentation and fermentation media. However, our result is not best butanol productivity but it is still need to optimization the fermentation condition of the mutants such as pH, medium and temperature etc.

Table 2 Butanol production in difference process

Culture	Process	Medium	N_2 fusing for an aerobic condition	Butanol production (g/L)	pH control	reference
C. cochlearium	batch	Glucose-containing medium	Beginning ferment	0.022-0.156	control	Matthias et al. ((1984
C. cochlearium	batch	Phosphate-carbonate medium	Beginning ferment	0.0518	control	Ploypailin Sujarawee (2016)
C. acetobutylicum TISTR 1462	batch	P 2medium	Beginning ferment	11.37±0.26	No control	Soonthon and Apisit(2012)
Wild type L6-01 L6-02 L6-03	**batch**	**Phosphate-carbonate medium**	**Always**	**0.01782 0.02106 0 0.01701**	**No control**	**This research**

Acknowledgments

This research supported by chemical engineering research funding 2017 and engineering faculty research funding วจร. ๓/๒๕๕๘.

References

[1] Palmer Rogers, Neil Palosaari, (1987), *Clostridium acetobutylicum* Mutants That Produce Butyraldehyde and Altered Quantities of Solvents, Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, 2761-2766

[2] Ploypailin Simma, Sujarawee Harisombat, (2016), Production of butanol by ABE fermentation from Clostridium Sp., Department of chemical engineering, Faculty of engineering, Burapha University, 24-35

[3] Sang Yup Lee, Jin Hwan Park, Seh Hee Jang, Lars, K. N., Jaehyun Kim,.& Kwang S. J, (2008), Fermentative butanol production by clostridia, Biotechnology and bioengineering, 210-211

[4] Mohammad B. Habibi Najafi, Parnian Pezeshki, (2013), bacterial mutation; types, mechanism and mutant detection methods: a review, Department of Food Science & Technology, Ferdowsi University of Mashhad, Mashhad, Iran, 628-637

[5] Teck Khiang Chua(a), Da-Wei Liang, Chao Qi(a), Kun-Lin Yang(b), Jianzhong He(a), (2013), Characterization of a butanol–acetone-producing Clostridium strain and identification of its solventogenic genes, Department of Civil and Environmental Engineering, National University of Singapore(a), Singapore, Department of Chemical and Biomolecular Engineering, National University of Singapore, Singapore(b), 372–378

[6] Gottwald, M., Hippe, H.,& Gottschalk, G. (1984). Formation of n-butanol from glucose by strains of the "clostridium tetanomorphum" group. Appl Environ. Microbiol. 3: 573-576

[7] Dr.Mohini Joshi, Dr.Deshpande J.D, (2010), polymerase chain reaction: methods, principle and application, Department of Anatomy, Rural Medical College, Pravara Institute of Medical Sciences, Loni, Maharashtra, India, Department of Community Medicine, Rural Medical College, Pravara Institute of Medical Sciences, Loni, Maharashtra, India, International Journal of Biomedical Research, 81-97

[8] Anjana Munshi, (2012), DNA Sequencing – Methods and Applications, Department of Molecular Biology,Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, India, 1-4

[9] T.C. Ezeji, N. Qureshi, H.P. BlaschekButanol fermentation research: upstream and downstream manipulations, Chem. Rec., 4 (2004), pp. 305-314

[10] Y.L. Lin, H.P. BlaschekButanol production by a butanol-tolerant strain of *Clostridium-Acetobutylicum* in extruded corn broth

Appl. Environ. Microbiol., 45 (1983), pp. 966-973

[11] Lan, E. I., & Liao, J. C. (2011). Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metabolic engineering*, *13*(4), 353-363.

[12] Francisca Lameiras, (2012),Butanol production in Lactic acid bacteria, Department of Chemical and Biological Engineering Systems & Synthetic Biology, Chalmers University of technology, 22-23

[13] V.V. Zverlov, O. Berezina, G.A. Velikodvorskaya, W.H.SchwarzBacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery Appl Microbiol Biotechnol, 71 (2006), pp. 587-597

[14] Laoong-u-thai, Y., Srinakorn, P., Srisertpol, P., (2015) "Enhancing Biogas Production from Shrimp Pond Sediment with additive" International conference on chemical and Biochemical , Paris, France, July 20-22, 2015