

# A tandem process for the production of levan and bioethanol by microbes

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## ABSTRACT

The purpose of the present study is to investigate the feasibility of a tandem production of levan and ethanol by microbial fermentation using sucrose substrate. The tandem process involves fermentation of *Bacillus subtilis* (natto) Takahashi in sucrose medium to produce levan, separation of levan product from glucose by-product by ultrafiltration and fermentation of the glucose remnant from levan production by *Z. mobilis* to produce ethanol. In the production of levan, the levan production was carried out in a 5-l fermenter using the optimal conditions developed previously in the shake-flask experiments. After cultivation of *B. subtilis* (natto) Takahashi for 48 h, 60-70 g l<sup>-1</sup> of levan was produced in medium containing 250g l<sup>-1</sup> sucrose, which was about 50 % yield on available fructose. After removing the cells, the fermentation broth was concentrated by ultrafiltration. The concentrated retentate, which was very viscous, required addition of 95 vol.% cold ethanol to give high yield with the total recovery of levan. The product thus obtained is consisted of high and low molecular weight levans. The filtrate containing glucose, fructose and unreacted sucrose was used for ethanol fermentation. Fractionation of levans of low and high molecular weight is necessary, because levans with different molecular weights are needed for different purposes. Attempts were made to carry out the fractionation of levans in the concentrated retentate by ultrafiltration through a membrane of 300, 000 MWCO, before their precipitation by alcohol. The cell-free solution was first cycled through the Tami system with the membrane of 300,000 MWCO. The first concentrated retentate, required addition of 95 vol.% cold ethanol to give high yield with the total recovery of high molecular weight levan. The first filtrate was further cycled through the membrane of 5,000 MWCO. The second concentrated retentate was harvested with the total recovery of low molecular weight levan by the addition of 95 vol.% cold ethanol. The second filtrate containing glucose, fructose and unreacted sucrose was used for ethanol fermentation. The filtrate which contained roughly 150 g l<sup>-1</sup> of hexose was diluted, supplemented with yeast extract, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgSO<sub>4</sub> · 7H<sub>2</sub>O, KH<sub>2</sub>PO<sub>4</sub> and used for alcohol fermentation by *Z. mobilis*. Nutritional and operational factors were investigated for optimal ethanol production; the results showed that incubation of *Z. mobilis* in the medium containing glucose remnant diluted two-fold, supplemented with Yeast extract 1%, KH<sub>2</sub>PO<sub>4</sub> 0.1%, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.1%, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.05%, at 30 °C, pH 6.5, standing for 48 h gave the highest 31.84 g l<sup>-1</sup> (Ey 93.78%, YP/S 0.49 g/g) of bio-ethanol. The tandem process developed in this study is an eco-friendly process in that the sucrose substrate was fully utilized without any waste of by-products in the process; in addition, two invaluable environmental-friendly biomaterials (levan and ethanol) were produced. Furthermore, the amount of alcohol required for levan recovery could be reduced to a fourth of that generally used in the conventional precipitation.

Keywords : *Bacillus subtilis*, *Zymomonas mobilis*, ultrafiltration, levan, ethanol

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## REFERENCES

- 1、陳立達. 利用固定化技術生產果糖聚合物之研究. 2009. 大葉大學碩士論文.
- 2、游芸悌. 以納豆菌生產生物性高分子研究. 2004. 大葉大學碩士論文.
- 3、廖國森. 納豆菌生產果糖聚合物之研究. 2007. 大葉大學碩士論文.
- 4、Abate, C., Callieri, D., Emilio Rodriguez and Garro, O. Ethanol production by a mixed culture of flocculent strains of *Zymomonas mobilis* and *Saccharomyces* sp. *Applied Microbiology and Biotechnology*. 1996. Volume 45: 580~583.
- 5、Albert, A. and Graf, D. Metabolic state of *Zymomonas mobilis* in glucose-, fructose-, and xylose-fed continuous culture as analysis by <sup>13</sup>C and <sup>31</sup>P-NMR Spectroscopy. *Archives of Microbiology*. 1999. Volume 171: 371~385.
- 6、Amutha, R. and Gunasekaran, P. Production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus*. 2001. *Journal of Bioscience and Bioengineering*. Volume 92: 560~564.
- 7、Ananthalakshmy, V.K. and Gunasekaran P. Isolation and characterization of mutants from levan-producing *Zymomonas mobilis*. 1999. *Journal of Bioscience and Bioengineering*. Volume 87: 214~217.
- 8、Arvidson, S.A., Rinehart B.T. and Maria, F.G. Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp. *Carbohydrate Polymers*. 2006. Volume 65: 144~149.
- 9、Avigad, G. Levan in whistler, methods in carbohydrate chemistry. Academic Press. 1965. Volume: 161~165.
- 10、Bai, F.W., Anderson, W.A. and Moo, Y.M. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances*. 2008. Volume 26: 89~105.
- 11、Barati, J.C. and Bu'Lock, J.D. *Zymomonas mobilis*: a bacterium for ethanol production. *Biotechnology Advances*. 1986. Volume 4: 95~115.
- 12、Biedrzycka, E. and Bielecka, M. Prebiotic effectiveness of fructans of different degrees of polymerization. *Trends in Food Science & Technology*. 2004. Volume 15: 170~175.
- 13、Bringer, S. and Finn, R.K. and Sahn, H. Effect of oxygen on the metabolism of *Zymomonas mobilis*. *Archives of Microbiology*. 1984. Volume 139: 376~381.
- 14、Buchholz, S.E. and Eveleigh, D.E. Transfer of plasmids to an antibiotic-sensitive mutant of *Zymomonas mobilis*. 1986. *Applied and Environmental Microbiology*. Volume 52: 366~370.
- 15、Carey, V.C. and Ingram, L.O. Lipid composition of *Zymomonas mobilis*: effect of ethanol and glucose. *Journal of Bacteriology* 1983. Volume 154: 1291~1300.
- 16、Clark, M.A., Roberts, E.J. and Garegg, P.J. New compounds from microbiological products of sucrose. *Carbohydrate Polymers*. 1997. Volume 34: 425.
- 17、Cummings, J.H., Roberforid, M.B., Andersson, H., Barth, C., Ferro-Luzzi, A., Ghos, Y., Gibney, M., Hermosen, K., James, W.P., Korver, O., Lairon, D., Pascal, G. and Voragen, A.G. A new look at dietary carbohydrate: chemistry, physiology and health. Paris Carbohydrate Group. *European Journal of Clinical Nutrition*. 1997. Volume 51: 417~423.
- 18、Demain, A.L. and Solomon, N.A. Biology of industrial. Microorganisms. 1985.
- 19、Edelman, J. and Jefford, T.G. The mechanism of fructan metabolism in higher plants as exemplified in *Helianthus tuberosus*. *New Phytologist*. 1968. Volume 67: 517~531.
- 20、Esser, K. and Karsch, T. Bacterial ethanol production: Advantages and disadvantages. 1984. *Process Biochemistry*. Volume 19: 116~121.
- 21、Elisashvili, V.I. Levan synthesis by *Bacillus* sp. *Applied Biochemistry and Microbiology*. 1984. Volume 20: 82~87.
- 22、Fiordaliso, M., Kok, N., Desager, J.P., Goethals, F., Deboyser, D., Roberfroid, M. and Delzenne, N. Dietary oligofructose lowers triglycerides, phospholipids and cholesterol in serum and very low density lipoproteins of rats. *Lipids*. 1995. Volume 30: 163~167.
- 23、Fuchs, A., De Bruijn, J.M. and Nideveld, C.J. Bacteria and yeasts as possible candidates for the production of inulinases and levanases. *Antonie van Leeuwenhoek*. 1985. Volume 51:333~351.
- 24、Gunasekaran, P. and Raj, K.C. Ethanol fermentation technology-*Zymomonas mobilis*. *Current Science*. 1999. Volume 77: 56~68.
- 25、Han, Y.W. Microbial levan. *Advances in Applied Microbiology*. 1990. Volume 35: 171~194.
- 26、Hestrin, S. and Avigad, G. The mechanism of polysaccharide production from sucrose. *Biochemical Journal*. 1958. Volume 38: 2~10.
- 27、Hestrin, S., Shapiro, A.S. and Aschner, M. The enzymic production of levan. *Biochemical Journal*. 1943. Volume 37: 450~456.
- 28、Imam, G.M. and Allah, N.M. Fructose, a new soil conditioning polysaccharide isolated from the metabolites of *Bacillus polymyxa* AS-1 and its clinical applications. *Egyptian Journal of Botany*. 1974. Volume 17: 19~26.
- 29、John, G., Eberhardt, I., Zeitz, A. and Hellendoorn, L. and Schugerl, K. Coimmobilized aerobic/anaerobic mixed cultures in shaken flasks. *Journal of Biotechnology*. Volume 46: 209~219.
- 30、Jung, K.E., Lee, S.O., Lee, J.D. and Lee, T.H. Purification and characterization of a levanbiose-producing levanase from *Pseudomonas* sp. NO. 43. *Biotechnology and Applied Biochemistry*. 1999. Volume 29: 263~268.
- 31、Kannan, T.R., Sangiliyandi, G. and Gunasekaran, P. Improved ethanol production from sucrose by a mutant of *Zymomonas mobilis* lacking sucrases in immobilized cell fermentation. *Enzyme and Microbial Technology*. 1998. Volume 22: 179~184.
- 32、Karsch, T., Stahl, U. and Esser, K. Ethanol production by *Zymomonas* and *Saccharomyces*, advantages and disadvantages. *Applied Microbiology and Biotechnology*. 2004. Volume 18: 387~391.
- 33、Kasapis, S., Morris, E.R., Gross, M. and Rudolph, K. Solution properties of levan polysaccharide from *Pseudomonas syringae* pv. *Phaseolicola*, and its possible primary role as a blocker of recognition during pathogenesis. *Carbohydrate Polymers*. 1994. Volume 23: 55~64.
- 34、Keith, J., Wiley, B., Ball, D., Arcidiacono, A., Zorfass, D., Mayer, J. and Kaplan, D. Continuous culture system for production of biopolymer levan using *Erwinia herbicola*. *Biotechnology and Bioengineering*. 1991. Volume 38: 557~560.
- 35、Kenjitanaka, Z.D. Investigation of the utility of pineapple juice and pineapple waste material as low-cost substrate for ethanol fermentation *Zymomonas mobilis*. *Journal of Bioscience and Bioengineering*. 1999. Volume 87: 642.
- 36、Kesava, S.S., Rakshit, S.K. and Panda, T. Production of ethanol by *Zymomonas mobilis*: The effect of batch step-feeding of glucose and relevant growth factors. *Process Biochemistry*. 1994. Volume 30: 41~47.
- 37、Lee, W.C. and Huang, C.T. Enhancement of ethanol production from sucrose by *Zymomonas mobilis* by the addition of immobilized invertase. *Enzyme and Microbial Technology*. 1995. Volume 17: 79~84.
- 38、Lee, W.C. and Huang, C.T. Modeling of ethanol fermentation using *Zymomonas mobilis* ATCC 10988 grown on the media containing glucose and fructose. *Biochemical Engineering Journal*. 2000. Volume 4: 217~227.
- 39、Leibovici, J., Kopel, S., Siegal, A. and Gal-Mor, O. Effect of tumor inhibitory and stimulatory doses of levan, alone and in combination with cyclophamide, on spleen and lymph nodes. *International Journal of Immunopharmacology*. 1986. Volume 8: 391~403.
- 40、Long, L.W., Stivala, S.S. and Ehrlich, J. Effect

of pH on the biosynthesis of levan and on the growth of *Streptococcus Salivarius*. *Archives of Oral Biology*. 1975. Volume 20: 503~507. 41、 Loos, H., Kramer, R., Sahm, H. and Sprenger, G.A. Sorbitol promotes growth of *Zymomonas mobilis* in environments with high concentration of sugar: evidence for a physiological function of glucose-fructose oxidoreductase in osmoprotection. *Journal of Bacteriology*. 1994. Volume 176: 7688~7693. 42、 Mantsala, P. and Puntala, M. Comparison of levansucrase from *Bacillus subtilis* and from *Bacillus amyloliquefaciens*. 1982. *Microbiology*. Volume 13: 395~399. 43、 Mohagheghi, A., Ruth, M. and Schell, D.J. Conditioning hemicellulose hydrolysates for fermentation: Effect of overliming pH on sugar and ethanol yields. 2006. *Process Biochemistry*. Volume 41: 1806~1811. 44、 Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*. 2005. Volume 96: 673~686. 45、 Mosier, N.S., Ladisch, C.M. and Ladisch, M.R. Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. *Biotechnology and Bioengineering*. 2002. Volume 79: 610~618. 46、 Newbrun, E. and Baker, S. Physico-chemical characteristics of the levan produced by *streptococcus salivarius*. *Carbohydrate Research*. 1968. Volume 6: 165~170. 47、 Newbrun, E., Lacy, R. and Christie, T.M. The morphology and size of the extracellular polysaccharides from oral streptococci. *Archives of Oral Biology*. 1971. Volume 16: 863~872. 48、 Ohta, A. and Baba, S. and Takizawa, T. and Adachi, T. Effects of fructooligosaccharides on the absorption of magnesium in the magnesium-deficient rat model. *Journal of Nutritional Science and Vitaminology*. 1994. Volume 40: 171~180. 49、 Park, J.M., Kwon, S.Y., Song, K.B., Kwak, J.W., Lee, S.B., Nam, Y.W., Shin, J.S., Park, Y.I., Rhee, S.K. and Paek, K.H. Transgenic tobacco plants expressing the bacterial levansucrase gene show enhanced tolerance to osmotic stress. *Journal of Microbiology and Biotechnology*. 1999. Volume 9: 213~218. 50、 Phelps, C.F. The physical properties of inulin solutions. *Biochemical Journal*. 1965. Volume 95: 41~47. 51、 Pontis, H.G. and Del Gampillo, E. Fructans. In Dey P.M. and Dixon R.A., *Biochimica of Storage Carbohydrates in Green Plants*. 1985. 205~227. 52、 Roberfroid, M.B. Functional effects of food components and the gastrointestinal system: chicory fructooligosaccharides. *Nutrition Reviews*. 1996. Volume 54: 38~42. 53、 Rebro?, M., Rosenberg, M., Stloukal, R. and Kri?tofikova, L. High efficiency ethanol fermentation by entrapment of *Zymomonas mobilis* into Lentikats. 2005. *Letters in Applied Microbiology* Volume 41: 412~416. 54、 Rogers, P.L., Lee, K.J., Skotnicki, M.L. and Tribe, D.E. Ethanol production by *Zymomonas mobilis*. *Advances in Biochemical Engineering*. 1982. Volume 23: 37~84. 55、 Rogers, P.L., Lee, K.J. and Tribe, D.E. High productivity ethanol fermentations with *Zymomonas mobilis*. 1980. *Process Biochemistry*. Volume 15: 7~11. 56、 Sahm, H., Meyer, S.B. and Georg, A.S. The genus *Zymomonas*. *Prokaryotes*. 2006. Volume 5: 201~221. 57、 Sato, S., Koga, T. and Inoue, M. Isolation and some properties of extracellular D-glucosyltransferases and D-fructosyltransferases from *Streptococcus mutans* serotypes c, e, and f. *Carbohydrate Research*. 1984. Volume 134: 293~304. 58、 Seo, J.S., Chong, H., Park, H.S., Yoon, K.O., Jung, C., Kim, J.J., Hong, J.H., Kim, H., Kim, J.H., Kil, J.I., Park, C.J., Oh, H.M., Lee, J.S., Jin, S.J., Um, H.W., Lee, H.J., Oh, S.J., Kim, J.Y., Kang, H.L., Lee, S.Y., Lee, K.J. and Kang, H.S. The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. 2005. *Nature Biotechnology*. Volume 23: 63~68. 59、 Shih, I.L., Yu, Y.T., Shieh, C.T. and Hsieh, C.Y. Selective production and characterization of levan by *Bacillus subtilis* ( Natto ) Takahashi. *Journal of Agricultural and Food Chemistry*. 2005. Volume 53: 8211~8215. 60、 Skotnick, M.L., Lee, K.J., Tribe, D.E. and Rogers, P.L. Comparison of ethanol production by different *Zymomonas* strains. *Applied and Environmental Microbiology*. 1981. Volume 41: 889~893. 61、 Song, K.B., Belghith, H. and Rhee, S.K. Production of levan, a fructose polymer, using an overexpressed recombinant levansucrase. *Annals of the New York Academy of Sciences*. 1996. Volume 799: 601~607. 62、 Stivala, S.S. and Bahary, W.S. Some dilute-solution parameters of the levan of *streptococcus salivarius* in various solvents. *Carbohydrate Research*. 1978. Volume 67: 17~21. 63、 Striolo, A., Prausnitz, J.M., Bertuccio, A., Kee, R.A. and Gauthier, M. Dilute-solution properties of arborescent polystyrenes: further evidence for perturbed-hard-sphere behavior. *Polymer*. 2001. Volume 42: 2277~2715. 64、 Swatloski, R.P., Spear, S.K., Holbrey, J.D. and Rogers, R.D. Dissolution of cellulose with ionic liquids. *Journal of the American Chemical Society*. 2002. Volume 124: 4974~4975. 65、 Swings, J. and Ley, J.D. The biology of *Zymomonas*. *Bacteriol Review*. 1977. Volume 41: 1~46. 66、 Tanaka, K., Hilary, Z.D. and Ishizaki, A. Investigation of the utility of pineapple juice and pineapple waste material as low-cost substrate for ethanol fermentation by *Zymomonas mobilis*. *Journal of Bioscience and Bioengineering*. 1999. Volume 87: 642~646. 67、 Taomas, K.C., Hynes, S.H. and Ingledew, W.M. Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. *Process Biochemistry*. 1996. Volume 31: 321~331. 68、 Van Kranenburg, R., Boels, I.C., Kleerebezem, M. and de Vos, W.M. Genetics and engineering of microbial exopolysaccharides for food: approaches for the production of existing and novel polysaccharides. *Current Opinion in Biotechnology*. 1999. Volume 10: 498~504. 69、 Vijn, I. and Smeekens, S. Fructan: More than a reserve carbohydrate. *Plant Physiology*. 1999. Volume 120: 351~360. 70、 Wolff, D., Czapl, S., Heyer, A.G., Radosta, S., Mischnick, P. and Springer, J. Globular shape of high molar mass inulin revealed by static light scattering and viscometry. *Polymer*. 2000. Volume 41: 8009~8016. 71、 Xu, Q., Yajima, T., Saito, K., Ohshima, Y. and Yoshikai, Y. Levan (beta-2, 6-fructan), a major fraction of fermented soybean mucilage, displays immunostimulating properties via Toll-like receptor 4 signalling: induction of interleukin-12 production and suppression of T-helper type 2 response and immunoglobulin E production. *Clinical and Experimental Allergy*. 2006. Volume 36: 94~101. 72、 Zhang, Y.H. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of Industrial Microbiology and Biotechnology*. 2008. Volume 35: 367~375. 73、 Zhang, Y.H. and Lynd, L.R. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulose systems. *Biotechnology and Bioengineering*. 2004. Volume 88: 797~824.