

Study on Production of Levan by *Bacillus subtilis* DYU1 in a Fermentor

李奕宏、吳建一；顏裕鴻

E-mail: 9806223@mail.dyu.edu.tw

ABSTRACT

Levan is a polymer of fructose linked by α -1,6 fructofuranosidic bonds present in many plants and microbial products. Levan offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceuticals. Although many investigations on levan formation have been reported, they suffered the disadvantages of low yields and the contamination of impure products. Thus, levan has great potential if it can be produced less expensively. To further investigate the possibility of the use of *Bacillus subtilis* DYU1 for the efficient production of the levan product, we studied and describe in this study the factors affecting the production of levan by this bacterium and the purification and characterization of the products. After cultivation for 24 h, 40-50 g/L of levan was produced in medium containing 20% (w/v) sucrose by *B. subtilis* DYU1. The product consisted of two fractions with different molecular masses (2×10^7 and 1×10^4 Da), and the high molecular mass product (2×10^7 Da) predominated during fermentation process (~ 24 h), which could be easily separated by fractionation using an ethanol gradient. The products were well characterized by Gel permeation chromatography (GPC), Fourier transform infrared (FTIR), and Nuclear magnetic resonance (NMR).

B. subtilis DYU1, which was washed with sterile water, was used to produce levan from a variety of nitrogen substrates, including yeast extract, peptone, urea, NH_4Cl , NaNO_3 and sweet corn extract. The *B. subtilis* DYU1 strain was found to be able to assimilate the two inorganic nitrogen substrates examined (NH_4Cl and NaNO_3), whereas it grew less efficiently in organic nitrogen substrates (especially yeast extract and sweet corn extract). The levan concentration and production rate (VL) were also influenced by the sucrose concentration. The sucrose was available utilized preferentially for levan synthesis. Maximum levan concentration (85 g/L) and VL (2.350 g/L/h) were attained at 25% sucrose concentration. Levan production from *B. subtilis* DYU1 was also affected by various environmental factors, i.e. pH and temperature, as pH 7 and 37 °C were favorable for levan production.

Keywords : *Bacillus subtilis* DYU1、fermentation、fructan、levan

Table of Contents

封面內頁	
簽名頁	
授權書	iii
中文摘要	iv
英文摘要	vi
誌謝	viii
目錄	x
圖目錄	xiv
表目錄	xxv

1. 前言	1
2. 文獻回顧	4
2.1 Levan之簡介	4
2.1.1 Levan之結構	6
2.1.2 Levan來源及生產	10
2.1.3 Levan之特性	11
2.2 Levan生化合成機制	13
2.3 Levan生化降解機制	16
2.4 利用微生物生產levan	18
2.4.1 生產levan之菌株	19
2.4.2 基質的影響	25
2.5 利用純化之酵素合成levan	28
2.5.1 利用純化之酵素合成levan	32

2.5.2 環境因子的影響	34
2.5.3 金屬離子的影響	35
2.6 Levan流變學行為	41
2.6.1 牛頓流體	41
2.6.2 非牛頓流體	41
2.6.3 Levan流變學行為之影響因子	44
2.6.4 黏度與分子量之關係	46
2.7 Levan之應用	47
2.7.1 農業方面	48
2.7.2 食品方面	49
2.7.3 藥品與製藥方面	52
2.7.4 其他	55
3. 材料與方法	57
3.1 實驗材料	57
3.1.1 藥品	57
3.1.2 儀器設備	59
3.2 菌株培養	60
3.2.1 菌株來源	60
3.2.2 菌株活化	60
3.2.3 Levan生產培養	61
3.3 前培養殘留氮含量之去除方法	61
3.4 粗酵素液的製備與分析	62
3.5 氧氣質傳(KLa)之實驗	62
3.5.1 生物反應器	62
3.5.2 氧氣質傳係數(KLa)	62
3.6 分析方法	64
3.6.1 醣度分析	64
3.6.2 黏度	68
3.6.3 Levansucrase之活性測定	68
3.7 Levan之分析	68
3.7.1 高效能液相層析儀(HPLC)分析	68
3.7.2 膠體滲透層析(GPC)分析	69
3.7.3 核磁共振(NMR)分析	70
3.7.4 傅立葉紅外線光譜儀(FT-IR)分析	70
3.8 Levan之純化	71
4. 結果與討論	72
4.1 環境因子	72
4.1.1 不同培養溫度的影響	72
4.2 培養基組成	84
4.2.1 添加不同濃度之蔗糖的影響	84
4.2.2 添加不同濃度之NH ₄ Cl的影響	93
4.2.3 添加不同金屬離子的影響	103
4.2.4 添加兩種不同金屬離子的影響	111
4.2.5 添加不同CaCl ₂ 濃度的影響	118
4.2.6 添加不同氨基酸濃度的影響	126
4.2.7 添加不同濃度之糖蜜的影響	139
4.3 發酵槽實驗	146
4.3.1 不同攪拌速率的影響	146
4.3.2 不同曝氣速率的影響	155
4.4 饋料實驗	164
4.4.1 搖瓶之不同時間饋料的影響	164
4.4.2 發酵槽之饋料的影響	169
4.5 Levan純化方法之探討	172

4.5.1 不同比例之酒精與丙酮對純化的影響	172
4.6 純化後之levan的分析	177
4.6.1 藉由超過濾濃縮裝置分離levan之高低分子量	177
4.6.2 傅立葉轉換紅外線光譜儀(FT-IR)	185
4.6.3 核磁共振(NMR)	187
4.7 Levan溶液之流變學特性探討	189
4.7.1 pH對不同濃度之levan黏度的影響	189
4.7.2 溫度對不同濃度之levan黏度的影響	207
4.8 Levansucras之活性探討	220
4.8.1 不同溫度對酵素活性的影響	220
4.8.2 不同時間對酵素活性的影響	223
5. 結論	225
6. 參考文獻	225

圖目錄

Figure 1-1 Overall outline of this study	3
Figure 2-1 Chemical structure of various fructans	9
Figure 2-2 Biosynthesis of levansucrase	16
Figure 3-1 The standard calibration curve of polysaccharide concentration	65
Figure 3-2 The standard calibration curve of glucose concentration	66
Figure 3-3 The standard calibration curve of fructose concentration	66
Figure 3-4 The standard calibration curve of sucrose concentration	67
Figure 3-5 HPLC chromatogram of various saccharide concentration	67
Figure 3-6 The standard calibration curve of levan concentration	69
Figure 3-7 The standard calibration curve of molecular weight	70
Figure 4-1 Effect of different culture temperature on levan production by <i>B. subtilis</i> DYU1	78
Figure 4-2 Effect of different culture temperature on levan production and apparent viscosity by <i>B. subtilis</i> DYU1	79
Figure 4-3 Effect of different culture temperature on biomass and levan production by <i>B. subtilis</i> DYU1	80
Figure 4-4 Effect of different culture temperature on levan production rate and sucrose consume rate by <i>B. subtilis</i> DYU1	81
Figure 4-5 GPC chromatogram of various temperature on levan production by <i>B. subtilis</i> DYU1	82
Figure 4-6 Arrhenius plots for (a) specific growth rate and (b) volumetric levan production rate	83
Figure 4-7 The time course of levan production and sucrose consumption by <i>B. subtilis</i> DYU1 in fermentation medium containing various sucrose concentration	89
Figure 4-8 Effect of different sucrose concentration on levan production and apparent viscosity by <i>B. subtilis</i> DYU1	90
Figure 4-9 Effect of different sucrose concentration on biomass and levan production by <i>B. subtilis</i> DYU1	91
Figure 4-10 Effect of different sucrose concentration on levan production rate and sucrose consumption rate by <i>B. subtilis</i> DYU1	92
Figure 4-11 Effect of levan production from various NH ₄ Cl concentration by <i>B. subtilis</i> DYU1	97
Figure 4-12 Effect of various NH ₄ Cl concentration on levan production and apparent viscosity by <i>B. subtilis</i> DYU1 at various culture time	98
Figure 4-13 Effect of various NH ₄ Cl concentration on biomass and levan production by <i>B. subtilis</i> DYU1	99
Figure 4-14 Effect of various NH ₄ Cl concentration on levan concentration and sucrose consumption rate by <i>B. subtilis</i> DYU1	100
Figure 4-15 GPC chromatogram of various NH ₄ Cl concentration on levan production by <i>B. subtilis</i> DYU1	101
Figure 4-16 GPC chromatogram of various NH ₄ Cl concentration on levan production by <i>B. subtilis</i> DYU1 (continued)	102
Figure 4-17 Effect of different metal ions on levan production by <i>B. subtilis</i> DYU1	106
Figure 4-18 Effect of different metal ions on biomass and levan production by <i>B. subtilis</i> DYU1	107
Figure 4-19 Effect of different metal ions on levan production rate and sucrose consumption rate by <i>B. subtilis</i> DYU1	108
Figure 4-20 Effect of different metal ions on levan production and apparent viscosity by <i>B. subtilis</i> DYU1	109
Figure 4-21 GPC chromatogram of levan production for different growth time and different metal ions of <i>B. subtilis</i> DYU1	110
Figure 4-22 Effect of different metal ions on levan production by <i>B. subtilis</i> DYU1	114
Figure 4-23 Effect of different metal ions on levan production and apparent viscosity by <i>B. subtilis</i> DYU1	115

Figure 4-24 Effect of different metal ions on biomass and levan production by *B. subtilis* DYU1 116

Figure 4-25 Effect of different metal ions on levan production rate and sucrose consumption rate by *B. subtilis* DYU1 117

Figure 4-26 Effect of different CaCl_2 concentration (add phosphate buffer) on levan production by *B. subtilis* DYU1 121

Figure 4-27 Effect of different CaCl_2 concentration on levan production and apparent viscosity by *B. subtilis* DYU1 in the flask 122

Figure 4-28 Time course of levan production by *B. subtilis* DYU1 at various CaCl_2 concentration in the flask 123

Figure 4-29 Time course of levan production by *B. subtilis* DYU1 at various CaCl_2 concentration in the flask 124

Figure 4-30 GPC chromatogram of levan production for different CaCl_2 concentration of *B. subtilis* DYU1 125

Figure 4-31 Effect of different mixed amino acid solution (solution A and B) on levan production by *B. subtilis* DYU1 131

Figure 4-32 Effect of different mixed amino acid solution (solution A and B) on levan production and apparent viscosity by *B. subtilis* DYU1 132

Figure 4-33 Effect of mixed amino acid solution A on biomass and levan production by *B. subtilis* DYU1 133

Figure 4-34 Effect of mixed amino acid solution B on biomass and levan production by *B. subtilis* DYU1 134

Figure 4-35 Time course of levan production with mixed amino acid solution A by *B. subtilis* DYU1 in the flask 135

Figure 4-36 Time course of levan production with mixed amino acid solution B by *B. subtilis* DYU1 in the flask 136

Figure 4-37 Time course of levan production with mixed amino acid solution A by *B. subtilis* DYU1 in the flask 137

Figure 4-38 Time course of levan production with mixed amino acid solution B by *B. subtilis* DYU1 in the flask 138

Figure 4-39 The effect of different initial total sugar concentration on levan production by *B. subtilis* DYU1 in flask culture 142

Figure 4-40 The effect of different initial total sugar concentration on levan production by *B. subtilis* DYU1 in flask culture 143

Figure 4-41 Effect of different total sugar concentration on levan production rate and total sugar consumption rate by *B. subtilis* DYU1 in the flask 144

Figure 4-42 Effect of different total sugar concentration on levan production rate and total sugar consumption rate by *B. subtilis* DYU1 in the flask 145

Figure 4-43 Time course of *B. subtilis* DYU1 for levan production under various agitation rate in the 5L fermentator 149

Figure 4-44 Effect of different agitation rate on levan production and apparent viscosity by *B. subtilis* DYU1 150

Figure 4-45 Effect of different agitation rate on biomass and levan production by *B. subtilis* DYU1 151

Figure 4-46 Time course of levan production by *B. subtilis* DYU1 at various agitation rate in the 5L fermentator 152

Figure 4-47 Time course of levan production by *B. subtilis* DYU1 at various agitation rate in the 5L fermentator 153

Figure 4-48 GPC chromatogram of levan production for different agitation rate of *B. subtilis* DYU1 154

Figure 4-49 Time course of *B. subtilis* DYU1 for levan production under various aeration rate in the 5L fermentator 158

Figure 4-50 Effect of different aeration rate on levan production and apparent viscosity by *B. subtilis* DYU1 159

Figure 4-51 Effect of different aeration rate on biomass and levan production by *B. subtilis* DYU1 in the 5L fermentator 160

Figure 4-52 Time course of levan production by *B. subtilis* DYU1 at various aeration rate in the 5L fermentator 161

Figure 4-53 Time course of levan production by *B. subtilis* DYU1 at various aeration rate in the 5L fermentator 162

Figure 4-54 GPC chromatogram of levan production for different aeration rate of *B. subtilis* DYU1 163

Figure 4-55 Effect of feed batch on levan production by *B. subtilis* DYU1 in the flask 167

Figure 4-56 Effect of feed batch on levan production by *B. subtilis* DYU1 in the flask 168

Figure 4-57 Effect of feed batch on levan production by *B. subtilis* DYU1 in 5L fermentator 171

Figure 4-58 Purified levan by using various agents 174

Figure 4-59 GPC chromatogram of levan production for different alcohol concentration of *B. subtilis* DYU1 175

Figure 4-60 GPC chromatogram of levan production for different acetone concentration of *B. subtilis* DYU1 176

Figure 4-61 Separate high and low molecular weight from levan 180

Figure 4-62 ^{13}C NMR of different molecular weight of the levan produced by *B. subtilis* DYU1 181

Figure 4-63 ^1H NMR of different molecular weight of the levan produced by *B. subtilis* DYU1 182

Figure 4-64 HPLC chromatogram of levan with various molecular weight at different times 183

Figure 4-65 GPC chromatogram of levan with various molecular weight at different times 184

Figure 4-66 Infrared spectra of the polysaccharide produced by *B. subtilis* DYU1 186

Figure 4-67 ^{13}C NMR and ^1H NMR of the fructan produced by *B. subtilis* DYU1 188

Figure 4-68 Viscosity-shear rate profiles of different concentration aqueous solutions of the levan produced by *B. subtilis* DYU1 196

Figure 4-69 Shear stress-shear rate profiles of different concentration aqueous solutions of the levan produced by *B. subtilis* DYU1 197

Figure 4-70 Effect of pH on the viscosity of the aqueous solutions of the purified levan in various acid-base solution 198

Figure 4-71 $1/[\eta]_t - 1/[\eta]_0$ against time (min) in various acid-base solution 199

Figure 4-72 Rate constant (kb) vs pH at each different concentration of aqueous solutions of purified levan 200

Figure 4-73 GPC chromatogram of levan with various pH (pH 1-11) at different times 201

Figure 4-74 HPLC chromatogram of levan with various pH (pH 1 and 3) at different times 202

Figure 4-75 HPLC chromatogram of levan with various pH (pH 5 and 7) at different times 203

Figure 4-76 HPLC chromatogram of levan with various pH (pH 9 and 11) at different times 204

Figure 4-77 Effect of pH on the hydrolysis of the levan solution 205

Figure 4-78 HPLC chromatogram of levan with various pH (pH 1-11) after 24 h 206

Figure 4-79 Viscosity-shear rate profiles of different concentration aqueous solutions of the levan produced by *B. subtilis* DYU1 214

Figure 4-80 Shear stress-shear rate profiles of different concentration aqueous solutions of the levan produced by *B. subtilis* DYU1 215

Figure 4-81 Effect of temperature on the viscosity of the aqueous solutions of the levan produced by *B. subtilis* DYU1 216

Figure 4-82 $1/[\eta]_t - 1/[\eta]_0$ against time (min) at different temperature 217

Figure 4-83 Rate constant (kb) vs temperature at each different concentration aqueous solutions of levan produced by *B. subtilis* DYU1 218

Figure 4-84 Arrhenius plots of levan degradation at different levan concentration (by *B. subtilis* DYU1 from this study) 219

Figure 4-85 Temperature behavior of *B. subtilis* DYU1 Levansucrase 222

Figure 4-86 The time course of activity of crude levansucrase from *B. subtilis* DYU1 224

表目錄

Table 2-1 Levan production by various microorganisms 22

Table 2-2 The levansucrase product strain and its properties 30

Table 2-3 The effect of metal ions on the activity of levansucrase 38

Table 3-1 Composition of nutrient broth (NB) 60

Table 3-2 Composition of sucrose medium (SM) on levan production 61

Table 4-1 Amino acid composition of solution A and B 130

REFERENCES

1. Abdel-Fattah, A. F., Mahmoud, D. A. R. and Esawy, M. A. T. 2005. Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymic synthesis of levan and fructo-oligosaccharides. *Current Microbiology* 51: 402-407.
2. Allen, D. G. and Robinson, C. W. 1989. Hydrodynamic and mass transferr in *Aspergillus niger* fermentations in bubble column and loop bioreactors. *Biotechnology and Bioengineering* 34: 731-740.
3. Allen, D. G. and Robinson, C. W. 1990. Measurement of rheological properties of filamentous fermentation broths. *Chemical Engineering Science* 45: 37-48.
4. Ammar, Y. B., Matsubara, T., Ito, K., Iizuka, M., Limpaseni, T., Pongsawadi, P. and Minamiura, N. (2002) Characterization of a thermostable levansucrase from *Bacillus* sp. Th4-2 capable of producing high molecular weight levan at high temperature. *Journal of Biotechnology* 99: 111-119.
5. Archbold, H. K. 1938. Physiological studies in plant nutrition. VII. The role of fructoseans in the carbohydrate metabolism of the barley plant. *Annals of Botany* 2: 403-1938.
6. Arrhenius, S. 1889. On the reaction velocity of the inversion of cane sugar by acids. *Zeitschrift fur Physikalische Chemie*. 4: 226-232.
7. Arvidson, S. A., Todd Rinehart, B. and Gadala-Maria, F. 2006. Concentration regimes of solutions of levan polysaccharide from *Bacillus* sp. *Carbohydrate Polymers* 65: 144-149.
8. Atkins, P. 2001. *The elements of physical chemistry*. (3rd ed.). Oxford University Press Inc., New York.
9. Avigad, G. 1968. Levans. In Bikales, N. M. (Ed.), *Encyclopedia of polymer science and technology*. p. 711-718. John Wiley & Sons, Inc., New York.
10. Bae, I. Y., Oh, I.K. Lee, S. Yoo, S.H. Lee, H. G. 2008. Rheological characterization of levan polysaccharides from *Microbacterium laevaniformans*. *International Journal of Biological Macromolecules* 42: 10-13.
11. Barrow, K. D., Collins, J. G., Leigh, D. A., Rogers, P. L. and Warr, R. G. 1984. Sorbitol production by *Zymomonas mobilis*. *Applied Microbiology and Biotechnology* 20: 225-232.
12. Beker, M. J., Shvinka, J. E., Pankova, L. M., Laivenienks, M. G. and Mezharde, I. N. 1990. A simultaneous sucrose bioconversion into ethanol and levan by *Zymomonas mobilis*. *Applied Biochemistry and Biotechnology* 24/25: 265-274.
13. Bekers, M., Linde, R., Danilevich, A., Kaminska, E., Upite, D., Vigants, A. And Scherbaka, R. 1999. Sugar beet diffusion juice and syrup as media for ethanol and levan production by *Zymomonas mobilis*. *Food Biotechnology* 13: 107-119.
14. Bekers, M., Upite, D., Kaminska, E., Grube, M., Laukevics, J., Vina, I., Vigants, A. and Zikmanis, P. 2003. Fructan biosynthesis by intra- and extracellular *Zymomonas mobilis* levansucrase after simultaneous production of ethanol and levan. *Acta Biotechnologica* 1: 85-93.
15. Bekers, M., Upite, D., Kaminska, E., Laukevics, J., Grube, M., Vigants, A. And Linde, R. 2005. Stability of levan produced by *Zymomonas mobilis*. *Procsee Biochemistry* 40: 1535-1539.
16. Bekers, M., Vigants, A., Laukevics, J., Toma, M., Rapoport, A., Zikmanis, P. 2000. The effect of osmo-induced stress on product formation by *Zymomonas mobilis* on sucrose. *International Journal of Food Microbiology* 55: 147-150.
17. Belghith, H., Song, K. B., Kim, C. H. and Rhee, S. K. 1996. Optimal conditions for levan formation by an

overexpressed recombinant levansucrase. *Biotechnology Letters* 18: 467-472.18.Ben Tovim, R. And Hestrin, S. 1957. Patterns of levandegradation by extracellular enzymes in bacteria. *Bulletin of the Research Council of Israel. Section A, 6A*: 31019.Bender, M. M. and Smith, D. 1973. Classification of starch and fructosan-accumulating grasses as C-3 or C-4 species by carbon isotope analysis. *Journal of the British Grassland Society* 28: 97-100.20.Beveridge, T. J. and Graham, L. L. 1991. Surface layers of bacteria. *Microbiological Reviews* 55: 684-705.21.Biedrzycka, E. and Bielecka, M. 2004. Prebiotic effectiveness of fructans of different degrees of polymerization. *Trends in Food Science & Technology* 15: 170-175.22.Blake, J. D., Clarke, M. L., Jansson, P. E. and Mcneil, K. E. 1982. Fructan from *Erwinia herbicola*. *Journal of Bacteriology* 151: 1595-1597.23.Bodie, E. A., Schwartz, R. D. and Catena, A. 1985. Production and Characterization of a polymer from *Arthrobacter* sp. *Applied and Environmental Microbiology* 50: 629-633.24.Bothner, H., Waaler, T. and Wik, O. 1988. Limiting viscosity number and weight average molecular weight of hyaluronate samples produced by heat degradation. *International Journal of Biological Macromolecules* 10: 287-291.25.Calazans, G. M. T., Lopes, C. E., Lima, R. M. O. C., Franca, F. P. 1997. Antitumoural activities of levans produced by *Zymomonas mobilis* strains, *Biotechnology Letters* 19: 19-21.26.Ceska, M. 1971. Biosynthesis of levan and a new method for the assay of levansucrase activity. *Biochemical Journal* 125: 209-211.27.Chambert, R. and Petit-Glatron, M. F. 1989. Study of the effect of organic solvents on the synthesis of levan and the hydrolysis of sucrose by *Bacillus subtilis* levansucrase. *Carbohydrate Research* 191: 117-123.28.Chambert, R., Treboul, G. and Dedonder, R. 1974. Kinetic studies of levansucrase of *Bacillus subtilis*. *European Journal of Biochemistry* 41: 285-300.29.Chung B. H., Kim W. K., Song K. B., Kim C. H. and Rhee S. K. 1997. Novel polyethylene glycol/levan aqueous two-phase system for protein partitioning. *Biotechnology techniques* 11: 327-329.30.Clarke, M. A., Roberts, E. J. and Garegg, P. J. 1997. New compounds from microbiological products of sucrose, *Carbohydrate Polymer* 34: 425.