

摘要

本研究由高雄縣十三坑的溫泉露頭取得之溫泉水/底泥樣品，分離純化出一株嗜熱厭氧澱粉水解菌 strain S2302。Strain S2302 為桿菌，大小約 $0.8-1 \times 6.0-9.0\mu\text{m}$ ，革蘭氏陰性菌，不具移動性。生長介於 $60-80^{\circ}\text{C}$ 與最佳生長酸鹼度 pH 7.2。strain S2302 可利用的碳源有 mannose, starch, xylose, glucose, maltose, fructose, sorbose, sucrose, pullulan，不可利用 lactose, glycine, mannitol, neopeptone, carboxymethyl cellulose, raffinose, arabinose, galactose, rhamnose, cellobiose, glycerol, gelatin, sorbitol, esculin, xylan, cellulose, carbon monoxide 為碳源生長。利用葡萄糖為碳源發酵時之主要產物為 ethanol, acetic acid, butyric acid。Strain S2302 之生長可被 $20\mu\text{g/ml}$ polymyxin B 與 vancomycin 所抑制，但是無法被 $100\mu\text{g/ml}$ penicillin 與 ampicillin 所抑制。於培養基添加硫代硫酸鈉可增加 strain S2302 的生長速率及生物量。Strain S2302 於不同濃度的可溶性澱粉之比生長速率倒數對不同濃度的可溶性澱粉倒數分析，得到最大比生長速率為 0.345 h^{-1} 與半飽和速率常數為 0.613 g/L 。Strain S2302 可水解經由乾燥研磨過的米粉與甘藷粉，但是不能水解經由乾燥研磨過的玉米粉。Strain S2302 的最高產率為 0.1618 g 還原糖/g 可溶性澱粉，此時的轉換率為 99%。16S rDNA 分析，strain S2302 與

Caldanaerobacter 屬較為接近。初步測試 strain S2302 的 amylase 酵素比活性為 638.9 U/mg。

關鍵字：嗜熱菌、厭氧菌、澱粉水解菌、澱粉水解？

Abstract

A thermophilic anaerobic starch-hydrolyzing bacterium was isolated from Shihsankeng hot spring at Kaohsiung county. The isolate was a non-spore former and tentatively named strain S2302. Cell of strain S2302 was gram-negative, non-motile rod whose size was from 0.8-1 by 6.0-9.0 μm . Growth occurred between 60 and 80°C and the optimum pH was 7.2. Strain S2302 fermented substrates such as mannose, starch, xylose, glucose, maltose, fructose, sorbose, sucrose and pullulan. Lactose, glycine, mannitol, neopeptone, carboxymethyl cellulose, raffinose, arabinose, galactose, rhamnose, cellobiose, glycerol, gelatin, sorbitol, esculin, xylan and cellulose were not fermented. The predominant fermentation end products from glucose were ethanol, acetic acid and butyric acid. Growth of strain S2302 was inhibited by 20 $\mu\text{g/ml}$ polymyxin B and vancomycin, not by 100 $\mu\text{g/ml}$ penicillin and ampicillin. Thiosulfate was found to enhance the growth rate and cell yield of strain S2302. Analysis of growth kinetics of strain S2302 showed the μ_{max} was 0.345 h^{-1} and K_s was 0.613 g/L . Analysis of the 16S rDNA sequence revealed that strain S2302 was closely related to the members of genus *Caldanaerobacter*. Strain S2302 could hydrolyze

crude starch of ground rice and sweet potato, but not crude starch of corn.

Strain S2302 exhibited a highest reducing sugar production yield of

0.1618 g / g soluble starch and the conversion was 99%. The amylase

specific activity of strain S2302 was 638.9 U/mg.

Key words: thermophiles, anaerobes, starch-hydrolyzing, amylolytic
enzymes

目 錄

第一章 前言.....	1
第二章 文獻回顧.....	3
2.1 生質能.....	3
2.2 生物能源產生之方式.....	3
2.3 澱粉.....	8
2.4 嗜熱菌與耐熱酵素.....	10
2.4.1 嗜熱性細菌.....	10
2.4.2 產生耐熱酵素之嗜熱菌.....	12
2.4.3 耐熱酵素之應用.....	12
2.4.4 澱粉水解? 之種類與機制.....	14
2.4.5 Amylase 在工業上之應用.....	22
2.5 澱粉水解菌.....	26
2.5.1 <i>Thermotoga maritima</i>	30
2.5.2 <i>Pyrococcus furiosus</i>	30
2.5.3 <i>Thermoanaerobacter ethanolicus</i>	31
第三章 實驗方法與設計.....	33
3.1 實驗流程設計.....	33
3.2 菌株樣本採集.....	33

3.3 菌種之純化、培養、選取與保存.....	33
3.3.1 嗜熱厭氧培養基.....	33
3.3.2 菌株之選取與保存.....	35
3.4 菌種鑑定.....	36
3.4.1 鑑別染色	36
3.4.2 菌株之 16S rDNA 鑑定.....	36
3.4.2.1 菌株 Genomic DNA 之萃取.....	38
3.4.2.2 聚合? 連鎖反應.....	39
3.4.2.3 TA cloning.....	40
3.4.2.4 限制內切? 反應.....	42
3.4.2.5 16S rDNA 定序與分析.....	43
3.5 菌株之生理生化特性分析.....	43
3.5.1 菌相觀察.....	43
3.5.2 最佳生長條件.....	44
3.5.3 不同碳源測試.....	45
3.5.3.1 蛋白質測試.....	46
3.5.3.2 還原糖含量測試.....	47
3.5.4 抗生素感受度.....	48
3.5.5 過氧化氫? 測試.....	49

3.5.6 硫代硫酸鈉與硫酸鹽還原能力測試.....	50
3.5.7 硝酸還原測試.....	51
3.5.8 產乳酸測試.....	52
3.5.9 Alanine 測試.....	52
3.5.10 氫氣測試.....	54
3.5.11 揮發性有機酸測試.....	54
3.6 澱粉? 酵素活性分析.....	56
3.7 不同澱粉來源植物水解測試.....	56
3.7.1 總糖濃度之定量.....	57
3.7.2 澱粉濃度測定.....	57
3.8 不同基質濃度對生長之影響.....	57
第四章 結果與討論.....	59
4.1 研究菌株之選取.....	59
4.2 菌相觀察.....	59
4.3 菌種鑑定.....	62
4.3.1 革蘭氏染色.....	62
4.3.2 16s rDNA 序列分析.....	62
4.3.3 <i>Caldanaerobacter</i> 屬.....	65
4.4 生化特性分析.....	69

4.4.1 最佳生長條件.....	69
4.4.2 基質利用模式.....	74
4.4.3 抗生素感受性測試.....	78
4.4.4 過氧化氫? 測試.....	79
4.4.5 硫代硫酸鈉還原能力測試.....	81
4.4.6 代謝產物、氫氣與揮發性有機酸測試.....	81
4.4.7 L-alanine 測試.....	82
4.4.8 乳酸測試.....	86
4.5 研究菌株與相近菌株之比較.....	86
4.6 酵素分析.....	90
4.7 澱粉濃度對 strain S2302 生長之探討	92
4.8 不同來源澱粉水解測試.....	95
4.9 不同濃度澱粉水解測試.....	101
第五章 結論與建議.....	105
參考文獻.....	108
附錄	
附錄 1 蛋白質測試檢量線.....	116
附錄 2 還原糖含量測試檢量線.....	117
附錄 3 硫化氫測試檢量線.....	118

附錄 4 硫酸鹽檢量線.....	119
附錄 5 亞硫酸鹽檢量線.....	120
附錄 6 亞硝酸鹽檢量線.....	121
附錄 7 乳酸檢量線.....	122
附錄 8 alanine 測試檢量線與標準品停留時間.....	123
附錄 9 氫氣檢量線.....	124
附錄 10-1 GC-FID chromatogram of volatile acid standard mixture...	125
附錄 10-2 GC-FID chromatogram of ethanol.....	126
附錄 11 總糖檢量線.....	127
附錄 12 澱粉檢量線.....	128
附錄 13 Strain S2302 16S rDNA 定序結果.....	129

表 目 錄

Table 2.1 Different (bio)process strategies for biomass energy Production.....	4
Table 2.2 Bioconversion reactions and applications of thermostable enzymes.....	13
Table 2.3 Source microorganisms and properties of thermostable starch hydrolyzing enzymes.....	16
Table 3.1 Procedure and the results of Gram stain.....	37
Table 3.2 Gradient program for HPLC analysis of alanine.....	55
Table 4.1 Similarity index derived from 16S rDNA sequences of S2302, <i>Caldanaerobacter yonseiensis</i> , <i>Caldanaerobacter tengcongensis</i> , <i>Caldanaerobacter subterraneus</i> and <i>Caldanaerobacter</i> <i>pacificus</i>	68
Table 4.2 Characteristic comparison of the isolate strain S2302, <i>C.</i> <i>subterraneus</i> , <i>C. tengcongensis</i> , <i>C. yonseiensis</i> and <i>C.</i> <i>pacificus</i>	88
Table 4.3 Specific activity of Strain S2302, <i>Pyrococcus furious</i> , <i>Thermococcus Litoralis</i> , <i>Thermococcus profundus</i> DT5432, <i>Bacillus stearothermophilus</i> , <i>Lactobacillus manihotivorans</i> , <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Thermococcus</i> <i>hydrothermalis</i> and <i>Thermomyces lanuginosus</i>	91

圖 目 錄

Figure 2.1 Intricate food web of methanogenic anaerobic digestion. Several trophic groups of microorganisms work together to convert complex organic material into methane and carbon dioxide.....	6
Figure 2.2 Main conversion options for biomass to secondary energy carriers. Some categories represent a wide range of technological concepts as capacity ranges at which they are deployed.....	7
Figure 2.3 Structure of amylose and amylopectin.....	9
Figure 2.4 Schematic presentation of the action of amylolytic and pullulytic enzymes. Black circles indicate reducing sugars...	18
Figure 3.1 Flow chart of the experimental design.....	34
Figure 4.1 Specific growth rates of 7 isolated thermophilic anaerobic bacteria on glucose (5g/L).....	60
Figure 4.2 Photomicrographs of negative-stained S2320.....	61
Figure 4.3 Agarose gel electrophoresis of PCR-amplified 16S rDNA segment of strain S2302.....	63
Figure 4.4 Segments of 16S rDNA after TA cloning digested by restriction enzymes <i>Hinp</i> 1 and <i>Hinf</i> 1.....	64
Figure 4.5 Phylogenetic trees showing the relationship of strain S2302 with related organisms.....	67
Figure 4.6 Growth curves of strain S2302 under different temperatures.....	70
Figure 4.7 Specific growth rates of strain S2302 at different temperatures.....	71
Figure 4.8 Growth curves of strain S2302 under different pH's.....	72
Figure 4.9 Specific growth rates of strain S2302 at different pH.....	73
Figure 4.10 Growth curves of strain S2302 under different salt concentrations.....	75
Figure 4.11 Specific growth rates of strain S2302 at different salt concentrations.....	76
Figure 4.12 Effect of yeast extract on growth of strain S2302.....	77
Figure 4.13 Effect of various antibiotics on growth of strain S2302 with 5g/l glucose as substrate.....	80

Figure 4.14 Production of sulfide and cell growth from thiosulfate reduction by strain S2302.....	83
Figure 4.15 Hydrogen production from fermentation of various growth substrates by strain S2302.....	84
Figure 4.16 Gas chromatogram of volatile fatty acids produced from fermenting glucose by strain S2302.....	85
Figure 4.17 Specific growth rates of strain S2302 with different starch concentrations.....	93
Figure 4.18 Relationship of reciprocal of starch concentration and specific growth rate of strain S2302.....	94
Figure 4.19 Cell growth from fermentation of various growth substrates by strain S2302.....	98
Figure 4.20 Production of reducing sugar from fermentation of various growth substrates by strain S2302.....	99
Figure 4.21 Total sugar produced in culture medium from fermentation of various growth substrates by strain S2302.....	100
Figure 4.22 Growth curve of strain S2302 with different concentration of soluble starch.....	102
Figure 4.23 Production of reducing sugar (equivalent to glucose) from fermentation of different concentration starch by strain S2302.....	103
Figure 4.24 Change of starch concentration when strain S2302 was grown with different concentration of soluble starch.....	104