Metabolic Engineering of the Thermophilic Bacteria, *Bacillus stearothermophilus*, for Ethanol Production

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ABSTRACT

Thermophilic bacterium, *Bacillus stearothermophilus NUB3621*, was engineered to produce ethanol from glucose by introducing cloned thermostable pyruvate decarboxylase and alcohol dehydrogenase genes. A novel promoter sequence was screened and used for the enhancement of these two enzymes. Successful redirection of metabolic flux into ethanol was obtained. In addition, gene expression profiling using *Bacillus subtilis* DNA microarray was analyzed to overcome the intrinsic low glucose utilization of *B.stearothermophilus*. Many known and unknown genes were identified to be up or down regulated under glucose-containing media.

INTRODUCTION

Solar energy stored by the conversion of carbon dioxide into green plants represents the most abundant source of renewable energy in the world. The most economical process for ethanol production from cellulosic biomass has been identified as the SSF (Simultaneous Saccharification and Fermentation) process[1)]. However, discrepancy between the optimal temperatures of ethanol-producing microorganisms (30-37 °C) and cellulolytic enzymes (45-50°C) is major obstacle for the application of the SSF process to commercial process. There have been many efforts to raise the optimal temperature of the common ethanologenic bacteria and yeast up to 40-50 °C. In addition, many researches have been done for getting cellulolytic enzymes working well under physiological conditions. But all these approaches were not successful.

Metabolic engineering of non-ethanologenic bacteria, *E.coli* and *Klebsiella oxytoca*, have been successful to produce ethanol [2),3)],. Using this technique, non-ethanologenic thermophilic bacteria can be transformed to thermophilic ethanologenic bacteria. In this study, we engineered thermophilic bacteria, *Bacillus stearothermophilus NUB3621*, for ethanol production by introducing the genes for thermostable pyruvate decarboxylase and alcohol dehydrogenase. This thermophile showed limited glucose utilization. To overcome this characteristics, systematic analysis of gene expression profile using *Bacillus subtilis* genomic DNA microarray was done under glucose-containing media. The preliminary results are also discussed.

MATERIALS AND METHODS

E.coli XL1-Blue, GM2163, ER1647 were used as DNA manipulation host. All the DNA works were followed by the standard methods. *Bacillus stearothermophilus NUB 3621* was used as a transformation host for the final expression vectors. The pNW26 was used as expression vector for pdc and adh genes in *B.stearothermophilus*. Transformation of the expression vectors into *B.stearothermophilus* was done by the method described Welker et al., 1986. The modified LB media was used for the growth of *B.stearothermophilus*. The chromosomal DNA of the *B.stearothermophilus* XL-65-6 was used for the cloning of thermostable adh gene and screening for a novel strong promoter sequence. Cell mass, Glucose and enzyme activities for the pdc and adh were analyzed by the spectrophotometric method. Ethanol was measured by GC-FID.

The genomic DNA microarray and optimized RT primer set for the *Bacillus subtils* was purchased from the Sigma-Genosys. Total RNA was prepared by lysozyme-treated hot phenol method. The prepared RNA was purified by using Dneasy mini-kit (Qiagen). The RT reaction was done using ^{33}P-labelled dCTP. The scanned data from the Phosphoimage analyzer (Storm 840, Molecular Dynamics) were analyzed by determining the pixel density for each spot using ImageQuant (Version 5.0) software (Molecular Dynamics).

RESULTS AND DISCUSSION

1. Construction of the ethanologenic *B.stearothermophilus*

The chromosomal DNA from *B.stearothermophilus XL-65-6* was digested with *Sau 3AI*. The 2-4 kb fragments were ligated into the *BamHI* digested pUC18. The resulting ligation mixtures were transformed into *E.coli* and screened on aldehyde indicator plate. The colony showing intense rose-red color was selected for the cloned adh gene. The thermostable adh was purified and characterized to be MW=55 kDa, T_{opt}=60 °C, pH_{opt}=5.0, K_m=0.42 mM, V_{max}=136 U/mg. The pdc gene from *Zymomonas mobilis* showed good thermostability. These two genes were used for the construction of the ethanologenic *B.stearothermophilus*.

To express the adh and pdc gene efficiently in *B.stearothermophilus*, promoter probe vector, which lacks any promoter sequence from adh gene, was constructed to screen a novel powerful promoter. Random cleaved chromosomal DNA of *B.stearothermophilus XL-65-6* was introduced in this vector. Several new promoters were screened and one of them was sequenced and characterized. Using this novel promoter sequence, expression vector (pNW-PET) for pdc and adh genes were constructed as shown in Figure 1. By transforming this plasmid into *B.stearothermophilus NUB3621*, new ethanologenic thermophile, *B.stearothermophilus NUB3621 (pNW-PET)* was constructed.

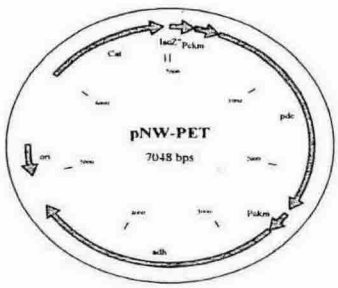

Figure 1. Construction of the expression vector, pNW-PET.

2. Expression of the pdc and adh genes and ethanol production

Expression of pyruvate decarboxylase in *B.stearothermophilus* was confirmed by the Western blot analysis. The quantitative analysis for pdc and adh activities showed that the two cistronic expression cassette was better than mono-cistronic configuration. The final expression level for pdc and adh was 0.35 U/mg-protein and 1.74 U/mg-protein, respectively.

When applied to ethanol fermentation, the *B.stearothermophilus NUB3621 (pNW-PET)* showed good ethanol yield in mLB media containing 2% glucose under pH-controlled condition as shown in Figure 2. However, only slight amount of glucose was utilized when this bacteria was cultured without pH-control. In addition, considerable amount of glucose was still not utilized even in pH-controlled fermentation.

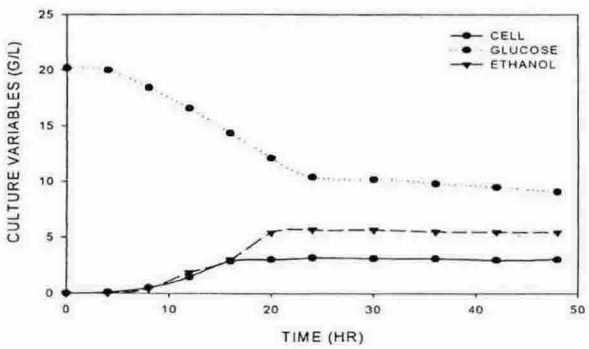

Figure 2. Time course of the pH-controlled batch fermentation.

3. Characterization of the glucose metabolism

To find out the reason for severe growth-inhibitory effect of glucose on *B.stearothermophilus NUB3621,* metabolite was analyzed using ^{1}H-NMR. Under non-pH-controlled cultivation, the major carbon flux from glucose was directed toward lactate accumulation at first few hours after inoculation with rapid drop of pH. Also, growth was ceased soon and cell lysis was started. After 24 hours, all the cells were found to be dead as shown in Figure 3. When the pH was controlled, glucose utilization was alleviated and metabolic flux went to ethanol accumulation instead of lactate, but the growth period was still quite short. This kind of growth-inhibitory effect was not found in case of using mLB media without glucose. To overcome inhibitory effect of glucose and increase glucose utilization in *B.stearothermophilus NUB3621 (pNW-PET),* systematic analysis was needed.

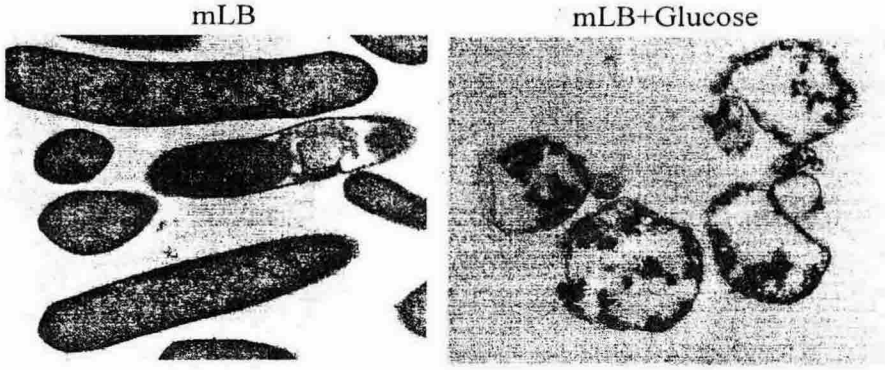

Figure 3. Growth inhibitory effect of glucose on *B.stearothermophilus NUB3621.*

4. Metabolic profiling for glucose utilization

To ease the inhibitory effect of glucose and increase glucose utilization in *B.stearothermophilus*, systematic analysis for all the ORFs at genomic level was made using DNA microarray of *B.subtilis*, which has all 4107 ORFs encoded in genome showing over 70 % sequence homology with *B.stearothermophilus*. Total RNA was prepared from both the cells grown in mLB and mLB containing glucose (2%) media under continuous cultivation. By reverse transcription reaction of mRNAs with the optimized primer sets, ^{33}P-dCTP-labelled transcriptome probe was prepared. These probes were hybridized with the *B.subtilis* DNA microarray. After scanning with phosphoimage analyzer, each spot was analyzed with the ImageQuant software for quantitative analysis. After normalization, each spot from the glucose-containing media for each ORF was compared with that from the culture without glucose. The expression ratio of whole ORFs were calculated as shown in Figure 4.

Many genes were found to be regulated by the presence of glucose. Among them, 42 genes were highly up regulated while 28 genes were significantly down regulated. Except for 32 genes out of these significant genes, all others were unknown genes. From this DNA array analysis, major repression site by glucose was found to be energy metabolic pathways such as the TCA cycle and electron transport chain. Interestingly, some genes of the glycolysis were also severely inhibited. The pentose phosphate pathway seemed to be activated by glucose. This might be due to the excess requirement for reducing capacity to supply energy by substrate level phosphorylation under the repression of the major energy production site. Some of the data were consistent with the previous biochemical or genetic data, but lots of data including the unknown genes was not possible to interpret with the known genetic control mechanisms. Some more detail analysis for these DNA array data will be discussed.

Using these expression profile data from DNA microarray analysis, systematic modification of *B.stearothermophilus NUB3621* as well as the identification of unknown genes revealed to be major regulation sites by glucose is under way. Some genes are being amplified while others

being knocked-out. From this kind of approach, systematic metabolic redirection or reprogramming may be possible.

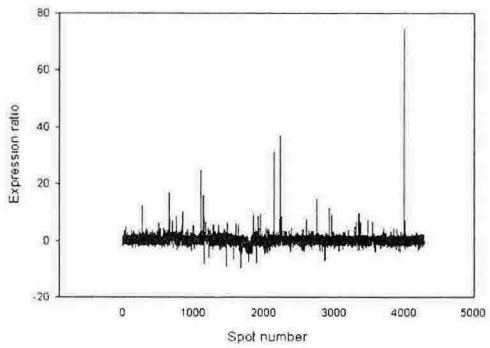

Figure 4. Expression profile using DNA microarray

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4. Chen Z., Wojcik S.F., Welker N.E., "Genetic analysis of *Bacillus stearothermophilus* by protoplast fusion" (1986), J. Bacteriol., 165(3), 994-1001.