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Periodic Change in DO Concentration for Efficient Poly-β-hydroxybutyrate Production Using Temperature-inducible Recombinant *Escherichia coli* with Proteome Analysis

Nor Aini Abdul Rahman[1], Yoshihito Shirai[1], Kazuyuki Shimizu[1]*, and Mohd Ali Hassan[2]

[1] Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan
[2] Department of Biotechnology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Abstract Recombinant *Escherichia coli* strain harboring the λp_R-p_L promotor and heterologus poly-β-hydroxybutyrate (PHB) biosynthesis genes was used to investigate the effect of culture conditions on the efficient PHB production. The expression of *phb* genes was induced by a temperature upshift from 33°C to 38°C. The protein expression levels were measured by using two-dimensional electrophoresis, and the enzyme activities were also measured to understand the effect of culture temperature, carbon sources, and the dissolved oxygen (DO) concentration on the metabolic regulations. AcetylCoA is an important branch point for PHB production. The decrease in DO concentration lowers the citrate synthase activity, thus limit the flux toward the TCA cycle, and increase the flux for PHB production. Since NADPH is required for PHB production, the PHB production does not continue leading the overproduction of acetate and lactate. Based on these observations, a new operation was considered where DO concentration was changed periodically, and it was verified its usefulness for the efficient PHB production by experiments.

Keywords: proteome analysis, recombinant *Escherichia coli*, PHB, enzyme activity, genes regulation

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates that are synthesized and accumulated intracellularly as an energy and/or carbon storage material by numerous microorganisms. PHAs are considered to be good candidates for biodegradable plastics and elastomers since they posses material properties similar to those of synthetic polymers currently in use and completely biodegradable after disposal [1,2].

The PHA biosynthesis genes of *Ralstolnia eutropha* H16 have been cloned in *Escherichia coli* and studied in detail. A high concentration of P(3HB) could be produced by recombinant *E.coli* in a relatively inexpensive medium. In comparison with *R. eutropha*, recombinant *E.coli* has several advantages such as fast growth, the ability of accumulating a large amount of polymer, the ability to utilize various carbon sources, well established high cell density culture techniques, and the lack of the PHA depolymerases. Furthermore, PHA recovery seems to be easier with recombinant *E.coli* not only because the cell which accumulates a large amount of PHA becomes fragile, but also the PHA granules synthesized by recombinant *E.coli* are larger and more crystalline than those synthesized by *R. eutropha* [3,4].

A major drawback to the industrial realization of bioplastic PHB production is its higher production cost compared to the conventional petrochemical plastics. The conventional approach is to find the appropriate culture condition based on many fermentation data. However, the approach limit the improvement of the fermentation process. The essential thing is to quantitatively understand the metabolic regulation in relation to culture conditions. We, therefore, made proteome analysis of recombinant *E. coli* based on two-dimensional electrophoresis (2DE) [5]. Proteome analysis is to study the protein expression properties such as expression levels, post-translational modification *etc.* in a large scale to obtain a global and integrated view for disease processes, cellular processes and networks at the protein level [6]. Proteome analysis by 2DE has been proposed as a powerful tool for investigating genomics functional [7]. The emerging field of proteomics has grown out of the mature technology of high quantification and increasingly refined technologies for the identification of separated proteins [8]. However, since the protein is identified based on isolelectric pH (pI) and molecular weight, it has to be made certain by using TOF-MS *etc.* In the present study, we measured the enzyme activities to ascertain the 2DE result.

In the present study, we used a recombinant *E. coli* to produce PHB and investigated the effect of culture conditions on the protein expression levels by applying 2DE and by measuring the enzymes activities for the central metabolic pathway. Fig. 1 shows the overall main metabolic pathways for recombinant *E. coli*, where

* **Corresponding author**
Tel: +81-948-29-7817 Fax: +81-948-29-7801
e-mail: shimi@bse.kyutech.ac.jp

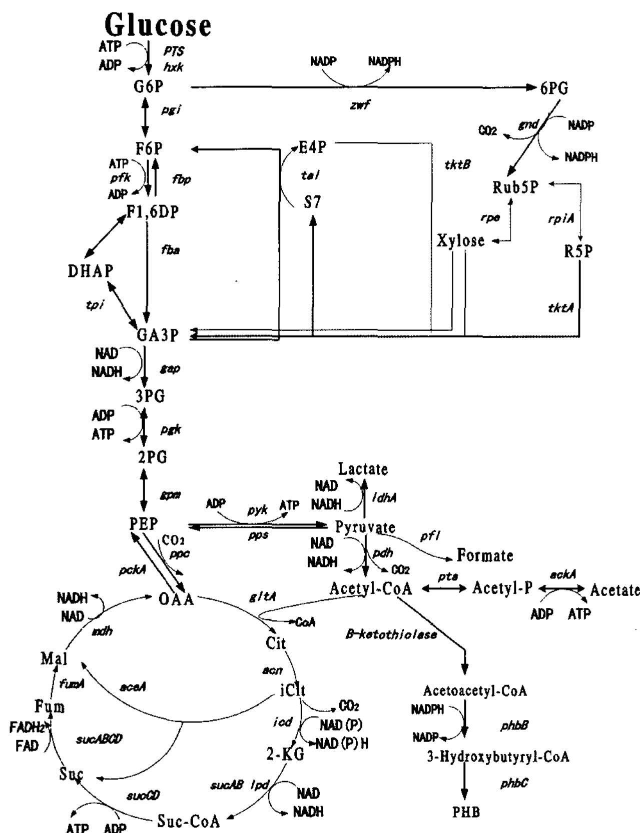

Fig. 1. Metabolic network of recombinant *E. coli* for PHB production.

AcCoA is an important intermediate for PHB production. It may be considered to redirect toward PHB synthetic pathway at AcCoA branch by blocking the pathway to TCA cycle by decreasing the DO concentration. In the present study, we investigated the effect of induction temperature, different carbon sources, and the dissolved oxygen (DO) concentration on the protein expression levels focusing on the efficient PHB production. Then we proposed a new culture operation based on the results on metabolic regulation.

MATERIALS AND METHODS

Microorganism and Culture Condition

Eschericia coli JM109 having plasmid pNDTM2 which contains λp_R-p_L promoter, temperature sensitive *cI 857* repressor, and *phb CAB* gene for PHB production [4] was used in the present study. The inoculum was prepared by transferring cells from a glycerol stock (0.1 mL) stored at –80°C to a 50-mL T-shaped tube containing 10 mL of modified LB medium. The modified LB medium contained in g/L: tryptone, 10; yeast extract, 5; NaCl, 5, Na_2HPO_4, 3; KH_2PO_4, 1.5; NH_4Cl, 1; $MgSO_4.7H_2O$, 0.25; and $CaCl_2.H_2O$, 0.02. The culture was incubated overnight at 30°C and 1 mL of culture was transferred into a 500-mL T-shaped flask containing 100 mL of the LB medium. Ampicillin (50 µg/mL) was added to maintain the plasmid. After cultivation, the culture broth was centrifuge at 5,000 g for 10 min at 4°C. The cell paste was suspended in a fresh medium and inoculated into the fermenter. Batch and fed-batch culture were performed in a 1-L fermenter (MDL B.E. Marubishi Co., Tokyo, Japan) with the working volume of 700 mL. The pH was maintained at 7.0 using 3 N NaOH and 2 N of HCl. The dissolved oxygen (DO) concentration was kept above 30% unless otherwise stated. The growth medium was LB medium plus glucose. The temperature was increased from 33°C to 38°C to induce PHB production for batch and fed-batch culture. The substrate feeding for the fed-batch culture was made manually based on the changing patterns of pH and DO concentration.

Chemicals and Enzymatic Assay

Cell concentration was determined by measuring the optical density (OD) of the culture broth using a spectrophotometer at 600 nm. The OD value was then converted to g DCW (dry cell weight)/L using the relationship obtained previously.

The concentration of organic acids was measured using HPLC (LC-10AC, Shimadzu Co., Tokyo, Japan) with a column of Shim-PAK SCR-102H. PHB concentration was determined by HPLC with a column TSK GEL SCX H^+ (Tohsoh Co., Tokyo, Japan) using 7 mM sulphuric acid as the mobile phase. For preparing the sample of PHB, 1 mL of the broth containing the cells were first centrifuged and the supernatant was discarded. One ml of concentrated H_2SO_4 was then added to break the PHB into its monomer. The mixture was placed at 100°C for 4 h. The glucose concentration was determined by a colorimetric method at 505 nm using a Glucose kit (Wako Co., Osaka, Japan).

For enzyme activity measurement, cells were harvested from the fermenter, washed and resuspended in disruption buffer containing 100 mM Tris-HCl buffer (pH 7.6) with 2 mM dithiothreitol (DTT) and disrupted using a sonicator (TOMY UD-201) for about 60 s. The cell debris was removed by centrifugation, and the resulting crude extracts were used for the measurement of the specific enzyme activities. The enzyme activity measurements were made using a thermostat spectrophotometer (U2000 A, Hitachi Co., Tokyo, Japan) at 30°C. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, and citrate synthase activities were determined with a modified method of Kyra *et al.* [9]. Phosphoenol pyruvate carboxylase, PEP carboxykinase and malic enzyme were based on the modified method by Peterson *et al.* [10]. Malate dehydrogenase was assayed with a modified method of Heini-Suner *et al.* [11], and the phosphoglucose isomerase was followed by the method of Dominguez *et al.* [12]. Acetate kinase was determined based on the method of Lamed and Zeikus [13], and isocitrate lyase

was followed the method of Maloy *et al.* [14]. Pyruvate kinase and lactate dehydrogenase were measured according to the modified method by Garrigues *et al.* [15]. PHA synthase was measured according to the method of Valentin and Steinbuchel [16]. The protein concentration was measured according to the Bradford method [17] using Protein Assay Rapid Kit (Wako, Co., Osaka, Japan) and bovine serum albumin as standard.

Isoelectric Focusing and 2D SDS PAGE

Isoelectric focusing (IEF) was performed in 18 cm, pH 3-10 nonlinear (NL) immobiline gels (Amersham Pharmacia Biotech. Co., Sweden). The immobiline gel strips, which have an IPG strips to improve reproducibility, are rehydrated overnight in a reswelling cassette (Amersham Pharmacia Biotech. Co., Sweden) with the combination of rehydration solution. After isoelectirc focusing, gels were equilibrated in two steps by adding 1% DTT and 2.8% iodoacetamide with SDS equilibration buffer stock solution according to the protocol. 2-D-PAGE was performed using Multiphor II flatbed system and ExcelGel XL SDS 12-14%T.

Proteins were detected using an ammoniacal silver stain, and all steps were carried out using Hoefer Automated Gel Stainer (Amarsham Pharmacia Biotech Co., Sweden). Silver stained gels were scanned on a Digital Image Stocker DS-30, FAS III (Toyobo Co., Osaka, Japan) to create computer images of each individual gel. To provide uniformity to all spots of individual gel, the same parameters were set to calibrate intensity, background substraction and normalization. Computer-assisted gel analysis software Image Master® 2D Elite was used for feature detection and matching, and corrected by visual inspection. For calibration of isoelectric pH (pI) and molecular weight (Mw), 2-D SDS PAGE standards (Bio-Rad Laboratories, USA) were comigrated with the sample. The normalized volume for a spot was calculated by dividing its volume by the total volume of the detected spots on the image. The characterization of spot changes was made on the basis of comparison with available proteome database such as SWISS-2DPAGE (www.expasy.org).

RESULTS AND DISCUSSION

Batch Cultivation

Fig. 2a shows the batch experimental result, where the temperature was changed from 33°C to 38°C at 6 h. The initial glucose concentration was 25 g/L. Fig. 2b indicates that acetate and lactate were formed initially and that the lactate was first assimilated and then acetate was consumed as the glucose concentration decreased. The final cell concentration was 12 g/L. The PHB was formed after the temperature upshift and the final PHB concentration was 6 g/L. It should be noted that no PHB production was observed after glucose depletion although acetate was present in the broth. The

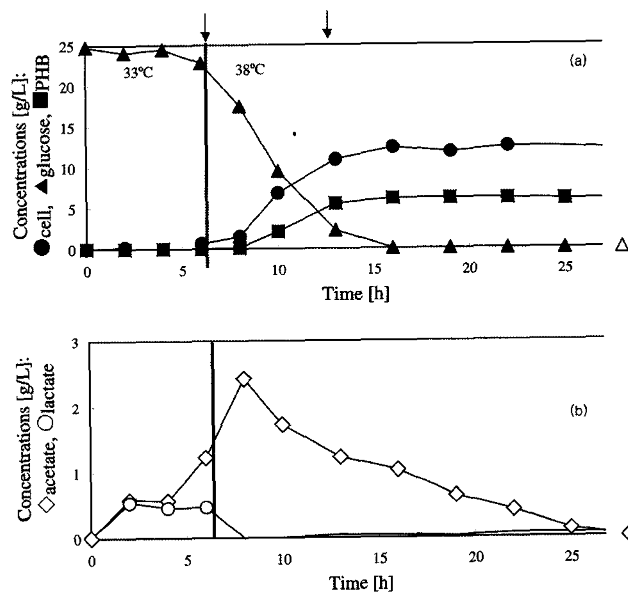

Fig. 2. Time course of batch recombinant *E.coli* cultivation. The line indicates the temperature upshift from 33°C to 38°C and the arrow represents the samples taken for enzyme activities measurement.

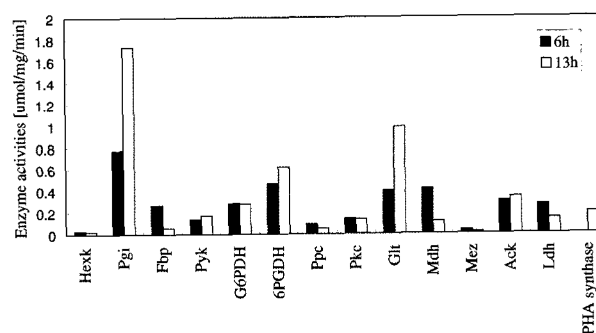

Fig. 3. Enzyme activities of recombinant *E.coli* for batch culture of utilized different carbon source.

enzyme activities were measured for cells grown at 6 h and 13 h, where glucose was assimilated as a main carbon source at 6 h, while acetate was utilized at 13 h. Fig. 3 shows how the enzyme activities changed between the two culture periods. The result shows that when the acetate was used as the main carbon source, the activity of citrate synthase was significantly increased and that of PEP carboxylase were decreased, which implies the gluconeogenesis at 13 h. For cells grown on acetate, it is considered that the anapleurotic reaction occurs through the glyoxylate bypass which generates malate and then oxaloacetate from acetylCoA by isocitrate lyase and malate synthetase. For substrate utilization, acetate is consumed by a proton symport, phosphorylated by acetokinase to acetyl phosphate and then to acetyl-CoA by a phosphotransacetylase [18,19]. Fig. 3 shows that acetate kinase activity slightly increased

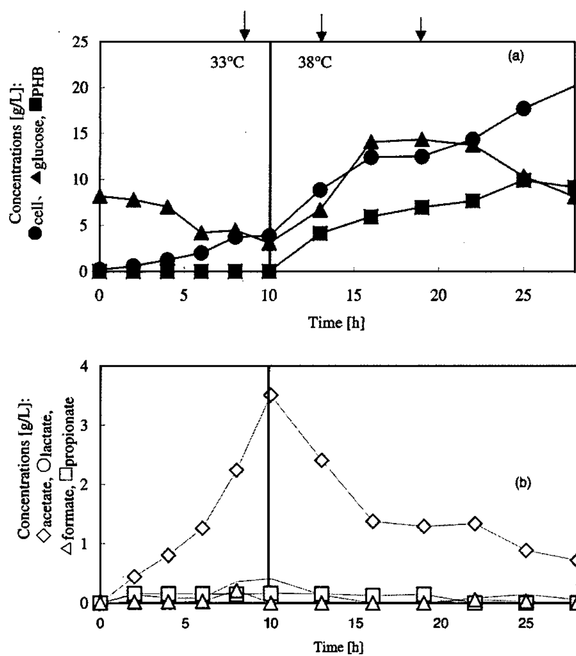

Fig. 4. Time course of PHB production in fed-batch culture by recombinant *E. coli.* The line indicates the temperature upshift from 33°C to 38°C and the arrow represents the samples taken for enzyme activities measurement and proteome analysis.

while lactate dehydrogenase decreased at 13 h, both of which correspond to the experimental result of Fig. 2. The activity of glucose-6-phosphate dehydrogenase slightly decreased at 13 h when acetate was used as the main carbon source. Higher level of NADPH and/or a higher NADPH/NADP ratio in the medium resulted in satisfactory synthesis of PHB in recombinant *E. coli* [20]. The activities of hexokinase, PEP carboxykinase and PEP carboxylase were low and not significantly changed for both cases. PHB producing gene, PHB synthase expressed after induction at 13 h.

Fed-batch Cultivation

Fig. 4 shows the fed-batch experimental result, where culture temperature was changed from 33°C to 38°C at 10 h and the glucose was fed manually. In this experiment, the final cell concentration was 14 g/L of DCW and the final PHB concentration increased up to 9 g/L. The samples were taken from the culture before and after induction at 8 h and 13 h, respectively, for enzyme activity measurement and proteome analysis based on 2DE. As in the batch culture, the trends of organic acids production were similar. Namely, the acetate was predominantly produced (the maximum concentration was around 3.5 g/L), while other organic acids such as propionic, lactic, and formic acids were very low throughout the fermentation. The formation of acetate has been suggested to be caused by an imbalance between glucose metabolism and respiration, a condition

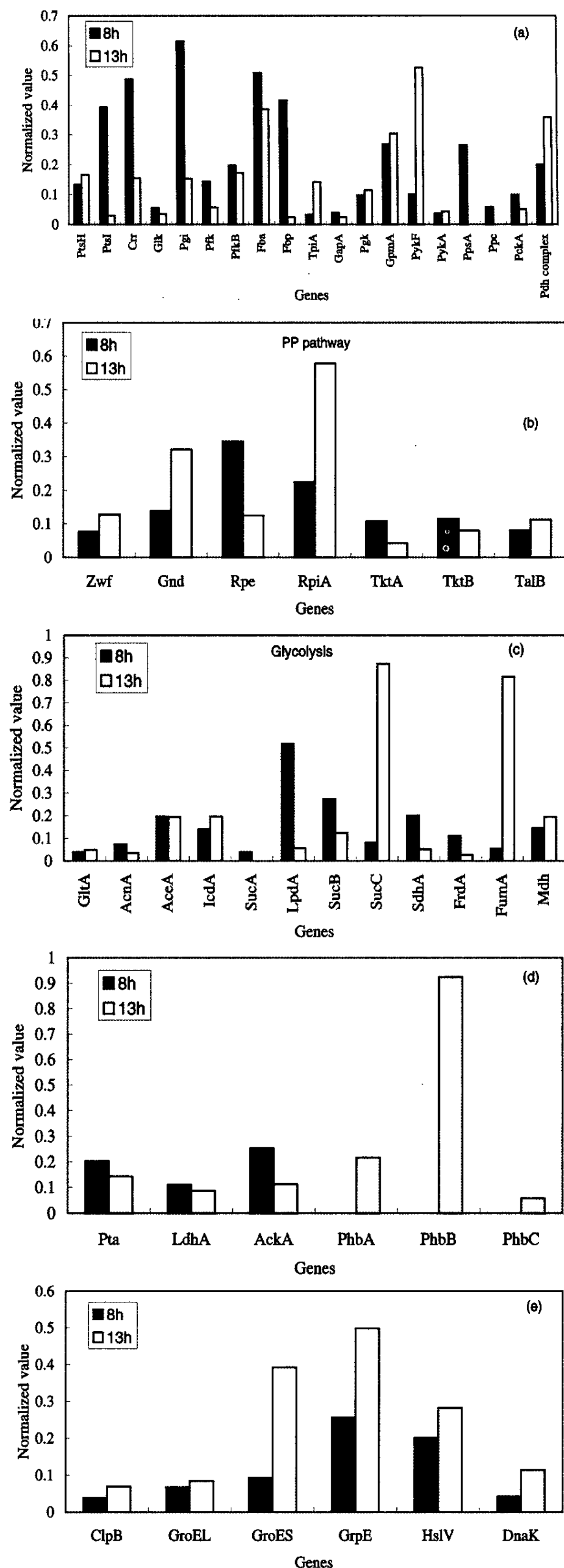

Fig. 5. Identified proteins by proteome analysis using 2DE before and after induction.

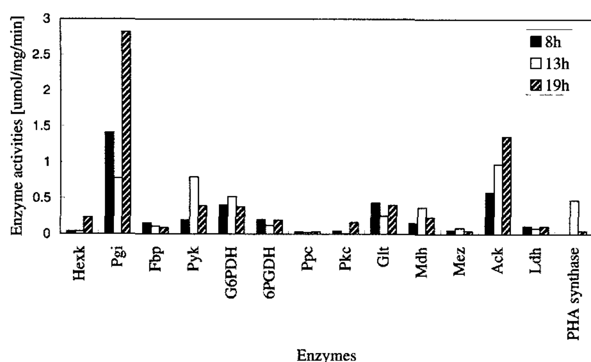

Fig. 6. Enzyme activities of recombinant *E. coli* for fed-batch culture before and after induction.

in which the influx of carbon into the cell exceeds the demands for biosynthesis due to the presence of excess NADH and the repression of tricaboxylic acid cycle enzymes [21]. Once the temperature was increased, the acetate concentration decreased even though there was still much glucose, which might be due to the fact that the PHB formation required acetyl-CoA, and the acetate was replenished for this. Fig. 5 shows how the protein expression levels changed based on 2DE, while Fig. 6 shows the results of enzyme activities. Both Figs. 5 and 6 indicate that phosphoglucose isomerase activity significantly decreased after induction. This phenomenon might be the result for enhancing the flux to PP pathway instead of utilizing glycolytic pathway at this point. It can be seen that glucose-6-phosphate dehydrogenase activity slightly increased. The other pentose phosphate pathway related genes such as *gnd, rpi* and *rpe* were shown to be increased after induction (see Fig. 5b) This may be explained by the fact of increased NADPH demand especially for PHB production. NADPH requirement for biosynthesis should be provided by the PP pathway or by the oxidation of isocitrate to α-ketoglutarate through an NAD(P)H-dependent isocitrate dehydrogenase [22] Since glucose was used as a carbon source for this experiment, the activity of malic enzyme was low. Malate dehydrogenase activity slightly increased after induction at 13 h and then decreased. Fig. 5 shows that the expression of pyruvate dehydrogenase (*pdh*) complex increased after induction. The *pdh* complex serves to interconnect the metabolic pathways of glycolysis and organic acids synthesis and to the TCA cycle as well as the PHB synthesis pathway. PHB synthase activity was high at 13 h and decrease at 19 h, which conincides with the experimental result of Fig. 4. The expression levels of *sucD, lpdA, sucA, sdhA* and *frdA* in TCA cycle decreased after induction, while there were no significant changes in *glt, acnA, aceA,* and *icd.* Fig. 5 shows also that the heat shock proteins increased after induction, where *groES, grpE, hslV* and *dnaK* were significantly upregulated. In general, heat shock proteins are synthesized in order to protect cells from external stresses such as sudden increase of temperature, UV radiation, virus infection and others [6]. Promoters

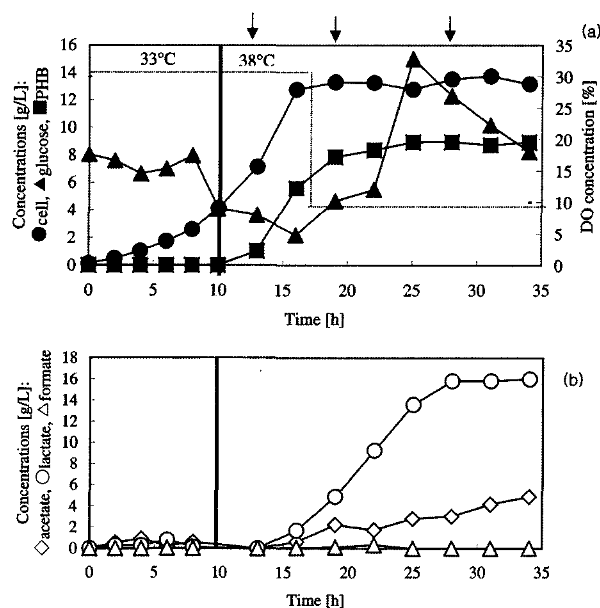

Fig. 7. Effect of the change in DO concentration on recombinant *E. coli* cultivation. The line at 10 h indicates the temperature upshift from 33°C to 38°C, and the arrows represent the timing of the samples taken for enzyme activities measurement and proteome analysis. The dotted line shows the change in DO concentration.

of major heat-shock genes such as *groES, groEL* and *dnaK, dnaJ* are the strongest found in *E. coli* [23]. PHB accumulation in the cells and higher temperature for induction may be considered as the stresses for the induction of the heat shock proteins.

Change in DO Concentration

Fig. 7 shows the fed-batch experimental result where the DO concentration was decreased from above 30% to about 10% at 17 h. The final cell concentration was around 13-14 g/L and the PHB concentration was around 9 g/L. Fig. 8 shows that most of the enzyme activities such as glucose-6-phophate dehydrogenase (*zwf*), 6-phosphogluconate dehydrogenase (*gnd*), phosphoenol pyruvate carboxylase (*ppc*) decreased when DO concentration was low both at 19 h and 28 h. The enzyme activity of lactate dehydrogenase increased as the DO conentration decreased, which corresponds to the experimental result of Fig. 7b. The lactic acid produced was up to 16 g/L while acetic acid was about 5 g/L. The enzyme activities such as pyruvate kinase (*pyk*), PEP carboxylase (*ppc*) and malic enzyme (*mez*) increased at 28 h. It can be seen that the enzyme activities such as citrate synthase as well as PEP carboxylase decreased as DO concentration was decreased. It should be noted from Fig. 7 that the decrease in DO concentration stopped the cell growth while PHB concentration was kept increasing but not for long period. The reason why the cell growth sopped may be due to the decreased

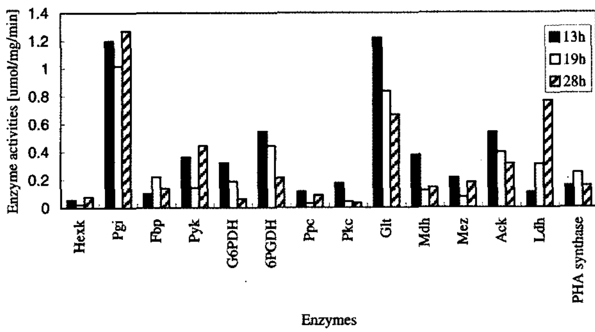

Fig. 8. Effect of the change in DO concentration on enzyme activities.

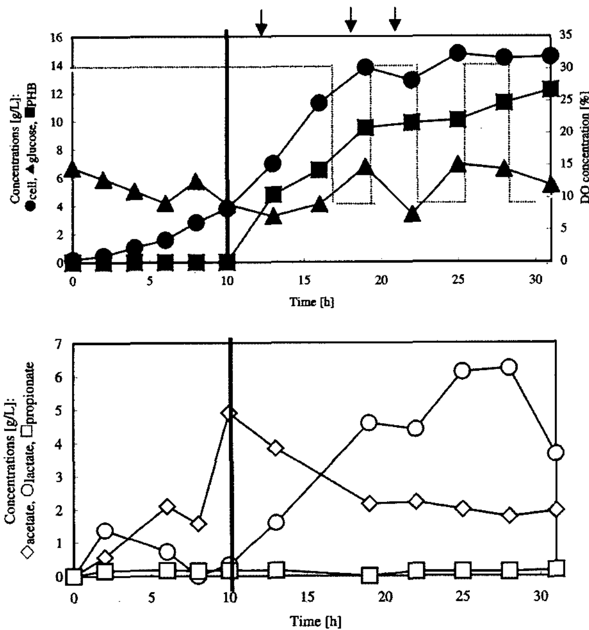

Fig. 9. Effect of periodic change in DO concentration on recombinant *E. coli* cultivation The line at 10 h indicates the temperature upshift from 33°C to 38°C and the arrow represents the samples taken for enzyme activities measurement and proteome analysis. The dotted line shows the change in DO concentration.

production of ATP by reducing the DO concentration. Moreover, the reason why the increase in PHB concentration was for short period after the decrease in DO concentration may be due to the limited supply of NADPH. Based on these observations, it may be considered to periodically change the DO concentration in order to enhance both cell growth and PHB production.

Periodic Change in DO Concentration

Fig. 9 shows such experimental result, where the final cell concentration became 14-15 g/L, and the PHB concentration increased up to around 12 g/L as expected. Enzyme activities were measured and 2DE was con-

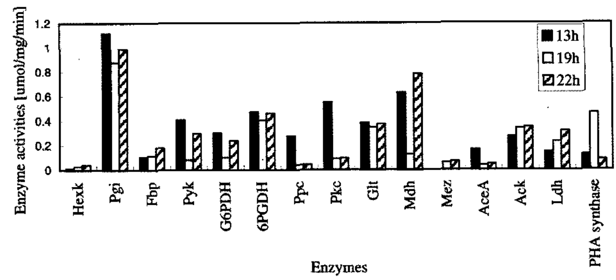

Fig. 10. Enzyme activities of recombinant *E. coli* under periodic change in DO concentration.

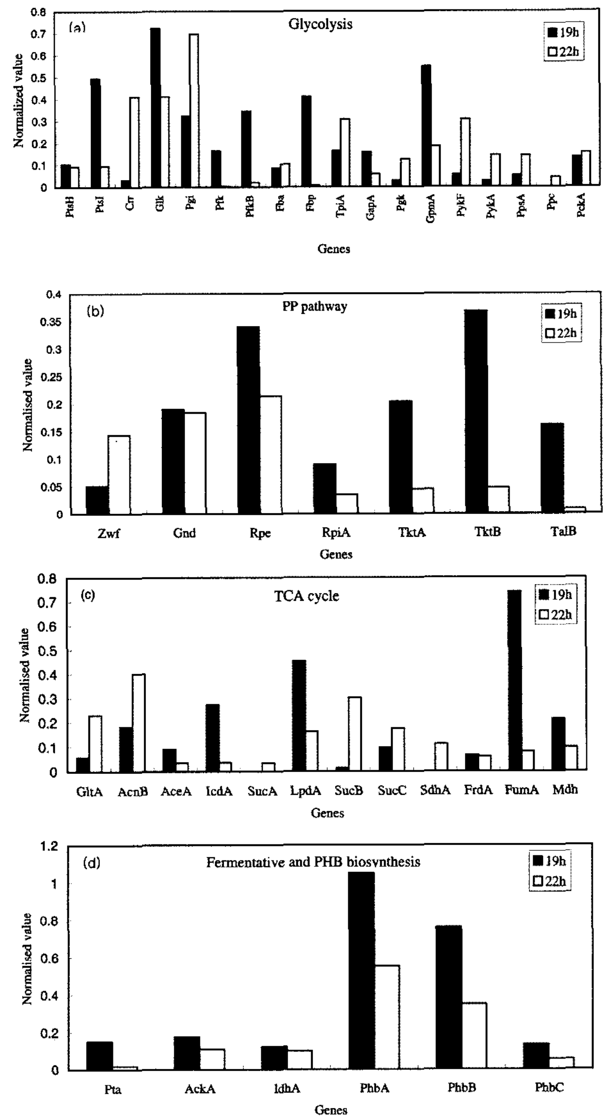

Fig. 11. Comparison of the protein expressions identified using 2DE for periodic change in DO concentration.

ducted for the samples taken at 19 h (low DO concentration) and at 22 h (high DO concentration). Figure 10 shows that the enzyme activities of glucose-6-phos-

phate dehydrogenase (*zwf*), 6-phosphogluconate dehydrogenase (*gnd*), pyruvate kinase (*pyk*), PEP carboxykinase (*pck*), malate dehydrogenase (*mdh*) and citrate synthase (*glt*) decreased as the DO concentration decreased. Namely, they were downregulated when the DO concentration was decreased (19 h) and then increased again after upshift of the DO concentration at 22 h. It can be said that the citrate synthase activity was affected by the DO concentration, which limits the flux from acetyl-CoA to TCA cycle. Lactic acid concentration was significantly increased up to 6 g/L as a result of decreased DO concentration. The lactate dehydrogenase activity increased as the DO concentration decreased. The abundance of this protein was also increased as the DO concentration decreased. The acetic acid concentration was maintained around 2 g/L during the periodic change in DO concentration. Interestingly, PHA synthase involved in PHB production increased as the DO concentration decreased. The proteome result of *phbC* coding for PHB synthase corresponds to the change in enzyme activity. β–ketothiolase encoded by *phbA* gene and acetoacetyl-CoA reductase encoded by *phbB* were expressed with the same trends as PHA synthase (*phbC*). The phosphoglucose isomerase activity as shown in Fig. 10 and the proteome result (*pgi*) as shown in Fig. 11b slightly decreased as the DO concentration decreased at 19 h. The 2DE result for *pykA* and *pykF* corresponds to the result of enzymatic assays where they increased when the DO concentration was increased.

CONCLUSION

The 2DE results are, in general, complimented with TOF-MS to ascertain several proteins. In the present research, we measured some of the enzyme activities. The comparison between 2DE result and the enzyme activity result is well correlated with some exceptions. It should be noted that 2DE data give global protein expression levels with only one operation, while enzyme activity measurements require different assay methods for different enzymes and are limited to enzymatic proteins. The present research demonstrates that 2DE result with enzyme activity measurement can be used to understand the metabolic regulation from the protein expression viewpoint. In particular, the present research indicates the usefulness of periodically changing the DO concentration for the efficient PHB production using recombinant *E. coli*.

NOMENCLATURE

AcCoA	acetyl-CoA
Acetyl-P	acetyl-phosphate
Cit	citrate
DHAP	dihydroxyacetone-phosphate
E4P	erytrose-4-phosphate
F6P	fructose-6-phosphate
F1,6P	fructose-1,6-diphosphate
GAP	glyceraldehyde-3-phosphate
Fum	fumarate
iCit	isocitrate
2-KG	2-ketoglutarate
Mal	malate
OAA	oxaloacetae
PEP	phosphoenolpyruvate
6PG	6-phospho-gluconate
3PG	3-phospho-glycerate
2PG	2-phospho-glycerate
R5P	ribose-5-phosphate
Rub5P	ribulose-5-phosphate
S7	sedoheptulose-7-phosphate
Suc	succinate
Suc-CoA	succinyl-CoA

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