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Change in Proteomic Profiles of Genetically Modified 1,3-Propanediol-Producing Recombinant *E. coli*

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The recombinant *E. coli* Δ6 mutant (*galR, glpK, gldA, ldhA, lacI, tpiA*) was used to produce 1,3-propanediol (PD) from glucose. The 1,3-PD production increased with feedback control of the glucose concentration using fed-batch fermentation. The maximum 1,3-PD concentration produced was 43 g/l after 60 h of fermentation. Glycerol production was minimized when controlling the glucose concentration at less than 1 g/l. The expression levels of seven enzymes related to the 1,3-PD production metabolism were compared during the cell growth phase and 1,3-PD production phase, and their expression levels all increased during 1,3-PD production, with the exception of alcohol dehydrogenase.

Keywords: 1,3-Propanediol, glycerol, proteomics, fed-batch fermentation

With the development of sustainable technology, renewable materials produced by agriculture as feedstock for industry and energy, and industrial bio-based processes will play a vital role in future biotechnology. In particular, biological resources and biodiversity from nature will have a significant impact by providing bio-based industrial feedstock and products from non-food crops [7, 15, 25]. 1,3-Propanediol (PD) is one of the key bio-based industrial feedstocks, and has a wide variety of applications in polymers, cosmetics, foods, lubricants, and medicines. 1,3-PD is also an important intermediate chemical in the synthesis of polyester. Thousands of tons of polyester are produced every hour worldwide, with polyethylene terephthalate (PET) comprising a major fraction of this amount, and 1,3-propanediol (PD) is a key intermediate in the synthesis of PET. Copolymerization with terephthalic acid leads to materials ideal for textiles, owing to their appropriate mechanical and electrostatic characteristics [6, 12, 19].

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Metabolic engineering (*i.e.*, the design, modification, and construction of biochemical pathways) is an important technology in the chemical, biochemical, and environmental industries. In addition, recent progress in metabolic engineering has also included classical fermentation, recombinant DNA technology, and protein engineering [13, 16]. Accordingly, this study used the conversion of glucose into 1,3-PD as a model biocatalytic conversion system [8, 10, 19, 22, 23, 27].

Microbial fermentation for 1,3-PD production has already been extensively studied in species belonging to the family Enterobacteriaceae (*Enterobacter* sp., *Klebsiella* sp., and *Citrobacter* sp.) or genus *Clostridium* sp. [1, 3, 5, 18, 20, 26]. In addition, 1,3-PD production by recombinant *E. coli* has attracted considerable interest, with a particular focus on the metabolic pathway in order to make a new metabolic pathway or increase the metabolic flux [2, 22, 23, 27], and the fermentation processes used in the production of glycerol and 1,3-PD have also been well studied [4, 9, 11, 14, 19]. However, no reports have yet focused on a proteomic analysis of the expression level of the relevant enzymes under different fermentation conditions.

Therefore, this study selected a recombinant *E. coli* as a model organism for the production of 1,3-PD and investigated its utilization of glucose for the production of 1,3-PD. Changes in the protein expression levels were detected in relation to two natural metabolic pathways (glucose to glycerol, and glycerol to 1,3-PD).

Materials and Methods

Recombinant Strain

The recombinant strain *E. coli* Δ6 mutant (*galR, glpK, gldA, ldhA, lacI, tpiA*) was used. This strain harbors plasmids containing the genes for glycerol formation and 1,3-PD transformation. The recombinant *E. coli* was cultured in a defined minimal medium (12.8 g/l $Na_2HPO_4 \cdot 7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 mg/l $CaCl_2$, and 1 mM $MgSO_4$) containing a final concentration of 0.3–0.5% (w/v) glucose, 50 μg/ml ampicillin, 100 μg/ml kanamycin, and 0.1 mM $CoCl_2$. Seed cultures for batch fermentation were prepared using two 100-ml

culture volumes in 500-ml Erlenmeyer flasks at 250 rpm and 37°C. After reaching the exponential growth phase, the cells were transferred to a batch fermentor with 3 l of the culture medium. When the cell density reached 2.0 (OD_{600}), 0.5 mM of IPTG was added to induce expression of the recombinant protein. After induction, 10 ml of the culture broth was harvested to measure the cell density, glucose, glycerol, acetate, and 1,3-PD.

Protein Preparation

For the analytical gels, 1 ml of the pulse-labeled cells was washed twice in 20 mM of a phosphate buffer at pH 7, and then lysed in 200 ml of a lysis buffer containing 8 M urea, 4% cholamidopropyl-dimethyl-ammonio-propane sulfonate, 0.8% ampholytes, pH 3–10 (Pharmalyte Amersham Pharmacia), 65 mM DTT, and a few grains of bromophenole blue. The incorporated radioactivity of the trichloroacetic acid precipitated proteins was determined using a scintillation counter. For the preparative gels, 250 ml of a suspension containing the cells was washed in a 20 mM phosphate buffer at pH 7, and resuspended in 8 ml of a breaking buffer (20 mM phosphate buffer, pH 7, 5% sucrose) containing a protease inhibitor mixture (Complete; Roche Diagnostics, Rotkreuz, Switzerland), 4 mg/ml of RNase, and 16 mg/ml DNase. The suspension was passed twice through a precooled French pressure cell at 1,000 psi and centrifuged at 120,000 ×*g*. The soluble proteins were then concentrated and washed twice with H_2O in an Amicon filtration cell using a membrane with a molecular weight cutoff of 10,000. Solid urea and a concentrated lysis buffer were added to the protein solution to give the same final concentration as that described above.

2D Gel Electrophoresis

The proteins were separated using 18-cm Immobiline DryStrips, pH 4–7 (Amersham Pharmacia) in the first dimension, and on continuous 12% SDS gels in the second dimension. The analytical and preparative gels were loaded with 10^6 cpm and 2 mg of protein, respectively. The radioactivity was then detected by storage phosphor imaging, and the preparative gels were stained with ammoniacal silver. The protein size (10–100 kDa) and isoelectric point range (pH 4–7) of the 2D gels were determined using 2D gel marker proteins (Bio-Rad).

Data Processing and Analysis

Samples from three independent labeling experiments were resolved on three independent 2D gels at each time point investigated. The 2D gel autoradiographs were matched and quantified by image analysis using Image Master software V 4.01 (Amersham Bioscience, Uppsala, Sweden). The data were then analyzed using S-PLUS (MathSoft, Cambridge, MA, U.S.A.) and Excel (Microsoft) as follows: (i) the spot intensities were converted to parts per million of the total gel intensity and normalized, (ii) spots were removed from the data set for a given time point in the case of being detected in only one of the three repeats, (iii) spots present on the gels of the nonsynchronous cultures were removed if they were not detected on any of the gels from the cell cycle time points and *vice versa*, and (iv) spots with the highest intensity <200 ppm showing a high experimental variation were removed. Finally, only the highly reproducible protein spots, determined by comparing the ExPASy protein server data, were used as the data set for the statistical analyses. The dried gel pieces were incubated in a trypsin digestion solution containing 12.5 ng/μl trypsin and 50 mM ABC with 2 mM $CaCl_2$ on ice for 45 min. After 1 h, the unabsorbed solution was removed and the same solution (without trypsin) added to cover the pieces, and then the solution was stored overnight at 37°C. The resulting peptides were extracted by sonication in 50% acetonitrile/0.1% TFA, and desalted using C18 Ziptips (Millipore). The peptide solution was prepared using an equal volume of a saturated α-cyano-4hydroxy-cinnamic acid solution in 50% CAN/ 0.1% trifluoroacetic acid on a MALDI-TOF-MS sample plate. The peptide mass fingerprints were then obtained from the MALDI-TOF (Voyager DE-STR; PE Biosystem, Framingham, MA, U.S.A.) using a 150–200 Hz pulsed nitrogen laser at 355 nm. The spectra were calibrated using a matrix and the tryptic autodigestion ion peaks as the internal standards. The peptide mass fingerprints were analyzed using Web-based software programs, and Mascot (Matrix Science Ltd., U.K.; http://www.matrix science. com), PeptIdent (Swiss Institute of Bioinformatics; http://www.expasy.ch/tools/peptident.html), and Knexus (Proteometrics Inc.; http://www.proteometrics.com) were used to analyze the MALDI data using the public databases NCBInr and SWISS-PROT/TrEMBL.

Analytical Procedure

The glucose concentration was measured using the DNS method. Samples of the fermentation broth were centrifuged (13,000 ×*g*, 5 min), and then 100 μl of the supernatant was mixed with 900 μl of a DNS solution and the mixture heated to 100°C for 15 min. After cooling, 1 ml of water was added and the absorption at 575 nm measured using a UV spectrophotometer (Shimadzu, Japan). Glucose was used as the standard.

High Performance Liquid Chromatography Analysis

The 1,3-PD, glycerol, and other fermentation by-products, such as lactic acid and acetic acid, were analyzed using a Shimadzu HPLC system (Japan) fitted with a refractive index detector and Bio-Rad Aminex HPX-87H organic acid column (Richmond, CA, U.S.A.). The column temperature was maintained at 30°C, and a 1.0 ml/min flow rate of 0.01 N sulfuric acid was used as the mobile phase. The samples were filtered through 0.45-μm Supor membranes (Gelman Sciences, Ann Arbor, MI, U.S.A.), and 20 μl was injected into the column.

RESULTS AND DISCUSSION

Effect of Controlling Glucose Level on Glycerol, 1,3-PD, and Acetic Acid

Fed-batch reactors are used widely in industrial applications, as they combine the advantages of both batch and continuous processes. Although the process is initiated as a batch process, it is prevented from reaching a steady state by the starting substrate feed when the initial glucose is consumed [17]. In this study, a simple fed-batch fermentation process was used to maximize the level of 1,3-PD production. The glucose level was maintained using feedback control based on measuring the glucose concentration and manipulating the glucose feed rate. A single fed-batch experiment was performed when controlling the glucose concentration between 5–10 g/l, while another fed-batch experiment was carried out when controlling the glucose concentration at a minimum level <1 g/l (*i.e.*, a limited glucose concentration).

With a high glucose concentration, the acetic acid production increased owing to a high glucose metabolic rate, whereas the level of acetic acid production decreased when decreasing

Table 1. Cross-species identification using public protein databases, and identification of proteins from the *E. coli* Δ6 mutant using specific protein database.

Code	Proteins	PI	MW (Da)	Sequence coverage (%)	Mascot score
3.1.3.11	Fructose-bisphosphatase	5.68	32,568	32	113
2.7.1.11	6-Phosphofructokinase	5.45	38,156	38	198
4.1.2.13	Fructose-P2-aldolase	5.55	40,651	40	98
1.2.1.12	Glyceraldehyde-3P dehydrogenase	6.28	36,748	24	142
2.7.2.3	Phosphoglycerate kinase	5.07	41,960	31	103
1.1.1.1	Alcohol dehydrogenase	5.17	25,083	48	86
2.7.1.30	Glycerol kinase	5.3	25,083	43	174
4.2.1.30	Glycerol dehydratase β	6.53	50,642	28	101
	Glycerol dehydratase small unit	6.66	24,300	36	71
1.1.1.202	1,3-Propanediol dehydrogenase	5.91	16,800	58	63

the glucose concentration. Glycerol was also accumulated with a high glucose concentration, which in turn decreased the level of 1,3-PD production owing to the inhibition of the reaction for 1,3-PD production. Thus, a high glucose level enhanced the production of glycerol, while decreasing the level of 1,3-PD production.

The 1,3-PD yield was approximately 30% when the glucose level was controlled between 5–10 g/l, yet increased to 38% with a low glucose concentration level. The increased yield with a low glucose concentration was attributed to a decrease in the formation of by-products as a result of the limited nutrient source inhibiting 1,3-PD formation.

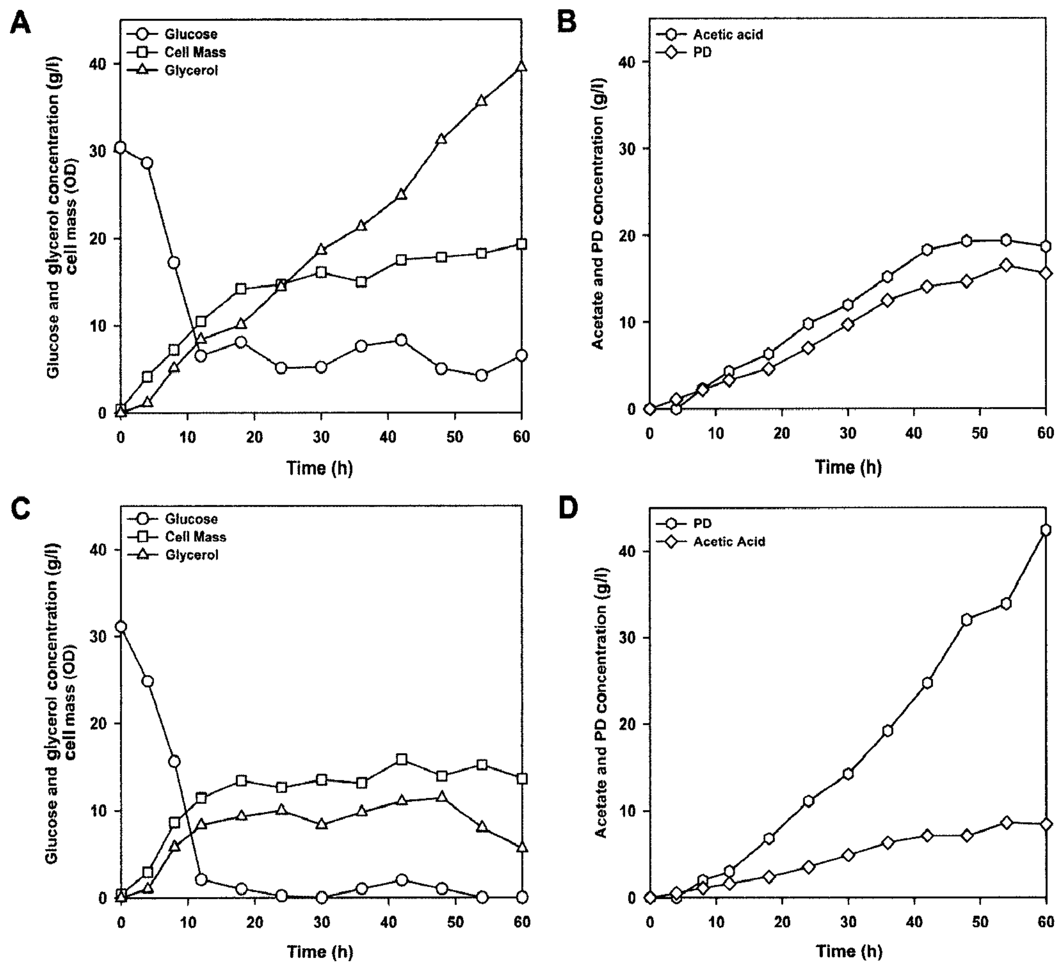

Fig. 1. Cell growth and 1,3-PD production when controlling feedback glucose concentration between 5–10 g/l.
A. Cell mass, glycerol, and glucose concentrations; **B.** 1,3-PD and acetic acid concentrations; **C.** Cell mass, glycerol, and glucose concentrations; **D.** 1,3-PD and acetic acid concentrations when reducing glucose level.

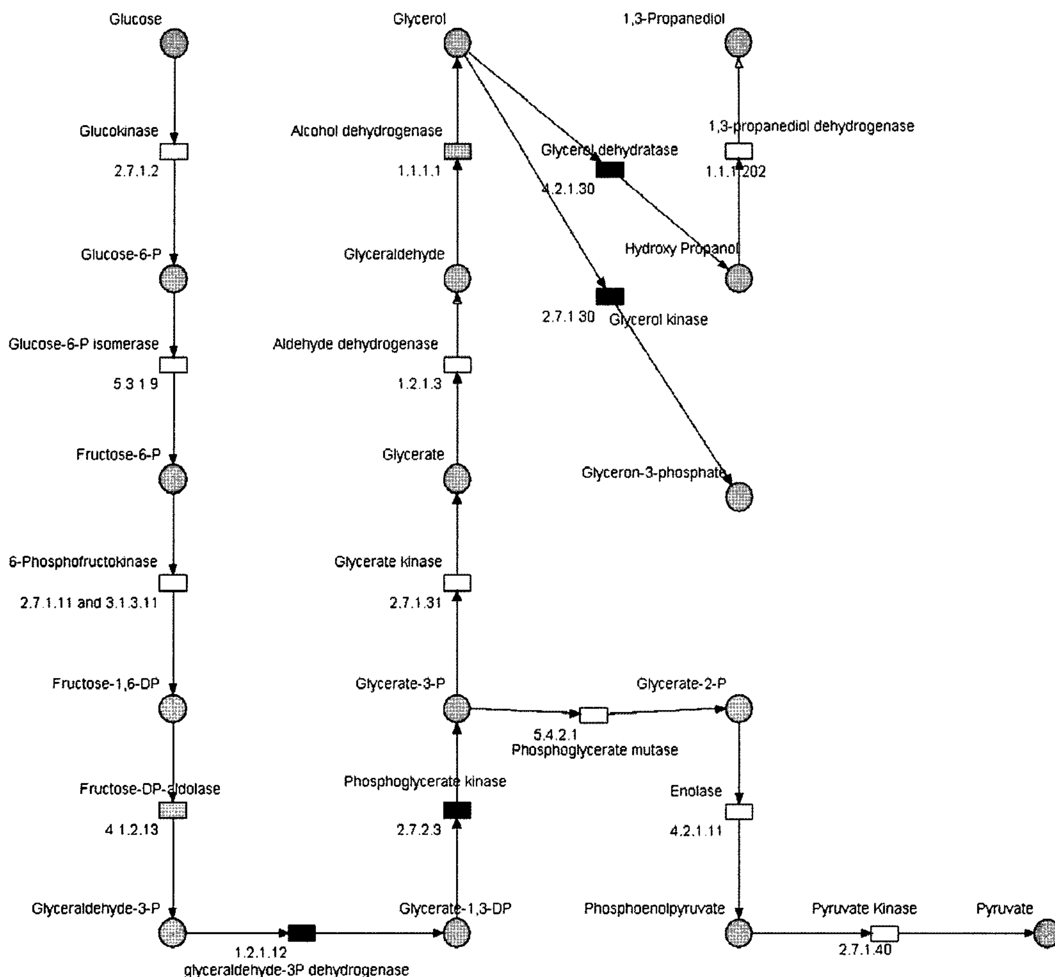

Fig. 2. 1,3-PD production metabolic pathway with glucose as carbon source.
An open rectangle in the enzyme notation indicates that the enzyme could not be identified in this study. The shade density represents the enzyme expression level (light, 1–2 fold; dark, 2–3; black, 3–4; pink, 0–1; blank, unknown).

Analysis of 1,3-PD Synthesis Metabolic Pathway

Information on gene expression at the protein level provides critical data on the metabolic activity of 1,3-PD metabolism, which is essential for understanding this nonlinear relationship and necessary for metabolic and cellular engineering efforts. For increased accuracy, the sample preparation and 2D gel electrophoresis were repeated three times, starting each time with an independent colony [21]. The Expert Protein Analysis System (ExPASy at http://www.expasy.ch/) was found to be an excellent resource for Internet-accessible proteome databases for protein identification. The functions of the proteins identified were assigned by comparing their sequences with those in the public protein database. Table 1 lists the homolog proteins with the highest sequence similarity under the corresponding annotation for each protein identified. Wang *et al.* [24]. reported that two subunits of glycerol dehydratase (E.C. 4.2.1.30) converted glycerol into hydroxyl glycerol, the beta subunit (MW 24.3 kDa, PI 6.53) and a small subunit (MW 16.8 kDa, PI 6.66). They also showed that the molecular mass and PI of 1,3-propanediol dehydrogenase (EC 1.1.1.202) were 41.5 kDa and 5.91, respectively. From these results, the beta subunits of glycerol dehydratase and 1,3-propanediol dehydrogenase were identified from the 2D gel.

Seven of the 11 enzymes associated with the metabolism of 1,3-PD synthesis from glucose were identified. Glycerol kinase phosphorylate glycerol was also identified in this study. Fig. 2 shows the enzyme expression level with the change in the cell state from growth to 1,3-PD production. Five of the 7 enzymes identified as participating in the metabolism of 1,3-PD synthesis were upregulated, and the level of 1,3-PD production was increased. As no energy metabolism-related enzymes from glycerate to pyruvate were identified, the changes in flux were monitored as the process moved from an energy metabolism to a production metabolism. The level of alcohol dehydrogenase expression during 1,3-PD production decreased to 20% of that during the cell growth phase, indicating that the alcohol dehydrogenase may have been strongly inhibited by the glycerol formation during fermentation. An open rectangle in the enzyme notation indicates that the enzyme could not be identified in this study, whereas the shade density represents the enzyme

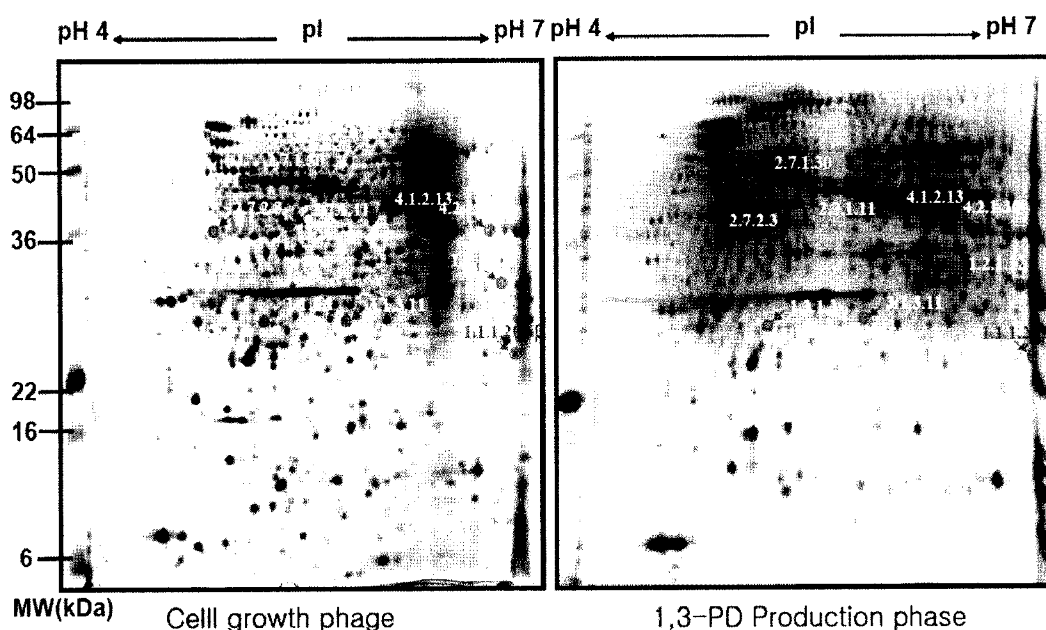

Fig. 3. Protein images of mutant *E. coli* Δ6 producing 1,3-PD separated by two-dimensional protein electrophoresis and stained with ammoniacal silver.

The pH gradient (horizontal direction) runs from pH 4–7, and the SDS-PAGE separation (vertical direction) was performed in a 12% gel. The key metabolic enzymes were identified and their expression levels measured.

expression level (light, 1–2 fold; dark, 2–3; black, 3–4). Two enzymes, glycerol dehydratase (from *dha* B) and 1,3-propanediol dehydrogenase (from *dha* T), showed a high level of expression during 1,3-PD production.

Proteomic studies integrating the protein expression level and metabolism have a great potential to accelerate metabolite production research, as the combination of a cellular biological technique and approach can provide extended information for the production of various metabolites.

This is the first report showing the changes in the proteomic profiles of a 1,3-PD-producing recombinant *E. coli* using 2D gel electrophoresis. Seven of the 11 enzymes involved

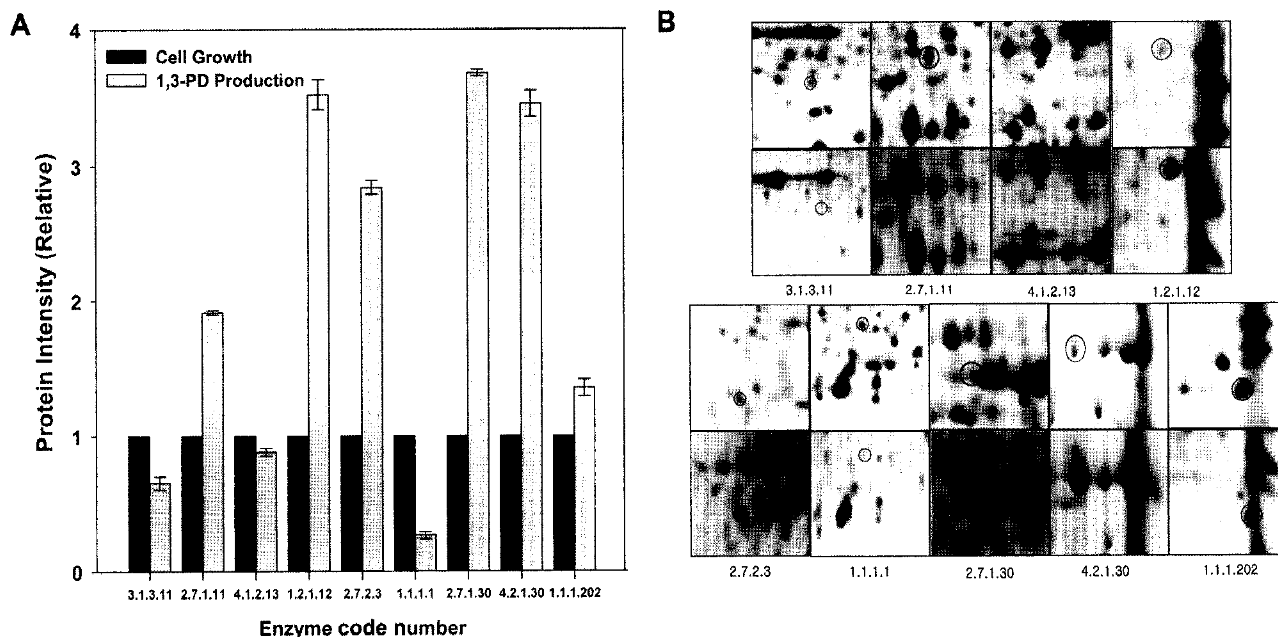

Fig. 4. Change in the enzyme expression level during the cell growth phase and 1,3-PD production phase.

A, Shaded bars represent the enzyme expression level during the cell growth phase, and hatched bars represent the enzyme expression level during the 1,3-PD production phase; **B**, Enlarged protein spots. The enzyme code numbers and their names are listed in Table 1.

in the 1,3-PD production metabolism were identified, plus their changes in expression from cell growth to 1,3-PD production were studied and presented. The major enzymes, except for alcohol dehydrogenase, were all overexpressed to enhance 1,3-PD production.

Glycerol, an intermediate compound for 1,3-PD production, was rapidly produced when the glucose concentration was >5 g/l and inhibited the production of 1,3-PD, which decreased to 35 g/l. Meanwhile, the level of 1,3-PD production increased when the glucose concentration was <1 g/l, which prevented the overproduction of glycerol. As a result, 43 g/l of 1,3-PD was produced after 60 h of fed-batch fermentation.

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