JOURNAL OF PROTEOMICS 89 (2013) 255–264

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Protein identification in two phases of 1,3-propanediol production by proteomic analysis

Natalia Comba González[a], *Andres F. Vallejo*[b], *Myriam Sánchez-Gómez*[b], *Dolly Montoya*[a,*]

[a]*Bioprocesses and Bioprospecting Group, Biotechnology Institute, Universidad Nacional de Colombia, Bogotá, Colombia*
[b]*Hormone Research Group, Universidad Nacional de Colombia, Bogotá, Colombia*

ARTICLE INFO

Article history:
Received 31 July 2012
Accepted 13 June 2013
Available online 28 June 2013

Keywords:
Clostridium sp.
Proteome
Two-dimensional electrophoresis
Mass spectrometry
1,3-Propanediol

ABSTRACT

Proteomic analysis by two-dimensional electrophoresis (2D)–mass spectrometry was used to identify differentially expressed proteins in the *Clostridium* sp. native strain (IBUN 158B) in two phases of the 1,3-propanediol (1,3-PD) production (lag phase and exponential growth phase). Intracellular protein fraction extraction conditions were standardised, as well as the 2D electrophoresis. Differences were found between both of the growth phases evaluated here. Thirty-two of the differentially expressed proteins were chosen to be identified by tandem mass spectrometry (MALDI TOF/TOF). The presence of four enzymes implicated in the 1,3-PD metabolic pathway was recorded: one from the reductive route (1,3-propanediol dehydrogenase) and three from the oxidative route (3-hydroxybutyryl-CoA dehydrogenase, NADPH-dependent butanol dehydrogenase and phosphate butyryl transferase). The following enzymes which have not been previously reported for *Clostridium* sp., were also identified: phosphoglycerate kinase, glucose 6-phosphate isomerase, deoxyribose phosphate aldolase, transketolase, cysteine synthetase, O-acetylhomoserine sulphhydrylase, glycyl-tRNA ligase, aspartate-β-semialdehyde dehydrogenase, inosine-5-monophosphate dehydrogenase, aconitate hydratase and the PrsA protein. The foregoing provides a novel contribution towards knowledge of the native strain for the purpose of designing genetic manipulation strategies to obtain strains with high production of 1,3-PD.

Biological significance
The article "Protein identification in two phases of 1,3-propanediol production by proteomic analysis" provides a novel contribution towards knowledge regarding the Colombian *Clostridium* sp. native strain (IBUN 158B) because this is a new approximation in comparative proteomics in two phases of the bacterial growth and 1,3-propanediol (1,3-PD) production conditions. The proteomic studies are very important to identify the enzymes that are expressed at different stages of production and therefore genes of interest in the genetic manipulation strategies; the results can be taken into account in future studies in metabolic engineering when optimising 1,3-PD production, in a cost-effective process having direct industrial applications.

* *Corresponding author at:* Institute of Biotechnology, Universidad Nacional de Colombia, AA 14490 Santafé de Bogotá, Colombia. Tel.: +57 1 3165000; fax: +57 1 3165415.
E-mail address: dmontoyac@unal.edu.co (D. Montoya).

1874-3919/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jprot.2013.06.011

1. Introduction

1,3-Propanediol (1,3-PD), also called Trimethylene Glycol, is an organic compound having a broad variety of uses; it is used as a monomer in the synthesis of several polyesters and polyurethanes (thereby improving their chemical and mechanical properties), in designing refrigerants and aqueous dyes, in making certain solvents, adhesives, detergents and cosmetics, as well as in producing biocides for treating waste water [1].

1,3-PD is produced via chemical routes, such as acrolein hydrolysis and ethylene oxide reaction with carbon monoxide and hydrogen. However, such processes are costly, toxic and not very profitable for industry [2]. Due to the growth in demand for 1,3-PD, mechanisms have been implemented for obtaining it as a product of bacterial fermentation by microorganisms from the genus *Clostridium* and the Enterobacteriaceae family which can be used as cost-effective substrates such as industrial glycerine (from the biodiesel industry) and biotransform them into 1,3-PD having superior yield/performance resulting from their chemical synthesis [3]. It is known that these microorganisms use glycerol via two parallel metabolic pathways; the oxidative route involves acids and solvents being obtained as products and adenosine triphosphate (ATP) being produced as well as enabling nicotinamide adenine dinucleotide ($NADH_2$) reducing agent which is then used in the other route (the reductive route) consisting of glycerol dehydration to 3-hydroxypropionaldehyde, followed by aldehyde reduction to 1,3-PD. About 25 known enzymes participate in both processes [4], although publications are lacking regarding differences in their level of expression during microorganism growth phases.

Studying proteins leads to important applications in the biotechnological field, bearing in mind that an organism's protein complement has a dynamic behaviour in response to a determined condition; this contrasts with what has happened with genetic information (i.e. a static pattern) [5]. The emergence of proteomics (having rapid and continuous evolution) enabled an overall analysis of proteins and their expression in a particular environment [6,7]. Studying proteins has implied two stages in most cases: separation of proteins by two-dimensional electrophoresis (2D) followed by the identification of the separated proteins by estimation of their molecular weight by mass spectrometry; this has enabled proteins to be separated, detected and identified in a determined condition [8,9].

The first proteomic studies in the field of *Clostridium* were centred on analysing *Clostridium acetobutylicum* response in environmental stress conditions, as well as during the acidogenesis to solventogenesis step [10,11]. Research has since been aimed at evaluating proteome expression in *Clostridium* species in specific environmental conditions [12–15]. Proteomics have also been used for studying the physiology and pathogenicity of strains like *Clostridium botulinum*, *Clostridium perfringes* and *Clostridium difficile* [16–18]. Proteome studies in clostridia have been aimed at optimising the methods used for preparing protein fractions [19], protein separation and their subsequent identification by using mass spectrometry (i.e. MALDI-TOF); however proteomic studies in anaerobe strains have some difficulties, since the integrity of the cells and the proteins is affected when the bacteria are exposed to environments that contain oxygen [20,21].

1,3-PD production from glycerol fermentation could be enhanced if the expression of the enzymes involved in the metabolic pathways used by *Clostridium* sp. were known. The interest in the fermentation technologies is to improve both the process and the microorganism. In the process studies the interest is to control the variables and lower the cost of the carbon source. In the microorganism, genetic manipulation strategies are built with the aim of designing high producing mutants. The proteomic studies are very important to identify the enzymes that are expressed at different stages of production and therefore genes of interest in the genetic manipulation strategies. 2D electrophoresis and mass spectrometry analysis were thus used for evaluating a native Colombian *Clostridium* sp. strain's (IBUN 158B) intracellular proteome expression comparing two points on the growth curve, one corresponding to the end of the latency period or lag period (cell adaptation stage to changes in physical–chemical environment) and the other to the end of the exponential growth period where 1,3-PD production reaches its highest level.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains studied were *Clostridium* sp. IBUN 158B and *Clostridium butyricum* DSM 2478 as reference strain, both from the Universidad Nacional de Colombia's Instituto de Biotecnología's (IBUN) strainbank in Bogotá. Pre-inoculums were prepared according to the methodology described by Montoya et al. [22]. The microorganisms were cultured in anaerobic conditions at 200 rpm, 37 °C, in TGY medium where the original carbon source (glucose) was replaced with glycerol (5 g/l yeast extract, 8.5 g/l tryptone, 1 g/l K_2HPO_4, 0.5 g/l L-cysteine and 50 g/l glycerol).

2.2. Fermentation

Anaerobic conditions were used in the fermentation reactor (RAF Plus Bioengineering AG) with constant bubbling with N_2, 4 L effective working volume and 10% inoculum; industrial culture medium resulting from modifying a previously standardised medium for *acetobutylicum* fermentation was used [23–25] (40 g/l glycerol, 2 g/l yeast extract, 4 g/l $(NH_4)_2SO_4$, 0.5 g/l L-cysteine, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, 4 mg/l biotin, 3 mg/l PABA, 4 ml/l mineral solution). The glycerol concentrations, vitamins and inorganic and organic nitrogen sources had been optimised in prior studies by our research group. Controlled conditions were maintained at 37 °C, 200 rpm and pH 7 [26].

Each fermentation product (about 4000 ml) was spun (Sorvall centrifuge) at 5000 *g* for 30 min to collect the biomass; the supernatant was then skimmed off and the cell pellet was suspended in 1 mM 0.9% EDTA-saline solution. This was spun three more times, following which the supernatants were discarded and the cell pellet was stored at −70 °C until being processed.

2.3. Acid and solvent determination

Fermentation products and substrates were quantified in conditions previously standardised by our group [27] using high-

performance liquid chromatography (HPLC) with a refractive index detector (Waters) and an HPX-87H AMINEX column (Bio-Rad) at 50 ºC, 5 mM sulphuric acid mobile phase, 0.6 ml/min flow and 21 minute run time. Varian Star Chromatography Workstation 4.0 software was used for calculating retention time, the slope of the straight line and linear ratio coefficients (R^2) for determining the concentrations of the compounds being evaluated, using lactic acid, butyric acid, acetic acid, methanol, glycerol and 1,3-propanediol patterns, in concentrations ranging from 1 to 50 g/l.

2.4. Cell lysis and obtaining intracellular protein extracts

About 150 mg biomass was suspended in phosphate buffer, pH 7.0. Ultrasound was used for cell lysis, involving high-frequency sound waves for rupturing bacterial cell walls, thereby facilitating protein extraction, disaggregation and solubilisation. The protocol described by Mao et al. [28], was modified in this study to ensure 5-second sonication cycles (Sonics Vibra Cell sonicator) at 40 W, for 20 min, with 5-second rest intervals. Cell remains from each protein extract were separated by centrifugation at 14,000 *g* for 15 min at 4 °C; cell were discarded and the supernatants were recovered in 1.5 ml Eppendorf centrifuge tubes which were then stored at −70 °C until being processed. Protein extracts from each bacterial strain under the different phases were subjected to one-dimensional electrophoresis in order to evaluate the cell lysis procedure and verifying protein integrity (date not shown). The methanol–chloroform method was used for cleaning the extracted proteins. The clean protein extract was then suspended in isoelectric focusing (IEF) buffer (7 M urea, 2 M Thiourea, 2% CHAPS (p/v), 0.2% DTT (p/v), 1% ampholytes (v/v) (Amersham, pH 3–7) and bromothymol blue dye).

3. Two-dimensional (2D) electrophoresis

3.1. Isoelectric focusing (IEF)

A colorimetric assay (Pierce 660) was used for protein quantification of each extract. Two-dimensional electrophoresis was performed according to the protocol developed by the Hormone Research Group, Universidad Nacional de Colombia and IEF conditions were standardised, bearing in mind the characteristics of the microorganism being studied. We performed two biological replicates and three technical replicates. For IEF, 300 μg protein samples were thus suspended in IEF buffer, until a final 325 μl volume was completed; this was used for rehydrating 18 cm immobilised pH gradient (IPG) strips, pH 4–7 (Bio–Rad). They were then incubated at room temperature for 1 h and covered with mineral oil (Bio-Rad). IEF was done in seven steps: 11 h at 50 V, 0.5 h at 250 V, 0.5 h at 1000 V, 1 h at 1000 V, 0.75 h at 4000 V, 1 h at 4000 V and 6.5 h at 10,000 V up to 75,000 Vh (maximum 50 μA per gel and constant 20 °C temperature). The IPG strips were stored at −70 °C or immediately prepared for the second dimension (SDS–PAGE).

3.2. Second dimension electrophoresis

Equilibrium was performed twice, first with buffer A (6 M urea, 2% SDS (p/v), 1.5 M Tris–Cl, pH 8.8 (0.375 M), 20% glycerol (v/v) and 130 mM DTT (20 mg/ml)) and then with buffer B (6 M urea, 2% SDS (p/v), 1.5 M Tris–Cl, pH 8,8 (0.375 M), 20% glycerol (v/v) and 135 mM iodoacetamide (25 mg/ml)). SDS–PAGE electrophoresis involved 20 × 20 cm gels at 12% concentration (30% acrylamide and 0.8% bisacrylamide); run conditions were 80 V, 0.02 A and 2 W, at room temperature, for around 18 h. The gels were fixed with fixing solution (50% methanol and 5% acetic acid) twice for 15 min and then stained overnight with Coomassie staining solution (20% methanol, 10% phosphoric acid, 10% ammonium sulphate and 0.125% Coomassie G-250).

3.3. Image analysis

The stained gels were scanned at 300 dpi resolution and analysed using PDQuest 8.0 software (Bio–Rad). The images were filtered according to the type of noise found. The number of spots on the gels was determined using the tools made available in the software being used, as well as matching, qualitative, quantitative, statistical and Boolean analysis for comparing the growth phases being evaluated. Regarding identifying proteins, the selected spots were analysed by mass spectrometry at the Instituto Pasteur, Uruguay. The selection criteria were based on the data reported in the pertinent literature concerning molecular weight and isoelectric point (Ip) for enzymes implicated in the 1,3-PD production metabolic pathway, protein spot intensity and their expression in one of the two growth phases being studied. Six of the selected spots were analysed by triplicate. For the identification, *C. butyricum* was chosen as reference strain due to genomic similarities with the native strain.

3.4. Protein identification by MS/MS

Peptide mapping was chosen for identifying proteins. The proteins extracted from the gels were sent to the Instituto Pasteur; these were treated with trypsin (sequencing grade, Promega) overnight at 37 °C. The peptides were extracted from the gel using 60% acetonitrile in 0.2% TFA and then vacuum concentrated, dried and desalinated using reverse phase C18 microcolumns (OMIX). Peptides were directly eluted from the microcolumns in the mass spectrometer sample plate with 3 μl matrix solution (alpha-cyano-4-hydroxycinnamic acid in 60% aqueous acetonitrile with 0.2% TFA). A MALDI-TOF/TOF 4800 (Applied Biosystems) in reflector mode was used for acquiring the digested samples' mass spectra which were externally calibrated using a standard peptide mix (Applied Biosystems). A collision was induced for mass dissociation in the selected peptides. The proteins were identified by searching local databases using m/z values and MASCOT software with the following search parameters: 0.08 monoisotopic mass tolerance, 0.25 Da mass tolerance fragment, methionine oxidation as possible modification, cysteine carbamidomethylation as fixed modification and permitted tryptic cleavage loss. For the identification, protein scores greater than 83 are significant ($p < 0.05$).

4. Results

4.1. Acid and solvent determination by HPLC

Fermentation with the *C. butyricum* DSM 2478 reference strain and the *Clostridium* sp. IBUN 158B native strain was carried out

in duplicate according to the aforementioned parameters; the first fermentation corroborated each phase's duration and this was followed by another whose resulting biomass was used for extracting the intracellular proteins. The latency phase was presented after 0 to 12 h had elapsed in both fermentations whilst the exponential growth phase occurred after 12 to 23 h of fermentation.

Production yield/performance was defined according to HPLC substrate (glycerol) and product (1,3-PD, acetate and butyrate) values (Table 1). As expected, 1,3-PD was only detected during the exponential growth phase, thereby explaining why it is a primary metabolite. It was also determined that the native strain led to greater yield in producing this solvent from glycerol (0.78 mol/mol). Regarding other products, both the reference and native strains produced a greater amount of acetate than butyrate; however, they both had lower yields than 1,3-PD (Table 1).

4.2. 2D electrophoresis

The conditions for the extraction of a representative protein fraction from *Clostridium* sp. IBUN 158B native strain were standardised in this study (Fig. 2a and b); a suitable IEF protocol for separating the proteins present in such fraction was also established. The correlation coefficients obtained (0.85–0.95) for replicate gels showed high reproducibility. The proteomic profiles obtained for the *Clostridium* sp. IBUN 158B native strain had an average of 132 spots were detected during exponential phase and 215 during latency phase; the *C. butyricum* DSM 2478 strain had 151 and 202 spots, respectively. Comparisons were made for establishing differences between the growth phases evaluated in the two bacterial strains (Figs. 1 and 2). The *Clostridium* sp. IBUN 158B strain had more spots exclusively expressed during the latency phase (30 spots) compared to the *C. butyricum* DSM 2478 strain (14 spots) (Fig. 1). In the native strain *Clostridium* sp. (IBUN 158B) more spots were exclusively expressed during latency phase (50 spots) compared to exponential growth phase (20 spots) (Fig. 2), the *C. butyricum* DSM 2478 strain had the same tendency.

4.3. Protein identification by MS/MS

Thirty-two spots were chosen to be analysed by mass spectrometry according to the intensity of the spots, their differential expression during one of the two growth phases evaluated here and bibliographic review of enzymes from the 1,3-PD route. Four enzymes implicated in the production route of the solvent of interest were identified; one was associated with the reductive route (1,3-propanediol dehydrogenase) and three with the oxidative route (3-hydroxybutyryl-CoA dehydrogenase, NADPH-dependent butanol dehydrogenase and phosphate butyryl transferase). A novel contribution made by the present study lay in identifying the following enzymes: phosphoglycerate kinase, glucose-6-phosphate isomerase, deoxyribose phosphate aldolase, transketolase, cysteine synthetase, O-acetylhomoserine sulphhydrylase, glycyl-tRNA ligase, glutamine synthetase, aspartate-β-semialdehyde dehydrogenase, inosine-5-monophosphate dehydrogenase, aconitate hydratase and the PrsA protein. Given that genomic studies have reported such enzymes for *Clostridium* sp., no records were found regarding their biochemical and structural characterisation. Enzymes associated with metabolic pathways, such as glycolysis, were also identified as well as enzymes implicated in protein synthesis and cellular metabolic processes (pentose phosphate route, pyruvate assimilation route, nucleotide synthesis and degradation, protein folding, polysaccharide synthesis, tricarboxylic acid (TCA) or Krebs' cycle and electron transport). Table 2 summarises the aforementioned proteins' main characteristics.

Table 1 – Solvent and acid concentrations for native and reference strains after 23 and 21 hours of fermentation, respectively, in an industrial setting and in controlled temperature and pH conditions: (Y) yield, (PD) 1,3-propanediol, (but) butyrate, and (acet) acetate.

Parameter	*Clostridium* sp. IBUN 158B native strain	*Clostridium butyricum* DSM 2478 reference strain
Glycerol consumed (g/l)	17	19
1,3-PD produced (g/l)	11	10
Y PD/s (mol/mol)	0.78	0.64
Y but/s (mol/mol)	0.04	0.04
Y acet/s (mol/mol)	0.12	0.11

5. Discussion

5.1. Acid and solvent profiles

Clostridium sp. batch fermentation is characterised by an initial phase (acidogenic phase) during which acetate, butyrate, CO_2 and hydrogen are produced. Acids are formed in parallel to growth; these are primary metabolites since ATP is produced by substrate phosphorylation in both the acetic acid and butyric acid production routes and is then used in biosynthesis. Reducing equivalents ($NADH_2$) are produced during this stage; if they are not transferred to an external electron acceptor then they become oxidised in the reductive route to produce 1,3-PD. As expected, the solvent of interest is produced in parallel to microorganism growth (exponential phase), having greater yield (0.78 ymol p/s) than that obtained in other research by this group (0.67 and 0.72 ymol p/s) (unpublished data). This may be explained as *Clostridium* sp. needs oxidised cofactors produced during 1,3-PD production, as well as ATP, for glycerol biosynthesis [4].

The results also revealed low yields regarding acid production (Table 1); greater acetate than butyrate production was recorded in all fermentations (Table 1), mainly promoting 1,3-PD synthesis since (regardless of biomass generation) one out of each three glycerol molecules is used for producing acetate and the others for two NADH2 molecules which could go by the reductive route. On the contrary, two glycerol molecules and two NADH2 molecules are consumed in the butyrate route, this being a competitive route for 1,3-PD production [2].

5.2. Proteins identified by mass spectrometry

Analysis of the 2D gels obtained in this study led to differences between the proteins synthesised during the growth phases evaluated here (Figs. 1 and 2); around fifty spots were exclusively

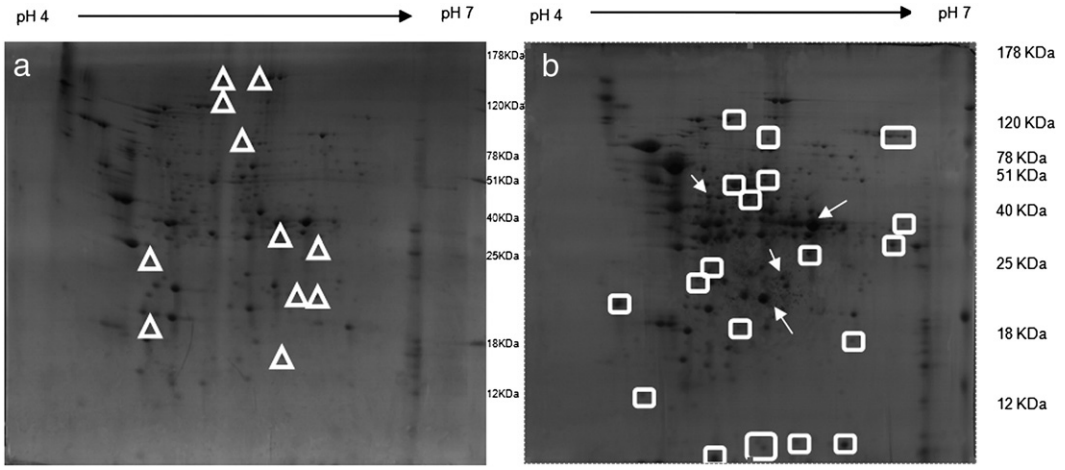

Fig. 1 – Spots exclusively expressed during latency phase. 2D-electrophoresis (12% polyacrylamide gels). a) △, intracellular proteins of the *Clostridium butyricum* DSM 2478 reference strain. b) □, intracellular proteins of the *Clostridium* sp. IBUN 158B native strain. → indicates proteins implicated in the 1,3-PD metabolic pathway: 1,3-propanediol dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, NADPH-dependent butanol dehydrogenase and phosphate butyryl transferase. Proteins (300 μg) extracted by ultrasound, in phosphate buffer with 10 mM Pefabloc. 1D-electrophoresis: strips pH 4–7 (18 cm). Blue silver stained.

expressed in the phase lag, as the enzymes required for cell growth, the use of nutrients present in the culture medium and the production of essential metabolites absent in it all occur during this stage [29]. The share of enzymes identified in the *Clostridium* sp. IBUN 158B native strain's metabolic pathways highlighted phosphoglycerate kinase, glucose 6-phosphate isomerase, deoxyribose phosphate aldolase, transketolase, cysteine synthetase, O-acetylhomoserine sulphhydrylase, glycyl-tRNA ligase, aspartate-β-semialdehyde dehydrogenase, inosine-5-monophosphate dehydrogenase, aconitate hydratase and the PrsA protein as no reports could be found concerning their characterisation in *Clostridium* sp.

5.3. *Enzyme implicated in the reductive route for 1,3-propanediol synthesis*

1,3-Propanediol dehydrogenase (EC 1.1.1.202) was identified; this enzyme reduces a 3-hydroxypropionaldehyde molecule to form 1,3-PD [30,31]. Reports have stated that most of this enzyme's activity occurs at the end of the exponential phase where maximum 1,3-PD production happens [32]. However, the presence of the enzyme in both growth phases evaluated was verified in the 2D gels obtained in this study (Fig. 1b). The foregoing has been supported by research aimed at characterising the enzyme, establishing that it is induced in

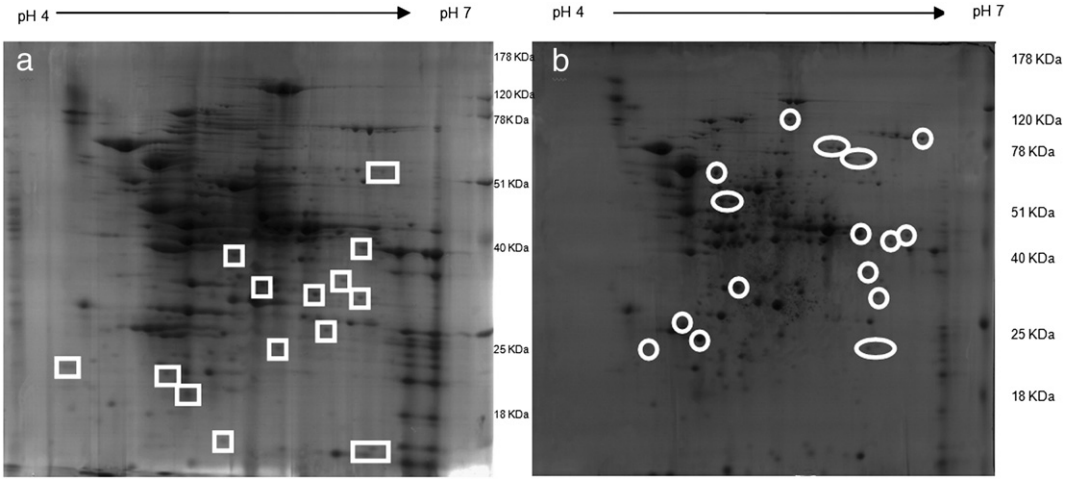

Fig. 2 – Spots exclusively expressed during latency and exponential phases. 2D-electrophoresis (12% polyacrylamide gels). a) □, intracellular proteins of the *Clostridium* sp. IBUN 158B native strain, expressed during exponential phase. b) ○, intracellular proteins of the *Clostridium* sp. IBUN 158B native strain, expressed during latency phase. Proteins (300 μg) extracted with ultrasound, in phosphate buffer with 10 mM Pefabloc. 1D-electrophoresis: strips pH 4–7 (18 cm). Blue silver stained.

Table 2 – Characteristics of the peptides identified in each protein by mass spectrometry. Lag (latency phase). Exp (exponential growth phase). Protein scores greater than 83 are significant ($p < 0.05$).

Protein identified	Apparent molecular mass (kDa)	Apparent isoelectric point (IP)	Mascot score	% coverage	Phase	Function
Reductive route: 1,3-propanediol synthesis						
1,3-Propanediol dehydrogenase	42	5.66	137	41	Lag, Exp.	1,3-PD metabolism
Oxidative route: 1,3-propanediol synthesis						
3-Hydroxybutyryl-CoA dehydrogenase	30	5.37	207	59	Lag, Exp.	Butyryl-CoA synthesis
Phosphate butyryl transferase	32	5.54	167	41	Lag, Exp.	Butyryl-phosphate synthesis
NADPH-dependent butanol dehydrogenase	43	5.08	162	52	Lag.	Butanol synthesis
Carbohydrate metabolism						
Phosphoglycerate kinase	42	5.11	472	64	Lag, Exp.	3-Phosphoglycerate and ATP synthesis
Transketolase	33	5.58	210	64	Lag, Exp.	Sugar metabolism
6-Phosphofructokinase	34	5.35	159	52	Lag, Exp.	Fructose-1,6-biphosphate synthesis
2,3-Biphosphoglycerate-independent phosphoglycerate mutase	56	5.06	287	50	Lag, Exp.	2,3-Phosphoglycerate-and 2-phosphoglycerate synthesis
Fructose-1,6-biphosphate aldolase, class II	31	5.08	392	74	Lag, Exp.	Glyceraldehyde-3-phosphate, dyhydroxyacetone-phosphate synthesis
Glucose-6-phosphate isomerase	50	5.16	243	45	Lag, Exp.	Fructose-6-phosphate synthesis
Glyceraldehyde-3-phosphate dehydrogenase	36	5.36	54	54	Lag, Exp.	1,3-Biphosphoglycerate synthesis
Triose-phosphate isomerase	17	5.01	108	23	Lag, Exp.	Glyceraldehyde-3-phosphate synthesis
Pyruvate ferredoxin oxydoreductase	127	5.46	389	34	Lag, Exp.	Oxidative decarboxylation of pyruvate
Deoxyribose phosphate aldolase	24	5.26	284	69	Lag, Exp.	Glyceraldehyde-3-phosphate and acetaldehyde synthesis
Protein synthesis						
Cysteine synthetase	32	5.53	133	65	Lag, Exp.	Cysteine synthesis
Glutamine synthetase	72	5.40	140	29	Lag, Exp.	Glutamine synthesis
Aspartate-semialdehyde dehydrogenase	40	5.98	142	44	Lag, Exp.	Aspartate-β-semialdehyde synthesis
O-acetylhomoserine sulphhydrylase	47	5.45	205	23	Lag.	Methionine synthesis
Glycyl-tRNA synthetase	54	5.09	227	29	Lag.	Glycine-tRNA binding (translation)
DNA-dependent RNA polymerase	35	4.87	361	55	Lag, Exp.	Transcription
S2 ribosomal protein	26	6.38	182	34	Lag, Exp.	30S subunit binding
L10 ribosomal protein	18	6.02	137	53	Lag, Exp.	70S subunit binding
Other metabolic pathways						
Inosine-5-monophosphate dehydrogenase	52	6.08	122	30	Lag, Exp.	Guanine nucleotide synthesis
Peptidylprolyl isomerase	48	4.74	381	41	Lag, Exp.	Peptide bond isomerisation
DnaK chaperone family	66	4.74	458	58	Lag, Exp.	Intermediate protein folding
GrpE chaperone family	23	4.51	293	50	Lag, Exp.	Intermediate protein folding
PrsA foldase protein	28	4.94	216	50	Lag, Exp.	Protein folding
Glucose-1-phosphate adenylyltransferase	16	5.08	162	52	Lag.	ADP-glucose synthesis
Aconitate hydratase	69	5.35	168	31	Lag, Exp.	Isocitrate synthesis
Conserved hypothetical protein	35	6.41	257	30	Lag.	
Electron transfer protein	32	5.42	309	65	Lag.	Electron transport

anaerobic conditions and when glycerol is present in the medium (i.e. fermentation conditions used in this study). Similarly, it is known that this enzyme's synthesis in the reductive route ultimately occurs to prevent accumulation of the intermediate-3-hydroxypropionaldehyde produced from glycerol (a toxic compound at cellular level) [33].

5.4. *Enzymes implicated in oxidative route synthesis of 1,3-propanediol*

3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) was one of the enzymes identified here; it catalyses acetoacetyl-CoA reduction by NADH, thereby producing 3-hydroxybutyryl-CoA,

an important intermediary for butyrate and butanol production [34,35]. Studies concerning the enzyme's activity in batch cultures have described that this becomes increased during the exponential growth phase and then remains constant once growth ceases [36]. Even though butyrate levels may have been low in this study (Table 1), this was produced by the microorganism parallel to cell growth (data not shown); the presence of 3-hydroxybutyryl-CoA was also detected in both phases evaluated here (latency and exponential growth phases).

The other enzyme implicated in the oxidative route has phosphate butyryl transferase (EC 2.3.1.19) which catalyses the reaction converting butyryl-CoA to butyryl-phosphate (an important intermediary for butyrate synthesis) [37–39]. It seems that phosphate butyryl transferase activity is much greater in acid-producing strains than in some solvent-producing ones. This enzyme has been widely studied in *Clostridium* sp. and has been purified in *C. acetobutylicum*, *C. acetobutyricum*, *Clostridium saccharoacetobutylicum*, *C. butyricum* and *Clostridium sporogenes* [36].

NADPH-dependent butanol dehydrogenase was also identified. It is associated with the final step in the butanol formation route; a butyraldehyde molecule is reduced to butanol in this step in a reaction in which the cofactor NADPH becomes oxidised [40,41]. It seems that this is an inducible enzyme, achieving its greatest mayor activity during solvent-production phase. Some reports have dealt with its characterisation in *C. acetobutylicum*, a strain recognised as producing butanol; two types of butanol dehydrogenase having different cofactors (an NADPH-dependent one and NADH) have been determined in it [42,43].

5.5. *Enzymes implicated in carbohydrate metabolism*

Most enzymes identified to date are implicated in carbohydrate metabolism, particularly regarding glycolysis. The proteins associated with the glycolytic process were: 6-phosphofructokinase (EC 2.7.1.11) [44], fructose biphosphate aldolase class II (EC 4.1.2.13) [45], triose-phosphate isomerase (EC 5.3.1.1) [46], glyceraldehyde-3- phosphate dehydrogenase (NADP+) (EC 1.2.1.12) [47,48] and 2,3-biphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1) [49,50]. Also were identified the enzymes glucose-6-phosphate isomerase (EC 5.3.1.9) [51,52] and phosphoglycerate kinase (EC 2.7.2.3) [53], uncharacterised in the genus *Clostridium* sp.

Apart from the enzymes implicated in glycolysis, proteins associated with the carbohydrate metabolism were identified, this is the case of the pyruvate ferredoxin oxydoreductase (EC 1.2.7.1) [54,55] and the glucose-1-phosphate adenyltransferase (EC 2.7.7.27) [56,57]. Additionally the presence of the enzymes deoxyribose-phosphate aldolase (EC 4.1.2.4) [58,59] and transketolase (EC 2.2.1.1) [60,61], uncharacterised in the genus *Clostridium* sp., was recorded.

5.6. *Enzymes implicated in protein synthesis*

The enzymes associated with amino acid synthesis were identified: aspartate-β-semialdehyde dehydrogenase (EC 1.2.1.11) [62,63] (uncharacterised in *Clostridium* sp.) and glutamine synthetase (EC 2.7.7.42) [64,65]. Two of the proteins identified are implicated in inorganic sulphur assimilation: the cysteine synthetase (EC 2.5.1.47) [66,67] and the O-acetylhomoserine sulphhydrylase (EC 2.5.1.49) [68,69] (no reports of this type of enzymes have been found regarding *Clostridium* sp.).

On the other hand, the transcription-associated enzyme DNA-dependent RNA polymerase (EC 2.7.7.6) [70] and the glycine-tRNA ligase (EC 6.1.1.14) were identified [71]; however, no reports for these enzymes have been found for *Clostridium* sp. Finally, two of the proteins identified in this group constitute the ribosome and are known as ribosomal protein S2 and L10 [72,73].

5.7. *Enzymes implicated in other metabolic pathways*

Three cell chaperones were identified as part of other important metabolic pathways for cell functioning: peptidylprolyl isomerase [74,75], DnaK [76] and GrpE [76,77]. The presence of such genes encoding these molecular chaperons has been demonstrated in *C. acetobutylicum* and it seems that their expression occurs in normal growth conditions, in situations of stress due to high temperatures or during the solvent formation phase [78]. The protein PrsA was also identified; it participates in the secretion of synthesised proteins in the cytoplasm [79,80] (no studies or reports for this enzyme have been found for the genus *Clostridium*).

The enzyme inosine-5′-monophosphate-dehydrogenase (EC 1.1.1.205) (which has not been reported in *Clostridium* sp.) was identified as part of another cell metabolism, specifically de novo biosynthesis of guanine nucleotides [81,82]. Finally, the presence of the enzyme aconitate hydratase (EC 4.2.1.3), which is the second enzyme in the tricarboxylic acid (TCA) or Krebs' cycle, was recorded [83]. Even though Krebs' cycle may not occur in anaerobic bacteria in the same way as in aerobic organisms, all of this pathway's biosynthetic intermediates are produced, whether in an incomplete form of the cycle (characteristic of anaerobic organisms) or by anaplerotic reactions [84,85]. However, no reports or studies related to this enzyme's characterisation and/or purification were found concerning *Clostridium* sp.

6. Conclusions

Using two-dimensional electrophoresis coupled to mass spectrometry analysis led to differences being established regarding proteins expressed during *Clostridium* sp. IBUN 158B native strain latency and exponential growth phases, in 1,3-PD production conditions. Identifying thirty-two differentially expressed proteins by mass spectrometry demonstrated the presence of enzymes associated with the production of the solvent of interest (1,3-propanediol dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase, NADPH-dependent butanol dehydrogenase and phosphate butyryl transferase), as well as enzymes implicated in the metabolism of sugars, in protein synthesis and other metabolic pathways enabling cell functioning. It should be stressed that even though phosphoglycerate kinase, glucose-6-phosphate isomerase, deoxyribose phosphate aldolase, transketolase, cysteine synthetase, O-acetylhomoserine sulphhydrylase, glycyl-tRNA ligase, aspartate-β-semialdehyde dehydrogenase, inosine-5-monophosphate dehydrogenase, aconitate hydratase and

the PrsA protein have been reported for *Clostridium* sp. based on genome analysis, no studies could be found in the bibliographic review about their biochemical and structural characterisation. This means that the material reported here provides a novel contribution towards knowledge regarding the *Clostridium* sp. IBUN 158B native strain which can be taken into account when optimising 1,3-PD production, in a cost-effective process having direct industrial applications.

Acknowledgements

This work was done in collaboration with the Hormone Research Group, Universidad Nacional de Colombia. The authors would like to thank the Universidad Nacional de Colombia's postgraduate interfaculty Microbiology programme, Colciencias' Young Researchers and Innovators' programme and the Instituto de Biotecnología.

REFERENCES

[1] Papanikolaou S, Fakas S, Fick M, Chevalot I, Galiotou M, Komaitis M, et al. Biotechnological valorization of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: production of 1,3-propanediol, citric and single cell oil. Biomass Bioenergy 2008;32:60–71.
[2] Saxena R, Anand P, Saran S, Isar J. Microbial production of 1,3-propanediol: recent developments and emerging opportunities. Biotechnol Adv 2009;27:895–913.
[3] Fonseca P, Ferreira T, Cardozo G, Zarur M. Glycerol valorization: new biotechnological routes. Food Bioprod Process 2009;30: 1–8.
[4] Biebl H, Menzel K, Zeng A, Deckwer W. Microbial production of 1,3-propanediol. Appl Microbiol Biotechnol 1999;52:289–97.
[5] Ogorzalek L, Hayes R, Yung Y, Hung F, Ramachandran P, Kim N, et al. Top-down, bottom-up, and side-to-side proteomics with virtual 2-D gels. Int J Mass Spectrom 2005;240:317–25.
[6] Cordwell S. Technologies for bacterial surface proteomics. Curr Opin Microbiol 2006;9:320–9.
[7] Cho W. Proteomics technologies and challenges. Genomics Proteomics Bioinformatics 2007;5(2):77–85.
[8] Anderson N, Matheson A, Steiner S. Proteomics: applications in basic and applied biology. Curr Opin Biotechnol 2000;11: 408–12.
[9] Natarajan S, Xu C, Cregan P, Caperna T, Garrett W, Luthria D. Utility of proteomics techniques for assessing protein expression. Regul Toxicol Pharmacol 2009;54:532–6.
[10] Terracciano J, Rapaport E, Kashket E. Stress and growth phase-associates proteins of *Clostridium acetobutylicum*. Appl Environ Microbiol 1989;1988:54.
[11] Pich A, Bahl H. Purification and characterization of the DNA-dependent RNA polymerase from *Clostridium acetobutylicum*. J Bacteriol 1991;173(6):2120–4.
[12] Narberhaus F, Pich A, Bahl H. Synthesis of heat shock proteins in *Thermoanaerobacterium thermosulforigenes* EM1 (*Clostridium thermosulfurogenes* EM1). Curr Microbiol 1994;29:13.
[13] Denger K. Anaerobic desulfunation of 4-tolylsulfonate and 2-(4-sulfophenyl) butyrate by a *Clostridium* sp. Appl Environ Microbiol 1996;62:1526.
[14] Flengsrud R, Skjeldal L. Two dimensional gel electrophoresis separation and N-terminal sequence analysis of proteins from *Clostridium pasterianum* W5. Electrophoresis 1998;19: 802.
[15] Shaffer S. Changes in protein synthesis and identification of proteins specifically induced during solventogenesis in *Clostridium acetobutylicum*. Electrophoresis 2002;23:110.
[16] McClane B, Rood J. Clostridial toxins involved in human enteric and histotoxic infections. In: Bahl H, Dürre P, editors. *Clostridia*: biotechnology and medical applications. Weinheim: Wiley-VCH Verlag GmbH; 2001. p. 169.
[17] Mukherjee K. Proteins released during high toxin production in *Clostridium difficile*. Microbiology 2002;148:2245.
[18] Karlsson S. Expression of *Clostridium difficile* toxins A and B and their sigma factor TcdD is controlled by temperature. Infect Immun 2003;71:1784–6.
[19] Schwarz K, Fiedler T, Fischer R, Bahl H. A Standard operating procedure (SOP) for the preparation of intra-and extracellular proteins of *Clostridium acetobutylicum* for proteome analysis. J Microbiol Methods 2007;68:396–402.
[20] Muetzelburg M, Hofmann F, Pinch J. Identification of biomarkers indicating cellular changes after treatment of neuronal cells with the C3 exoenzyme from *Clostridium botulinum* using the iTRAO protocol and LC–MS/MS analysis. J Chromatogr B 2009;877:1344–51.
[21] Everley R, Mott T, Toney D, Croley T. Characterization of *Clostridium* species utilizing liquid chromatography/mass spectrometry of intact proteins. J Microbiol Methods 2009;77: 152–8.
[22] Montoya D, Spitia S, Silva E, Schwarz W. Isolation of mesophilic solvent-producing clostridia from Colombian sources: physiological characterization, solvent production and polysaccharide hydrolysis. J Biotechnol 2000;79:117–26.
[23] Parra C, López L, Granados J, Dumar J, Montoya D, Ferreira S, et al. Evaluación de cepas de *Clostridium acetobutylicum* para el desarrollo de la fermentación acetobutílica. Rev Colomb Ciencias Quim Farm 1995;24:40–4.
[24] Buitrago G, Moreno N, Montoya D, Cabrera N, Cruz L. Estudio del efecto de la temperatura en la fermentación acetobutílica. Ing Investig 1996;3:114–22.
[25] Montoya D, Ramos J, Silva E, Buitrago G, Sierra J. Optimización de un medio de cultivo industrial para la fermentación acetobutílica (ABE). Rev Col Biotechnol 1999;2(2):37–42.
[26] Pérez X. Estandarización de la concentración de glicerol industrial y de la fuente de nitrógeno en el medio de cultivo para la producción de 1,3-propanodiol utilizando una cepa nativa de *Clostridium* sp. MSc Microbiology thesis Universidad Nacional de Colombia; 2009 70.
[27] Montoya D, Cárdenas D, Aragón O, Aristizabal F, Pulido C, Suárez Z. Evaluación de la producción de 1,3-propanodiol por cepas nativas de *Clostridium* sp. mediante fermentación a partir de glicerol USP y glicerol industrial. Rev Colomb Ciencias Quim Farm 2006;35(1):120–37.
[28] Mao S, Luo Y, Zhang T, Li J, Bao G, Zhu Y, et al. Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. J Proteome Res 2010;4(6):3046–61.
[29] Madigan M, Martinko J, Dunlap P, Clark D. Crecimiento Microbiano. Brock biology of microorganisms. 5th ed. San Francisco: Pearson education; 2009 137–67.
[30] Johnson E, Lin E. *Klebsiella pneumoniae* 1,3-propanediol: NAD + oxydoreductase. J Bacteriol 1987;169(5):2050–4.
[31] Vega M, Foster M. 1,3-Propanediol:NAD + oxydoreductase of *Lactobacillus brevis* and *Lactobacillus buchneri*. Appl Environ Microbiol 1992;58(6):2005–10.
[32] Hongwen C, Baishan F, Zongding F. Optimization of process parameters for key enzymes accumulation of 1,3-propanediol production from *Klebsiella pneumoniae*. Biochem Eng J 2005;25: 47–53.
[33] Chen Z, Hongjuan L, Dehua L. Metabolic pathway analysis of 1,3-propanediol production with a genetically modified *Klebsiella pneumonia* by overexpressing and endogenous

NADPH-dependent alcohol dehydrogenase. Biochem Eng J 2011;54:151–7.
[34] Hartmanis M, Gatenbeck S. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. Appl Environ Microbiol 1984;47(6):1277–83.
[35] Youngleson J, Jones D, Woods D. Homology between hydroxybutyryl and hydroxyacyl coenzyme A dehydrogenase enzymes from *Clostridium acetobutylicum* fermentation and vertebrate fatty acid β-oxidation pathways. J Bacteriol 1989;171(12):6800–7.
[36] Gheshlaghi R, Scharer J, Moo-Young M, Chou C. Metabolic pathways of clostridia for producing butanol. Biotechnol Adv 2009;27:764–81.
[37] Valentine R, Wolfe R. Purification and role of phosphotransbutyrylase. J Biol Chem 1960;235(7):1948–52.
[38] Wiersenborn D, Rudolph F, Papoutsakis E. Phosphotransbutyrylase from *Clostridium acetobutylicum* ATCC 824 and its role in acidogenesis. Appl Environ Microbiol 1989;55(2):317–22.
[39] Huang H, Liu H, Gan Y. Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. Biotechnol Adv 2010;28:651–7.
[40] Walter K, Bennett G, Papoutsakis E. Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isozyme genes. J Bacteriol 1992;174(22):7149–58.
[41] Vasconcelos I, Girbal L, Soucaille P. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grow in chemostat culture at neutral pH on mixtures of glucose and glycerol. 1994;176(3):1443–50.
[42] Dürre P, Kuhn A, Gottwald M, Gottschalk G. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl Environ Microbiol 1987;26:268–72.
[43] Petersen D, Welch R, Rudolph F, Bennett G. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J Bacteriol 1991;173(5): 1831–4.
[44] Kotzé J. Glycolytic and related enzymes in clostridial classification. Appl Environ Microbiol 1969;18(5):744–7.
[45] Cooper S, Gordon L, Mcsweeney S, Thompson A, Naismith J, Qamar S, et al. The crystal structure of a class II fructose-1,6-bisphosphate aldolase shows a novel binuclear metal-binding active site embedded in a familiar fold. Structure 1996;4:1303–15.
[46] Rentier F, Mande S, Moyens S, Terpstra P, Mainfroid V, Goraj K, et al. Cloning and over-expression of the triosephosphate isomerase genes from psychrophilic and thermophilic bacteria: structural comparison of the predicted protein sequences. J Mol Biol 1993;229:85–93.
[47] Schreiber W, Dürre P. Differential expression of genes within the *gap* operon of *Clostridium acetobutylicum*. Anaerobe 2000;6: 291–7.
[48] Iddar A, Valverde F, Serrano A, Soukri A. Expression, purification, and characterization of recombinant non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*. Protein Expr Purif 2002;25(202):519–26.
[49] Graña X, Pérez de la ossa P, Broceño C, Stöcker M, Garriga J, Puigdoménech P, et al. 2,3-Biphosphoglycerate-independent phosphoglycerate mutase is conserved among different phylogenic kingdoms. Comp Biochem Physiol 1995;112B(2): 287–93.
[50] Chander M, Setlow B, Setlow P. The enzymatic activity of phosphoglycerate mutase from gram-positive endospore-forming bacteria requires Mn^{2+} and is pH sensitive. Can J Microbiol 1998;44(8):759–67.
[51] Widjaja A, Shiroshima M, Oka T, Yasuda M, Ogino H, Miyatake K, et al. The kinetics and mechanism of a reaction catalyzed by *Bacillus stearothermophilus* phosphoglucose isomerase. J Ferment Bioeng 1998;86(3):324–31.
[52] Richards G. Structural and functional analyses of phosphoglucose isomerase from *Vibrio vulnificus* and its lysyl aminopeptidase activity. Bioenergetics 2004;1702:89–902.
[53] Mizuno Y, Mifuyo O, Yao Y, Shibasaki R, Takahashi R, Tokuyama T. Purification and comparison of phosphoglycerate kinases from nitrifying bacteria. J Ferment Bioeng 1998;86(4): 346–50.
[54] Moulis J, Davasse V, Meyer J, Gaillard J. Molecular mechanism of pyruvate–ferredoxin oxydoreductase based on data obtained with the *Clostridium pasteurianum* enzyme. FEBS Lett 1996;380:287–90.
[55] Lee K, Lillehoj H, Li G, Park M, Jang S, Jeong W, et al. Identification and cloning of two immunogenic *Clostridium perfringes* proteins, elongation factor Tu (EF-Tu) and pyruvate: ferredoxin oxydoreductase (PFO) of *C. perfringes*. Res Vet Sci 2011;91:80–6.
[56] Preiss J, Crawford K, Downey J, Lammel C, Greenberg E. Kinetic properties of *Serratia marcescens* adenosine 5′-diphosphate glucose pyrophosphorylase. J Bacteriol 1976;127(1):193–203.
[57] Ballicora M, Iglesias A, Preiss J. ADP-glucose pyrophosphorylase, a regulatory enzyme for bacterial glycogen synthesis. Microbiol Mol Biol Rev 2003;67(2):231-225.
[58] Sgarrella F, Poddie F, Meloni M, Sciola L, Pippia P, Tozzi M. Channelling of deoxyribose moiety of exogenous DNA into carbohydrate metabolism: role of deoxyriboaldolase. Comp Biochem Physiol A 1997;117(2):253–7.
[59] Heine A, Luz J, Wong C, Wilson I. Analysis of the class I aldolase binding site architecture based on the crystal structure of 2-deoxyribose-5-phosphate aldolase at 0.99 Å resolution. J Mol Biol 2004;343:1019–34.
[60] Mitra R, Woodley J, Lilly M. *Escherichia coli* transketolase-catalyzed carbon-carbon bond formation: biotransformation characterization for reactor evaluation and selection. Enzyme Microb Technol 1998;22:64–70.
[61] Jaromi R, Morris P, Martinez R, Dalby P. Structural stability of *E. coli* transketolase to temperature and pH denaturation. J Biotechnol 2011;155:209–16.
[62] Moore R, Bocik W, Viola R. Expression and purification of aspartate β-semialdehyde dehydrogenase from infectious microorganisms. Protein Expr Purif 2002;25:189–94.
[63] Faehnle C, Le Coq J, Liu X, Viola R. Examination of key intermediates in the catalytic cycle of aspartate-β-semialdehyde dehydrogenase from a Gram-positive infectious bacteria. J Biol Chem 2006;28(41): 31031–40.
[64] Usdin P, Zappe H, Jones D, Woods D. Cloning, expression, and purification of glutamine synthetase from *Clostridium acetobutylicum*. Appl Environ Microbiol 1986;52(3):413–9.
[65] Krishnan I, Ramji D. *Clostridium pasteurianum* glutamine synthetase mechanism: evidence for active site tyrosine residues. FEBS Lett 1985;185(2):267–71.
[66] Albanesi D, Mansilla M, Schujman E, De Mendoza D. *Bacillus subtilis* cysteine synthetase is a global regulator of the expression of genes involved in sulfur assimilation. J Bacteriol 2005;187(22):7631–8.
[67] Feldman A, Wirtz M, Ruediger H, Wade R. A mechanistic model of the cysteine synthase complex. J Mol Biol 2009;386: 37–59.
[68] Gophna U, Bapteste E, Ford W, Biran D, Ron E. Evolutionary plasticity of methionine biosynthesis. Gene 2005;355:48–57.
[69] Kumar D, Gomez J. Methionine production by fermentation. Biotechnol Adv 2005;23:41–61.
[70] Pich A, Narberhaus F, Bahl H. Induction of heat shock proteins during initiation of solvent formation in *Clostridium acetobutylicum*. Appl Microbiol Biotechnol 1990;33:697.
[71] Diaz Y, Aude J, Nitschké P, Chiapello H, Landés C, Risler J. Evolution of genes, evolution of species: the case of

aminoacyl-tRNA synthetases. Mol Biol Evol 1998;15(11):1548–61.
[72] Vishwanath P, Favaretto P, Hartman H, Mohr S, Smith T. Ribosomal protein-sequence block structure suggest complex prokaryotic evolution with implications for the origin of eukaryotes. Mol Phylogenet Evol 2004;33:615–25.
[73] Laalami S, Grentzmann G, Bremaud L, Cenatiempo Y. Messenger RNA translation in prokaryotes: GTPase centers associated with translational factors. Biochimie 1996;78:577–89.
[74] Göthel S, Schmid R, Wipat A, Carter N, Emmerson P, Harwood C, et al. An internal FK506-binding domain is the catalytic core of the prolyl isomerase activity associated with the *Bacillus subtilis* trigger factor. Eur J Biochem 1997;244:59–65.
[75] Maier R, Eckert B, Scholz C, Lilie H, Schmid F. Interaction of trigger factor with the ribosome. J Mol Biol 2001;314:1181–90.
[76] Teter S, Houry W, Ang D, Tradler T, Rockabrand D, Fischer G, et al. Polypeptide flux through bacterial Hsp 70: DnaK cooperates with trigger factor in chaperoning nascent chains. Cell 1999;97:755–65.
[77] De angelis M, Gobbetti M. Environmental stress responses in *Lactobacillus*: a review. Proteomics 2004;4:106–22.
[78] Sugimoto S, Higashi C, Yoshida H, Sonomoto K. Construction of *Escherichia coli dnaK*-deletion infected by ΛDE3 for over-expression and purification of recombinant GrpE proteins. Protein Expr Purif 2008;60:31–6.
[79] Bahl H, Muller H, Behrens S, Joseph H, Narberhaus F. Expression of heat shock genes in *Clostridium acetobutylicum*. FEMS Microbiol Rev 1995;17:341–8.
[80] Sarvas M, Colin H, Bron S, Dijl J. Post-translocational folding of secretory proteins in Gram-positive bacteria. Biochim Biophys Acta 2004;1694:311–27.
[81] Zhang X, He K, Duan Z, Zhou J, Yu Z, Ni Y, et al. Identification and characterization of inosine 5-monophosphate dehydrogenase in *Streptococcus suis* type 2. Microb Pathog 2009;47:267–73.
[82] McLean J, Hamaguchi N, Belenky P, Mortimer S, Stanton M, Hedstrom L. Inosine 5-monophosphate dehydrogenase binds nucleic acids *in vitro* and *in vivo*. Biochemical 2004;379:243–51.
[83] Jordan P, Tang Y, Bradbury A, Thomson A, Guest J. Biochemical and spectroscopic characterization of *Escherichia coli* aconitases (AcnA and AcnB). Biochem J 1999;344(3):739–46.
[84] Baumbargt M, Bott M. Biochemical characterisation of aconitase from *Corynebacterium glutamicum*. J Biotechnol 2011;154:163–70.
[85] Tsuchiya D, Shimizu N, Tomita M. Cooperativity of two active sites in bacterial homodimeric acotinases. Biochem Biophys Res Commun 2008;379:485–8.