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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*

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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).

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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-