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SHORT COMMUNICATION

Shotgun proteomic monitoring of *Clostridium acetobutylicum* during stationary phase of butanol fermentation using xylose and comparison with the exponential phase

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Abstract Economically viable production of solvents through acetone–butanol–ethanol (ABE) fermentation requires a detailed understanding of *Clostridium acetobutylicum*. This study focuses on the proteomic profiling of *C. acetobutylicum* ATCC 824 from the stationary phase of ABE fermentation using xylose and compares with the exponential growth by shotgun proteomics approach. Comparative proteomic analysis revealed 22.9% of the *C. acetobutylicum* genome and 18.6% was found to be common in both exponential and stationary phases. The proteomic profile of *C. acetobutylicum* changed during the ABE fermentation such that 17 proteins were significantly differentially expressed between the two phases. Specifically, the expression of five proteins namely, CAC2873, CAP0164, CAP0165, CAC3298, and CAC1742 involved in the solvent production pathway were found to be significantly lower in the stationary phase compared to the exponential growth. Similarly, the expression of fucose isomerase (CAC2610), xylulose kinase (CAC2612), and a putative uncharacterized protein (CAC2611) involved in the xylose utilization pathway were also significantly lower in the stationary phase. These findings provide an insight into the metabolic behavior of *C. acetobutylicum* between different phases of ABE fermentation using xylose.

Keywords Butanol · ABE fermentation · *Clostridium acetobutylicum* · Shotgun proteomics · Xylose

Introduction

Clostridium acetobutylicum is a gram positive, spore forming, strictly anaerobic bacterium that produces butanol, acetone, and ethanol from various carbohydrates through acetone–butanol–ethanol (ABE) fermentation process [12]. Although *C. acetobutylicum* is capable of degrading most of the sugars present in the plant biomass, hexoses and especially glucose is the most preferred substrate over pentose such as xylose [27]. Glucose is the most abundant sugar and xylose is the second most abundant sugar of lignocellulose found in the plant cell wall [6]. The bioconversion of these two sugars into solvents is essential for efficient and economic butanol production [34]. During ABE fermentation, glucose is metabolized through glycolysis and xylose via pentose phosphate pathway (PPP) [14] and the *C. acetobutylicum* cells undergo an acidogenic phase producing butyrate and acetate in the exponential growth phase and then switch to solventogenic phase to produce ABE solvents at start of the stationary phase [35]. The efficiency of *C. acetobutylicum* in xylose utilization

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and solvent yields were significantly lower when compared to glucose [20], suggesting the need for an in-depth investigation on this organism. The genome sequence of *C. acetobutylicum* ATCC 824 strain was published [19] followed by the proteomic studies [13, 28, 31]. Moreover, the proteome reference map of *C. acetobutylicum* DSM 1731 strain using glucose substrate was reported [17]. Recently, we published the comparative shotgun proteomic analysis of *C. acetobutylicum* from butanol fermentation using glucose and xylose substrates [29]. However, no proteomic studies have been done so far to monitor the metabolic behavior of this model organism during its growth on xylose substrate, which constituted the aim of this study. In this study, we have identified the *C. acetobutylicum* proteins from the stationary phase of ABE fermentation using xylose and compared with proteins identified from the exponential phase which was recently published [29] to understand the metabolic behavior of this bacterium during growth.

Materials and methods

Strain and fermentation development

Clostridium acetobutylicum ATCC 824 was obtained from American Type Culture Collection (ATCC, Cedarlane Labs, ON, Canada) and was cultured using reinforced clostridial medium (RCM) [11] in an anaerobic chamber (Coy Laboratory Products Inc., MI, USA) at 37°C for 20–24 h. Shake flask fermentation of *C. acetobutylicum* was performed in a 250-ml anaerobic flask containing 100 ml of media consisting of (g/l) yeast extract (5.0), ammonium acetate (2.0), sodium chloride (1.0), KH_2PO_4 (0.75), K_2HPO_4 (0.75), cysteine $HCl{\cdot}H_2O$ (0.50), $MgSO_4$ (0.2), $MnSO_4{\cdot}H_2O$ (0.01), $FeSO_4{\cdot}7H_2O$ (0.01) and xylose (30.0) [25]. Before inoculation, the medium was autoclaved at 121°C for 15 min (cysteine $HCl{\cdot}H_2O$ was filter-sterilized through a 0.45-µm filter and added to the medium) and cooled to 35°C in an anaerobic chamber. The cell suspension was incubated at 37°C with shaking at 120 rpm and the growth was monitored with $OD_{600\ nm}$. Samples of 10 ml were harvested from the start of the inoculation in the fermentation experiment until the stationary phase for further proteomic analysis. All chemicals used in this study were supplied from Fisher (Fisher Scientific, Canada) and Sigma (Sigma-Aldrich, Canada), unless otherwise specified.

Cell lysis and protein extraction

The microbial cell pellets (~100 mg wet mass) from fermentation broth were processed through single tube whole cell lysis and protein digestion. Briefly, the cell pellet was resuspended in 1,000 µl of 6 M guanidine/10 mM dithiothreitol (DTT) with 50 mM Tris/10 mM $CaCl_2$ at pH 7.6 by vortexing every 10 min for the first hour and incubated at 37°C for 12 h to lyse cells and extrude proteins. The guanidine concentration was diluted with sixfold 50 mM Tris buffer/10 mM $CaCl_2$ and 5–10 µg sequencing grade trypsin (Promega, WI, USA) was added and incubated at 37°C for 12 h to digest proteins to peptides. A second aliquot of the same amount of sequencing grade trypsin was added and incubated at 37°C for another 6 h to ensure the digestion process. Then, 1 M DTT was added to a final concentration of 20 mM and incubated for another hour with gentle rocking at 37°C. The complex peptide solution was centrifuged at 10,000×*g* for 10 min to remove cellular debris and the supernatant was collected and cleaned using Sep-Pak plus (Waters Limited, ON, Canada). Using a Savant SpeedVac (Thermo Electron Corporation, Waltham, MA, USA), samples were concentrated to ~200 µl. For each LC–MS/MS analysis below, ~1/4 of the total sample was used based on the protocol followed by Verberkmoes [33].

Mass spectrometry

Samples were analyzed in technical duplicates through a 2-D nano-LC MS/MS system with a split-phase column [32] (~3–5 cm SCX and 3–5 cm C18) (Polymicro Technologies, AZ) on a LTQ (ThermoFisher Scientific, Fremont, CA, USA) with 22-h runs [16, 26]. The LTQ settings were as follows: all data-dependent MS/MS in LTQ (top five), two microscans for both full and MS/MS scans, centroid data for all scans and two microscans averaged for each spectrum, dynamic exclusion set at 1.

Proteome informatics

All MS/MS spectra were searched with the SEQUEST algorithm [4] against *C. acetobutylicum* Uniprot proteome databases [2] and filtered with DTASelect/Contrast [32] at the peptide level [Xcorrs of at least 1.8 (+1), 2.5 (+2), 3.5 (+3)]. Only proteins identified with two fully tryptic peptides from a 22-h run were considered for further biological study. An in-house script was used to extract protein identifications, peptides, spectra, and sequence coverage from DTASelect filtered output files and used in calculation of protein abundance determination.

Results and discussion

Our results present the first large-scale investigation of the *C. acetobutylicum* proteome during the stationary phase of ABE fermentation grown on xylose by shotgun proteomics

approach. This shotgun approach enabled us to detect proteins by matching peptide mass data to available genome sequence databases. All proteins in the non-redundant Uniprot proteome database (http://www.uniprot.org) using the keyword "*C. acetobutylicum*" that could match with the same set of peptides were included in the protein list. A total of 686 and 985 proteins were identified in these first and second mass spectrometry (MS) runs, respectively (Supplementary Tables 1 and 2). Very few proteomic studies have been conducted in *C. acetobutylicum* using one- and/or two-dimensional gel electrophoresis–mass spectrometry (1D/2D-GE–MS) technique. Besides, most of them were from *C. acetobutylicum* grown on glucose substrate and focused specifically on proteins involved in acidogenic and solventogenic pathways [28, 31]. A total of 564 proteins identified from the exponential growth of glucose utilized *C. acetobutylicum* DSM1731 strain were used to publish the proteome reference map [17]. Proteomic study on steady-state cells of glucose utilized *C. acetobutylicum* in chemostat culture reported 178 proteins from acidognesis and 205 proteins from solventogenesis using a gel-based mass spectrometry approach [13]. Recently, we published a comparative proteomic analysis of *C. acetobutylicum* from the exponential phase of ABE fermentation between glucose and xylose substrate [29]. In this short communication, we are presenting the gel-free shotgun-based whole proteome investigation of *C. acetobutylicum* ATCC 824 from the stationary phase of xylose utilized ABE fermentation that could potentially be used to understand this organism in-depth.

The overall false discovery rate (FDR) was estimated by doubling the number of peptides found from the reverse database and dividing the result by the total number of identified peptides from both real and reverse databases using the formula: $\%\text{fal} = 2[n_{\text{rev}}/(n_{\text{rev}} + n_{\text{real}})] \times 100$ where %fal is the estimated false discovery rate, n_{rev} is the number of peptides identified from the reverse database and n_{real} is the number of peptides identified from the real database [18, 22]. The FDR was calculated as 0.7 and 2.1% for the first and second MS runs, respectively. The relative abundances of the proteins identified during the MS analysis were estimated by calculating the normalized spectral abundance factors (NSAF). The NSAF for a protein is the number of spectral counts (SpC, the total number of MS/MS spectra) identifying a protein, divided by the protein's length (*L*), divided by the sum of SpC/L for all proteins in the experiment [5, 36]. The entire lists of proteins were sorted by averaged NSAF across the sample and its technical run. Comparing the NSAF data from the sample and its technical run resulted in highly reproducible data; R^2 values of 0.82 (Fig. 1). The mean standard deviation of all the identified proteins was found to be 0.16%. Based on the prediction of NSAF values, five most abundant proteins found to be present in *C. acetobutylicum* during the stationary phase of xylose utilized ABE fermentation process includes 10-kDa chaperonin, phophocarrier protein, 18-kDa heat shock protein, 60-kDa chaperonin, and regulators of stationary/sporulation gene expression (*abrB*) *B. subtilis* ortholog protein (CAC0310).

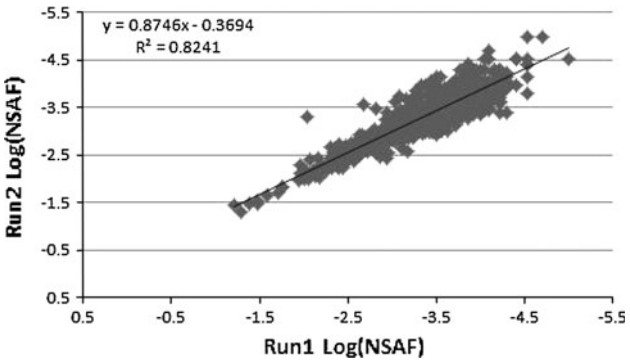

Fig. 1 Comparison of NSAF values. Stationary phase during ABE fermentation using xylose substrate, run 1 and run 2 NSAF values are plotted on a log scale. The *solid squares* represent individual proteins identified in the MS runs

Comparative proteomic analysis

The proteins identified in this study during the stationary phase of xylose utilized ABE fermentation were compared with our recently published study, which reported the identification of proteins during the exponential phase of xylose utilized ABE fermentation [29]. In this comparative analysis, a total of 882 proteins, which accounts for 22.9% of the predicted 3,848 ORFs in the *C. acetobutylicum* genome [15] were found to be present in *C. acetobutylicum* from ABE fermentation. A total of 716 proteins that covers 18.6% of the *C. acetobutylicum* genome were found to be commonly present during both the exponential and stationary phases. The number of proteins that were identified only during the exponential phase was found to be 109 and 57 proteins were unique to the stationary phase of ABE fermentation (Fig. 2).

Comparison of relative protein abundance based on the NSAF values showed that the most abundant proteins from both exponential and stationary phases are similar except stationary/sporulation gene expression regulator (*abrB*) *B. subtilis* ortholog protein (CAC0310). This CAC0310 protein is a transitional-stage gene regulator and is antagonistic to *Spo0A*, which is the master regulator of stationary-phase gene expression and sporulation in clostridia [1]. Stationary/sporulation gene expression regulator (*abrB*) *B. subtilis* ortholog protein (CAC0310), which is one of the five most abundant proteins identified from the stationary phase of xylose utilized ABE fermentation was found to be

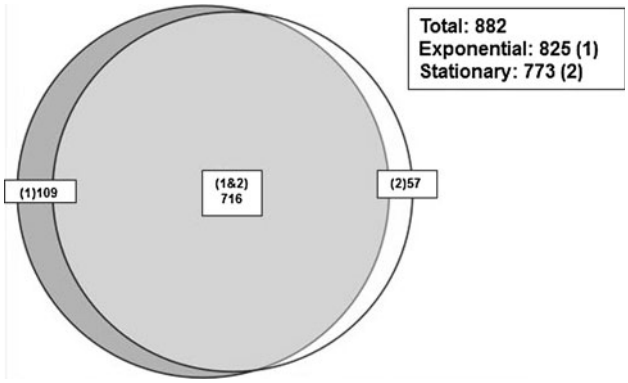

Fig. 2 Venn diagram of the proteins identified in *C. acetobutylicum* during exponential and stationary phase of ABE fermentation using xylose

less abundant in the exponential growth. Contrarily, proteomic analysis of *C. acetobutylicum* using glucose substrate showed that Spo0A protein is constitutively abundant from exponential to stationary phase [31].

To better understand the metabolic behavior of *C. acetobutylicum* during ABE fermentation using xylose sugar, the proteins identified from the stationary phase were functionally classified and compared with the exponential phase counterpart. A total of 621 proteins out of the 882 proteins identified were assigned to 82 pathways which can be classified into 18 categories involved in *C. acetobutylicum* based on the KEGG annotation database [15]. These include 543 proteins that were found to be commonly present in both the phases, while 53 and 25 proteins were identified only in exponential growth and stationary phase, respectively (Supplementary Table 3). The commonly identified proteins assigned to various pathways include carbohydrate metabolism (147), translation (76), amino acid metabolism (64), nucleotide metabolism (50), metabolism of cofactors and vitamins (27), energy metabolism (25), lipid metabolism (23), membrane transport (20), replication and repair (19), and metabolism of other amino acids (17). The number of functionally classified proteins in exponential phase was found to be more than in the stationary phase. This could be attributed mainly to the identification of proteins involved in carbohydrate metabolism such as starch and sucrose metabolism, and amino acid metabolism during the exponential growth phase of *C. acetobutylicum* and missing as it reaches the stationary phase.

The comparative proteomic analysis also revealed that all of the enzymes except α-acetolactate decarboxylase, involved in acid and solvent formation pathway of ABE fermentation, were identified from both exponential and stationary phases. These include the acetolactate synthase, pyruvate-formate lyase, acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, bifunctional acetaldehyde-CoA/alcohol dehydrogenase, NADH-dependent butanol dehydrogenase, alcohol dehydrogenase, phosphate butyryltransferase, butyrate kinase, butyrate-acetoacetate CoA-transferase, acetoacetate decarboxylase, phosphotransacetylase, and acetate kinase. This is the first study to identify the comprehensive set of proteins involved in acid and solvent formation pathway at the proteome level of *C. acetobutylicum* during the stationary phase of the ABE fermentation using xylose substrate.

Differentially expressed proteins

Clostridium acetobutylicum proteins identified from the stationary phase of ABE fermentation were examined for their differential expression in comparison to the exponential phase using PatternLab software [3]. A TFold pairwise analysis of proteins identified from both phases was carried out to find the differentially expressed proteins based on the spectral counting method (Fig. 3). A total of 17 (blue-dots) proteins were found to be differentially expressed with an absolute fold change >2.5, which is the established fold change cut-off and p values <0.05 were considered as statistically significant. Out of these 17 significantly differentially expressed proteins, the expression levels of 15 proteins were found to be higher in the exponential phase and only two proteins, namely DNA-binding protein and a 50S ribosomal protein, were found to be higher in the stationary phase of ABE fermentation (Fig. 4). Proteins such as aldehyde-alcohol dehydrogenase, pyruvate decarboxylase, transketolase, predicted flavoprotein met the fold-change cut-off but cannot be claimed to be statistically different (green-dots). Furthermore, eight proteins (orange-dots) did not meet the fold-change cut-off but were indicated as statistically different and about 382 proteins (red-dots) did not satisfy the fold-change or the statistical cut-offs.

We have found that the expression of two proteins, namely L-fucose isomerase-related protein (CAC2610) and xylulose kinase (CAC2612) involved in xylose utilization pathway, were found to be significantly lower in the stationary phase of ABE fermentation when compared to the exponential growth. Similarly, other proteins involved in the xylose utilization pathway such as transketolase and transaldolase were also found to be lower in expression compared to the exponential phase. However, they did not meet the fold change or cannot be claimed to be significantly differently expressed. During ABE fermentation, xylose is metabolized via PPP into glycolysis and converted to acetyl CoA [9]. L-Fucose isomerase-related protein (CAC2610) that potentially catalyzes the isomerization from xylose to xylulose, followed by its phosphorylation by xylulose kinase (CAC2612), are the two primary steps

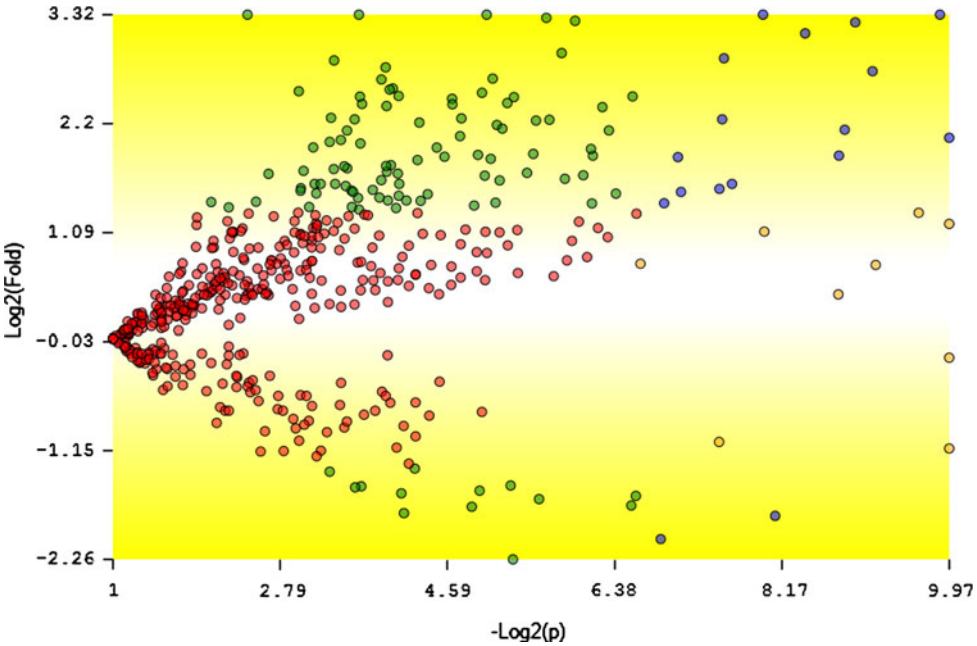

Fig. 3 TFold pairwise analysis of proteins identified between exponential and stationary phases of ABE fermentation using xylose substrate. Each protein is represented as a *dot* and is mapped according to its log2 (fold change) on the ordinate axis and its −log2 (*t* test *p* value) on the abscissa axis. *Blue-dot* proteins have *p* values of <0.05 and an absolute fold change >2.5, the established fold-change cut-off. *Orange-dot* proteins did not meet the fold-change cut-off but were indicated as statistically different. *Green-dot* proteins met the fold-change cut-off but cannot be claimed to be statistically different. *Red-dots* did not satisfy the fold-change or the statistical cut-offs (color figure online)

of xylose utilization in the PPP [7]. Decreased expression of CAC2610 and CAC2612 proteins may be due to the result of PPP being more active in the exponential growth phase compared to the stationary phase of ABE fermentation. In addition, the expression of a putative uncharacterized protein (CAC2611), which is a part of (CAC2610-CAC2612) operon in xylulose kinase as reported through a transcriptional study performed by Grimmler et al. [7], was also found to be significantly lower in the stationary phase.

Most noticeable differential expression of proteins between stationary and exponential phase of xylose utilized ABE fermentation were involved in the solvent production pathway of ABE fermentation process. This includes phosphate acetyltransferase, acetoacetate decarboxylase, acetyl CoA acetyl transferase, butyrate acetoacetate CoA transferase subunit-B, NADH-dependent butanol dehydrogenase-B. In parallel, the expression levels of butyrate acetoacetate CoA transferase subunit-A heterodimer and NADH-dependent butanol dehydrogenase-A homodimer were found to be lower in the stationary phase compared to the exponential phase. However, they did not meet the fold change or claimed to be significantly differentially expressed. Phosphate acetyltransferase (CAC1742) involved in the catalysis of acetyl CoA to acetyl phosphate leading to the production of acetate [10] and acetoacetate decarboxylase (CAP0165) [genes involved in the solvent formation resides on the plasmid and hence the prefix CAP] [19], which is essential for the solvent production that catalyzes the decarboxylation of acetoacetate to acetone [23], were found to be significantly lower in expression from the stationary phase in comparison to the exponential growth. In contrast, proteomic analysis of *C. acetobutylicum* using glucose reported that acetoacetate decarboxylase is up-regulated from exponential to stationary phase [31]. These findings were consistent with the protein-abundance analysis, which showed that (*abrB*) stationary/sporulation gene expression regulator protein (CAC0310) as the most abundant in stationary phase of xylose utilized ABE fermentation and Spo0A protein as the most abundant using glucose [31]. Moreover, these findings correlate well with the recent *C. acetobutylicum* genome expression analysis, which reported that the gene expression of *abrB* (CAC0310) was absent during the solventogenic growth itself [8].

Acetyl-CoA acetyl transferase (CAC2873), which catalyzes acetyl-CoA to acetoacetyl-CoA [21], is an important enzyme at the intersection in the pathway leading to the production of acids (acetate or butyrate) and solvents (acetone, butanol, ethanol) [30] was also significantly reduced in the stationary phase of xylose-utilized ABE fermentation. Furthermore, butyrate acetoacetate CoA transferase subunit B (CAP0164), involved in the solventogenic switch that allows *C. acetobutylicum* to uptake acids and produce solvents and the NADH-dependent butanol dehydrogenase B (CAC3298), which plays a key role in the production of butanol by converting butyraldehyde to butanol [24], were also found to be significantly lower in expression in the stationary phase compared to the exponential growth of ABE fermentation using xylose. On the other hand, transcriptional analysis of stationary-phase events in *C. acetobutylicum* using glucose reported that the expression of *thl* (CAC2873), *ctfB* (CAP0164), *bdhB* (CAC3298) genes increased continuously throughout the stationary phase [1]. This suggests that the metabolic

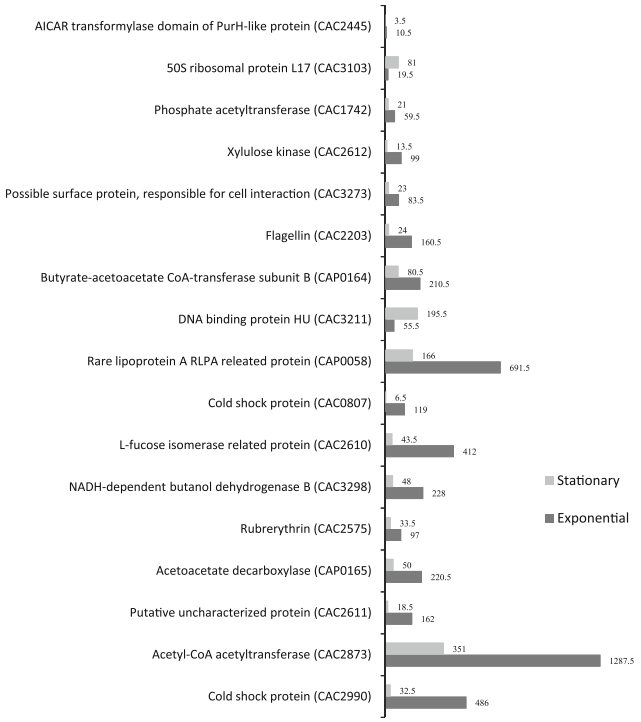

Fig. 4 Differentially expressed proteins identified between exponential and stationary phase of ABE fermentation using xylose substrate based on the spectral counting method with the spectral score shown at the end of each *bar*

behavior of *C. acetobutylicum* in terms of expression of proteins involved in the solvent production pathway is different during its growth between glucose and xylose substrates.

Overall, this study provided a global view of the *C. acetobutylicum* proteome during the stationary phase of ABE fermentation using xylose and the comparative analysis with its exponential phase provided evidence of how the proteomic profile changed during growth. The results presented here suggest that the expression of *C. acetobutylicum* proteins particularly involved in the solvent production is significantly lower in the stationary phase of xylose-utilized ABE fermentation when compared to the exponential phase and is contrary to the *C. acetobutylicum* grown on glucose substrate. We envision that this data serves as a base for future investigations in *C. acetobutylicum* and optimization of ABE fermentation. The varying nature of the proteomic profile of *C. acetobutylicum* during different phases of ABE fermentation should be further investigated. Specifically, the proteins involved in the production of acids and solvents in the ABE fermentation should be studied to complement the potential of genetic improvement of *C. acetobutylicum* leading to an economically viable solvent production process.

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