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ORIGINAL ARTICLE

Modified semi-continuous fermentation for resuscitating nongrowing cells during high-temperature gluconic acid production by *Acetobacter senegalensis*

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Keywords

acetic acid, co-feeding, gluconic acid, fermentation, NADH, oxidative stress, semi-continuous, viability.

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Abstract

Aims: The formation of metabolically inactive and nongrowing cells is an inevitable by-product of intensive fermentation. This study investigated whether co-feeding can be used to resuscitate nongrowing *Acetobacter senegalensis* cells to enable them to produce gluconic acid in successive fermentation runs at 38°C.

Methods and Results: In the first fermentation cycle, 75 g l^{-1} of glucose were converted to gluconic acid. Subsequently, however, stationary-phase cells were unable to initiate a new fermentation cycle. The majority of stationary-phase cells (97%) were nonculturable on glucose at 38°C. In addition, 54 and 41% of cells contained non-active cellular dehydrogenases and a compromised cell envelope respectively. Co-feeding stationary-phase cells with a mixture of ethanol, glucose and acetic acid for 7 h enabled these cells to grow on 75 g l^{-1} of glucose and produce gluconic acid. Additionally, 74% of cells contained active forms of cellular dehydrogenases after 7 h of co-feeding. However, co-feeding did not improve cell envelope integrity. Quantification of cellular NAD content showed that stationary-phase cells contained moderately reduced levels of total NAD (NADt) as compared with exponential-phase cells. Interestingly, the analysis of stationary-phase cells showed that co-feeding resulted in higher levels of NADt and NADH, suggesting that the regeneration of NADH is one of the limiting factors of glucose consumption. Expression of catalase and superoxide dismutase was increased in stationary-phase cells, but analysis of protein carbonylation and lipid peroxidation did not confirm an extensive oxidative stress.

Conclusions: Co-feeding with favourable nutrients may enable resuscitation of cells and utilization of less-favourable carbon sources in successive cycles.

Significance and Impact of the Study: This study proposed a unique method for resuscitation of nongrowing cells during high-temperature fermentation. By applying this method, cells can be used for consecutive fermentation cycles.

Introduction

Organic acids constitute a key group among the building block chemicals that can be produced by the microbial process. Because of their functional groups, organic acids are useful as starting materials for the chemical industry (Sauer *et al.* 2008). One example of such a chemical is gluconic acid, which is used in food, pharmaceutical and metal industry. High-grade gluconic acid is preferably produced by the microbial process (Rogers *et al.* 2013).

Most of the studies regarding gluconic acid production have been directed to proper micro-organism selection, bioprocess engineering and the development of the optimized bio-reactions. Generally, strain robustness to the

production environment is considered as one of the key characteristics of the successful microbial process (Cardona *et al.* 2007; Sauer *et al.* 2008). However, there are some problems in fermentation technology which cannot be simply solved by proper selection of micro-organism or metabolic engineering due to the exposure of biocatalyst to different kinds of stress (Alfenore *et al.* 2002; Saarela *et al.* 2004). Environmental stress such as nutrient depletion, osmotic pressure, pH and the presence of high concentrations of growth-inhibiting or toxic compounds can affect the cellular growth and survival (Bauer and Pretorius 2000). The failure of cells to adapt to stressful conditions during fermentation will lead either to cell death, reduced growth or to the formation of viable but non-culturable (VBNC) cells depending on the severity of the condition experienced (Bauer and Pretorius 2000; Saarela *et al.* 2004; Shafiei *et al.* 2013a).

Considering the economic constraint on an industrial scale, one approach for improving microbial viability and vitality (fermentative performance) is the use of thermo-tolerant micro-organisms which can maintain their performance in the face of temperature perturbation. However, because microbial environment undergoes rapid changes during fermentation, other strategies (such as different feeding strategy, use of different nutrients, and so on) are necessary for improving the viability of cells during fermentation. For example, in ethanol fermentation using *Saccharomyces cerevisiae*, final cell viability, ethanol titre and bioethanol production were improved significantly by vitamin feeding strategy (Alfenore *et al.* 2002; Frohman and Mira de Orduña 2013). Typical overflow metabolites such as acetic acid and lactic acid, can have adverse effects on cell viability, therefore by different feeding strategies, cell viability and productivity have been improved (Ito *et al.* 1991).

In the present study, *Acetobacter senegalensis* is used as a thermo-tolerant acetic acid bacterium isolated from mango fruit (Ndoye *et al.* 2007a). It was used for vinegar production and vinegar starter production (Ndoye *et al.* 2007b; Shafiei *et al.* 2013a; Shafiei *et al.* 2013b; Shafiei *et al.* 2014). In 2017, Shafiei and coworkers evaluated sodium gluconate production by *A. senegalensis* in the batch culture at 38°C; however, cells were not able neither to consume glucose nor to produce gluconic acid at the end of stationary phase (Shafiei *et al.* 2017). In addition, it was found that the cells sampled from stationary-phase exhibited improved growth on solid culture medium containing ethanol as the main carbon source (Shafiei *et al.* 2017).

Based on our previous findings (Shafiei *et al.* 2017), and the above-mentioned problems about cell viability change during fermentation, the main aim of the present study was to find a feeding strategy which enable nongrowing cells to resume growth on glucose, and produce gluconic acid in sequential fermentation cycles. Finally, based on single-cell analysis complemented with enzymatic activities and co-factor pool determination, we found physiological differences between cells growing in exponential-phase, in stationary-phase and resuscitated cells.

Materials and methods

Chemicals and enzymes

Hydrogen peroxide, bovine serum albumin (BSA), 2,6-dichlorophenolindophenol (DCPIP), phenazine methosulfate and propidium iodide (PI) were purchased from Sigma-Aldrich® (Darmstadt, Germany). Dimethyl sulfoxide was obtained from Fisher BioReagents® (Schwerte, Germany). All culture media components were purchased from Merck® (Darmstadt, Germany).

Microorganisms and culture media

Acetobacter senegalensis CWBI-B418 (LMG 23690T = DSM 18889T), a thermo-tolerant acetic acid bacterium isolated from mango fruit (Ndoye *et al.* 2007a), was used in this study. Three different culture media were used for culturing and enumerating *A. senegalensis*. GY agar (glucose-yeast extract) contained (per litre): 20 g glucose, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2PO_4$, 7·5 g yeast extract and 15 g agar. GYEA agar (glucose-yeast extract-ethanol-acetic acid) contained (per litre): 20 g glucose, 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2PO_4$, 7·5 g yeast extract and 15 g agar, which was supplemented with 3·5% (w/v) ethanol and 0·2 (w/v) acetic acid that were added aseptically to the culture media after sterilization.

Acetobacter senegalensis was grown on GYEA agar at 30°C. The colonies were then used for subsequent inoculations. The spread plate technique described previously by Shafiei et al. (Shafiei *et al.* 2013a) was used for indirect enumeration of bacteria grown on GYEA agar and GY agar. GY broth was used for preparing serial dilutions.

Preparation of inoculum

The inoculum was prepared by inoculating fresh colonies grown on GYEA agar into a 5-l baffled flask containing 850 ml of GYEA broth, which then incubated for up to 20 h at 30°C on a shaker (130 rev min^{-1}) until an OD_{540nm} of approximately 0·9 was reached.

Semi-continuous fermentation

Semi-continuous fermentation was performed in a 15-l laboratory-scale bioreactor (Bio Lafitte, Poissy, France), with a working volume of 10 l. GY broth was used for

production of gluconic acid in the bioreactor, which contained (per litre): 1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2PO_4$, 7·5 g yeast extract and 75 g glucose. The bioreactor was continuously sparged throughput the entire fermentation period with sterilized air at a flow rate of 1 vvm. A polarographic (Ingold) oxygen sensor was used to measure dissolved oxygen in the liquid phase. A setpoint of 30% was fixed based on the response of this probe. Two marine blade impellers (A315) were used to stir the bioreactor. An electrode pH meter and NaOH (1·5 N) and H_3PO_4 (1·5 N) were used to maintain the pH at 5·5 ± 0·1. The initial agitation speed was 150 rev min^{-1}. The culture medium was sterilized at 121°C for 35 min in the bioreactor. Glucose was separately sterilized and was added to the bioreactor after cooling. The prepared inoculum (850 ml) was inoculated into a 9·2-l of sterilized GY under aseptic conditions.

Following inoculation, two different fermentation modes were used. In conventional semi-continuous fermentation, once the glucose level dropped to 2 ± 0·5 g l^{-1}, 7·5 l of the culture volume (75% of the working volume) were removed and replaced with 7·5 l of fresh GY medium containing 100 g l^{-1} glucose. Fermentation was conducted at 38°C.

In modified semi-continuous fermentation, once the glucose concentration dropped to 1·5 ± 0·5 g l^{-1}, 7·5 l of culture medium (75% of working volume) were removed and 1 l of fresh GY medium containing 8·7% (w/v) ethanol and 1·05% (w/v) acetic acid was added to the remaining medium (2·5 l). In this step, the final volume of the medium was 3·5 l, and ethanol and acetic acid concentrations were 2·5% (w/v) and 0·3% (w/v) respectively. Additionally, the fermentation temperature decreased to 30 ± 0·5°C. After reaching an OD_{540nm} of approximately 4·5–5, 6·5 l of fresh GY medium containing 116 g l^{-1} of glucose were added to the bioreactor. The temperature increased to 38 ± 0·5°C again.

Bioreactor exhaust gas was cooled in a condenser (2°C) and dried prior to the analysis of carbon dioxide concentrations with a Rosemount NGA 2000 analyzer (Baar, Switzerland). The precise gas flow rate was determined using a Saga digital flow meter (Ion Science, Cambridge, UK).

Samples were taken for analysis of cell growth, glucose consumption and gluconic acid production. Data are presented as the average of three independent fermentation cycles.

Measurement of biomass and fermentation products

Biomass was estimated based on the conversion of optical density (OD_{540nm}) to cell dry weight. Fermentation samples were subjected to centrifugation at 13 000 ***g*** for 10 min. The supernatant was filtered (0·45 μm) and the concentration of glucose and gluconic acid in the supernatant was determined by HPLC (Agilent 1110 equipped with a Supelcogel C610H column), as described previously (Shafiei *et al.* 2013a; Shafiei *et al.* 2017).

Analysis of *in vitro* enzymatic activities

Preparation of cellular extract

At the end of exponential phase, stationary phase and co-feeding phase, cells were harvested by centrifugation (5000 ***g***, 4°C, 10 min). Pelleted cells were washed twice with 50 mmol l^{-1} potassium phosphate buffer (KPB) (pH 5·5) containing 100 mmol l^{-1} phenylmethylsulfonyl fluoride. Cells were resuspended in the same buffer to a wet biomass density of 70 mg ml^{-1}. Cell suspensions were maintained at 4°C, then passed twice through a French pressure cell press at 16 000 psi.

Disrupted cells were centrifuged at 5000 ***g*** for 10 min. to remove cell debris, and then the supernatant was centrifuged at 90 000 ***g*** for 60 min. at 4°C. During all operations, cellular extracts were maintained at 4°C (Ndoye *et al.* 2006). The prepared extracts were then used for the experiments described in the following sections.

Additionally, the protein concentration of the cell extract was determined by the Bradford colorimetric assay using BSA as the standard (Li and Schellhorn 2007).

PQQ-glucose dehydrogenase assay

PQQ-glucose dehydrogenase activity was measured photometrically at 38°C in a dye-linked system containing DCPIP and phenazine methosulfate (Sode *et al.* 2002; An and Moe 2016). Briefly, a 1-ml reaction mixture containing 50 mmol l^{-1} KPB (pH 5·5), 50 μmol l^{-1} DCPIP, 2 mmol l^{-1} PMS, 10 μmol l^{-1} PQQ, 0·5 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} NaN_3 and 50 μg protein (cell extract) was prepared. The reaction was initiated by the addition of 20 mmol l^{-1} glucose. DCPIP-dependent reduction kinetics were recorded with a spectrophotometer at 600 nm. Specific enzymatic activity was expressed in units per milligram of protein, where 1 U is defined as 1 μmol DCPIP reduced per minute (An and Moe 2016).

PQQ-ethanol dehydrogenase assay

The activity of ethanol dehydrogenase was determined photometrically at 38°C using a previously described procedure (Ndoye *et al.* 2006). One unit of enzymatic activity was defined as the number of enzymes catalysing the oxidation of one μmole of ethanol or the reduction of one μmole of ferrocyanide per minute under the operating conditions. Specific activity was expressed as units per milligram of protein.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity was measured and calculated using the SOD assay kit, as described by the manufacturer (Sigma-Aldrich, 19160). Xanthine-xanthine oxidase is used to generate superoxide anion ($O^{2\bullet-}$), and highly water-soluble tetrazolium salt (WST) is used as an indicator of $O^{2\bullet-}$ production. SOD competes with WST for $O^{2\bullet-}$. The percent inhibition of WST reduction indicates the amount of SOD that is present (Weydert and Cullen 2009). Specific activity was expressed as percent inhibition of WST reduction per minute per milligram of protein.

Catalase activity assay

Hydrogen peroxide, the substrate for catalase, was diluted to a final concentration of 20 in 50 mmol l^{-1} KPB (pH 5·5). Substrate solution (750 μl) was added to 50 μl of cell extract, and the decrease in absorbance at 240 nm was measured over time at 30°C. One unit of catalase activity was defined as a one unit decrease in the absorbance (240 nm) per minute (den Besten *et al.* 2013). Specific activity was expressed as units per milligram of protein.

Flow cytometric analysis

Total cellular dehydrogenase activity and cell envelope integrity was analysed by two different methods. Fluorochrome solutions were prepared as described by Shafiei *et al.* (2013a). Cells were harvested by centrifugation (5000 ***g***, 10 min), washed with KPB solution (50 mmol l^{-1}, pH 5·5), then re-suspended in fresh GY medium to a cell concentration of 5×10^7 cells per ml. Dehydrogenase activity was analysed by mixing 450 μl of cell suspension with 50 μl CTC, followed by incubation at 38°C for 120 min on a shaker. Then, 5 μl of diluted thiazole orange was added to each sample, and was incubated for 5 min at 38°C in darkness. To analyse cell envelope integrity, 500 μl of cell suspension was mixed with 5 μl of PI solution, and was incubated at 38°C for 5 min. Finally, the cells were washed with KPB (50 mmol l^{-1}, pH 5·5) and re-suspended in fresh KPB.

All experiments were performed using a BD FACSCalibur™ flow cytometer. Data were analysed by CellQuest™ Pro software (Becton Dickinson European HQ, Erembodegem, Belgium).

Quantification of NADH and total NAD (NADt)

Total NAD (NADt) and NADH levels in exponential phase cells, stationary phase cells and co-fed cells were measured by a NAD^+/NADH assay kit (Abcam, ab65348), according to the manufacturer's instructions. Briefly, 1×10^7 cells per ml were washed with cold 50 mmol l^{-1} KPB (pH 5·5), followed by extraction with NAD^+/NADH extraction buffer via two freeze/thaw cycles. Active enzymes were removed by filtering the samples through a 10 kD spin column. Total NADt and NADH were detected in a 96-well plate and the colour was read at 450 nm. Protein quantification assay was performed as described in previous section. The concentration of NADt or NADH was expressed as pmol μg^{-1} protein.

Quantification of carbonylated proteins

Carbonylated proteins were detected with an enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Antwerpen, Belgium, ALX-850-312-KI 01), according to the manufacturer's specifications. Briefly, cellular extracts were reacted with dinitrophenylhydrazine (DNP), and then the protein was nonspecifically adsorbed to an ELISA plate. Unconjugated DNP and non-protein constituents were removed by washing. Adsorbed protein was probed with a biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase.

Lipid peroxidation assay using TBARS assay

An assay for detecting thiobarbituric acid reactive substances (TBARS) was used to quantify malondialdehyde (MDA) in the sample, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. The MDA assay was performed as previously described (Devasagayam *et al.* 2003). Briefly, cells (1×10^7 cells per ml in KPB containing butylated hydroxytoluene) were sonicated on ice. Homogenated cells were heated with TBA reagent. After cooling, the solution was centrifuged at 2100 ***g*** for 10 min, and the absorbance of the supernatant was determined at 532 nm.

Results

Semi-continuous production of gluconic acid

During two fermentation cycles at 38 ± 0·5°C, semi-continuous production of gluconic acid by *A. senegalensis* was evaluated. Following completion of the first cycle (33rd hour), cells were unable to utilize glucose for growth and gluconic acid production in the second cycle (34th–60th hours) (Fig. 1a). In addition, a comparison of the number of culturable cells to the total number of cells in different growth phases (Table 1) showed that 44% of exponential-phase cells and the majority of stationary-phase cells (97%) were unable to grow on GY agar containing glucose as the main carbon source, demonstrating that the number of non-culturable cells increased with time. Therefore, a modified semi-continuous process was designed for optimizing glucose utilization and gluconic

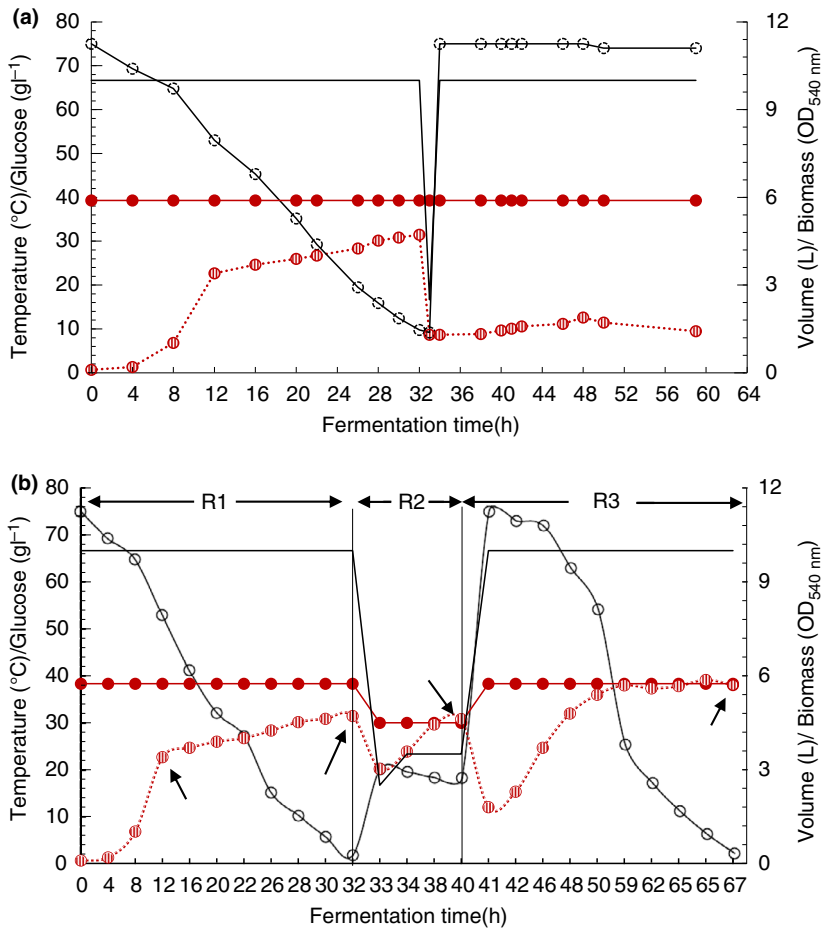

Figure 1 Gluconic acid production by *Acetobacter senegalensis* at high temperature (38 ± 0·5°C) using two different feeding strategies. The data represent three independent experiments that yielded similar results. (a) Conventional semi-continuous fermentation. After the first gluconic acid fermentation cycle (0–33rd hours), cells stopped consuming glucose (34th–60th hours). (b) Modified semi-continuous fermentation. A co-feeding fermentation cycle (R2, 33rd–40th hours) was performed between each gluconic acid fermentation cycle (R1 and R2). Fresh culture medium containing a mixture of ethanol, glucose and acetic acid was added to a portion of the cells. Then, the temperature was decreased to 30°C. This strategy prepared the cells for the second gluconic acid fermentation cycle (R3, 41st–59th hours). The arrows show the time intervals that samples were taken for further analyses. (—●—) temperature, (-⊙-) biomass (——) volume, (—⊙—) glucose. [Colour figure can be viewed at wileyonlinelibrary.com]

acid production by *A. senegalensis* during successive fermentation cycles (Fig. 1b).

Modified semi-continuous production of gluconic acid from glucose

Results of biomass production, glucose consumption, gluconate production and CO_2 production are shown in Fig. 2. During the initial 12 h of fermentation of the first batch, *A. senegalensis* achieved the maximum specific growth rate ($\mu_{max} = 0{\cdot}4\ h^{-1}$) and then continued to grow at a lower rate, reaching the maximum biomass of $2{\cdot}7\ g\ l^{-1}$. During the initial 10 h of the first fermentation cycle, only a small amount of glucose was consumed (approximately $7\ g\ l^{-1}$) and, consequently, a limited amount of gluconic acid was produced. The majority of gluconic acid ($50\ g\ l^{-1}$) was produced during the next 24 h, with a maximum specific production rate (q_p) of $1{\cdot}59\ h^{-1}$ within 12–16th hour, which gradually decreased towards the end of the cycle.

Following the first fermentation cycle (R1, 33rd hour), when the glucose concentration reached approximately $1–2\ g\ l^{-1}$, 1-l of fresh culture medium containing ethanol, glucose and acetic acid was added to a portion of the culture medium (2·5 l). As can be seen in Fig. 1(b), bacterial cells grew during 7 h and attained a final biomass of $2{\cdot}70\ g\ l^{-1}$ (OD_{540nm}: 4·7). At this stage of the fermentation (R2: co-feeding), ethanol was consumed by the cells as the preferred carbon source. However, a small amount of glucose was also consumed (Fig. 1b). In addition, concentration of acetic acid did not changed significantly during R2. Unexpectedly, as can be seen in Table 1, even after co-feeding cells with a mixture of ethanol, glucose and acetic acid (33rd–40th hours), only a small fraction of cells ($2{\cdot}36 \times 10^7$ cells per ml) was able to grow on GY agar containing glucose as the main carbon source at 38°C. In other words, 98% of the cells were unable to grow.

The second cycle (R3) of gluconic acid fermentation initiated at the 40th hour of fermentation by the addition of glucose to cells fed by a mixture of ethanol, glucose and acetic acid (Fig. 1b). Surprisingly, despite the low recovery rate of these cells on GY agar (Table 1), the cells grew well during the 41st–59th hours in a bioreactor

Table 1 Direct and indirect enumeration of *Acetobacter senegalensis* cells in different fermentation cycles

	Direct count[a]	Indirect count[b] on GY agar	Indirect count[b] on GYEA agar
Exponential-phase cells of first cycle (R1)[c]	$1{\cdot}63 \times 10^9$ ($\pm 2{\cdot}20 \times 10^8$)	$9{\cdot}1 \times 10^8$ ($\pm 1{\cdot}71 \times 10^8$)	$1{\cdot}28 \times 10^9$ ($\pm 2{\cdot}98 \times 10^8$)
Stationary-phase cells of first cycle (R1)[c]	$2{\cdot}22 \times 10^9$ ($\pm 1{\cdot}77 \times 10^8$)	$5{\cdot}81 \times 10^7$ ($\pm 7{\cdot}21 \times 10^6$)	$5{\cdot}92 \times 10^8$ ($\pm 4{\cdot}35 \times 10^7$)
Co-feeding cycle (R2)[c]	$1{\cdot}85 \times 10^9$ ($\pm 3{\cdot}27 \times 10^8$)	$2{\cdot}36 \times 10^7$ ($\pm 1{\cdot}78 \times 10^6$)	$5{\cdot}35 \times 10^8$ ($\pm 5{\cdot}16 \times 10^7$)

[a]Direct count means enumeration of cell by microscope.
[b]Indirect count means enumeration of colony forming unites on solid culture media incubated at 38°C. GY agar contained glucose as the main carbon source, whereas GYEA agar was supplemented with ethanol as the main carbon source.
[c]The sampling points are shown in Fig. 1(b).

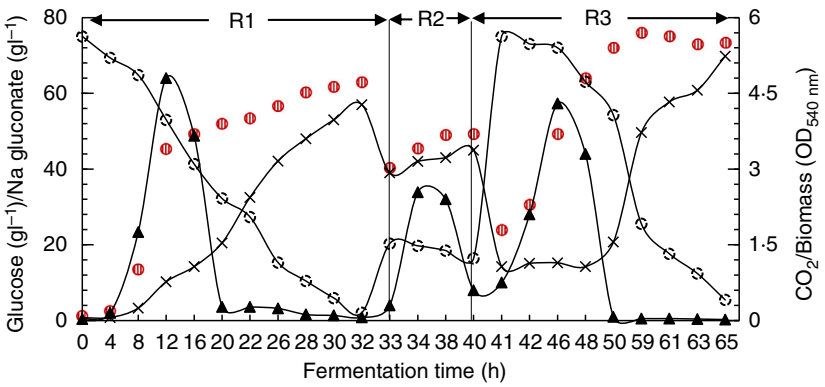

Figure 2 Comparison of CO_2 production, gluconate production and glucose consumption by *Acetobacter senegalensis* during different fermentation cycles (R1, R2 and R3). This figure is representative of three independent experiments that yielded similar results. Fermentation was performed in a 15-l bio-reactor with an initial glucose concentration of 75 g l^{-1}. CO_2 production increased during exponential phase (4th–12th, 33rd–38th and 41st–48th hours) and then sharply deviated from the peak starting at stationary phase. (—○—) glucose, (—▲—) CO_2, (—×—) sodium gluconate, (- -○- -) biomass. [Colour figure can be viewed at wileyonlinelibrary.com]

(Fig. 1b) and reached a biomass of 3·34 g l^{-1} (OD_{540nm}: 5·7). Noticeably, 28% of cells fed by a mixture of ethanol, glucose and acetic acid were able to grow at 38°C on GYEA agar containing a mixture of ethanol, glucose and acetic acid (Table 1). During the second gluconic acid fermentation cycle (R3), gluconic acid was continuously produced, with a slight decrease in the specific production rate (1·25 h^{-1}) as compared with the first cycle (R1). Consequently, the total duration for converting glucose to gluconic acid was slightly extended. The same yields ($Y_{p/s}$) of gluconic acid (>75%) were observed in all fermentation cycles. Moreover, in the first cycle, 1·25 g $l^{-1}$of 2-keto gluconate were produced as a by-product. In subsequent cycles, 2-keto gluconate accumulated to a final concentration of 2·55 g l^{-1}.

The CO_2 production profile of cells during growth and production phases indicated that CO_2 was mainly produced during the exponential phase. As can be seen in Fig. 2, a significant amount of CO_2 was produced when cells grew either on glucose (R1 and R3) or a mixture of ethanol, glucose and acetic acid (R2). Cells produced the majority of gluconate during stationary phase, whereas CO_2 production decreased substantially during stationary phase.

Physiological heterogeneity in the cell population during gluconic acid production

To investigate the factors that influence gluconic acid production during the modified semi-continuous process, activities of PQQ-glucose-dehydrogenases (PQQ-GDH) and PQQ-ethanol-dehydrogenase (PQQ-ADH) of *A. senegalensis* were determined during different fermentation phases. As shown in Fig. 3(a), the highest specific activity of PQQ-GDH was observed during stationary phase (1·53 μmole min^{-1} mg $protein^{-1}$). PQQ-GDH activity was approximately two-times lower (0·85 μmole min^{-1} mg $protein^{-1}$) during exponential phase as compared with stationary phase. Despite the availability of ethanol, PQQ-GDH exhibited higher activity during the co-feeding phase (Fig. 3a) as compared with the exponential phase. Quantification of PQQ-ADH in different phases (Fig. 3b) revealed that the activity of PQQ-ADH was significantly ($P < 0{\cdot}05$) higher during the exponential phase and co-feeding phase as compared to the stationary phase.

The percentage of CTC reducing cells, as an indication of total cellular dehydrogenases, was determined by flow

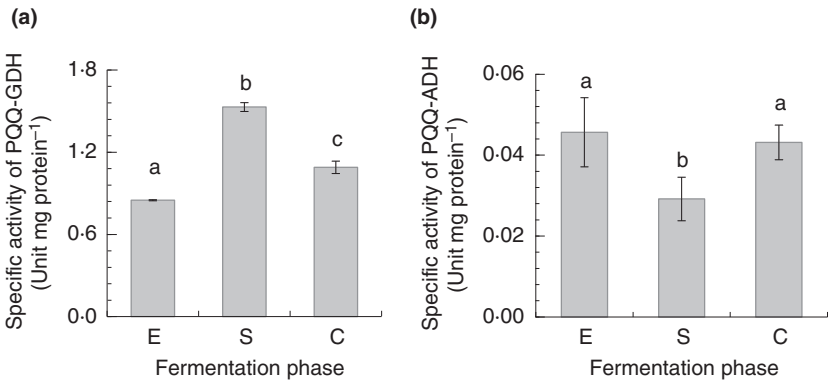

Figure 3 Specific activity of PQQ-glucose dehydrogenase (a) and PQQ-ethanol dehydrogenase (b) of *Acetobacter senegalensis* in three different phases of fermentation (E: exponential phase, S: stationary phase, and C: co-feeding phase). Error bars represent mean ± standard deviation. Sampling points are shown in Fig 1b by arrows. Mean values followed by the different letters indicate statistically significant differences ($P < 0{\cdot}05$).

cytometry. As shown in Fig. 4, 73% of exponential-phase cells were able to reduce CTC, indicating that cellular dehydrogenases were active in the majority of these cells. In contrast, only 46% of cells were capable of reducing CTC at the end of stationary phase (Fig. 4b). In other words, more than half of the cells (54%) contained inactive dehydrogenases. As previously mentioned, stationary-phase cells were unable to carry out additional glucose oxidation, even if fresh culture medium was provided.

Heterogeneity of the cell population based on cell envelope integrity during exponential, stationary, and co-feeding phases was quantified by flow cytometry using PI. It was observed that the majority of cells (92%) maintained their cell envelop integrity during exponential phase (Fig. 5a). However, 41% of stationary-phase cells had compromised cell membranes (Fig. 5b).

Interestingly, the addition of a mixture of ethanol, glucose and acetic acid (co-feeding phase) had a significant influence on the distribution of subpopulations (Fig. 4c). Co-feeding significantly increased the number of CTC-reducing cells, indicating that a mixture of ethanol, glucose and acetic acid improved cellular metabolism that resulted in ethanol oxidation and cell proliferation (Fig. 2). Unexpectedly, co-feeding cells with a mixture of ethanol, glucose and acetic acid did not decrease the percentage of cells with compromised membranes (Fig. 5c).

Total NAD (NADt) and NADH were quantified in cell extracts. As shown in Fig. 6, cells contained the highest amounts of NADt and NADH in exponential phase. Although NADt decreased moderately in stationary phase cells compared to exponential phase cells, NADH showed a threefold decrease. Remarkably, the NADH content of cells increased significantly by co-feeding cells with ethanol. These results suggest that *A. senegalensis* grown on glucose cannot regenerate the NADH pool. In contrast, cells fed with a mixture of ethanol, glucose and acetic acid are able to produce a higher concentration of NADH and regenerate NAD^+ to NADH.

Oxidative damage to protein and lipid

To evaluate whether variations in cell dehydrogenases and cell envelope integrity in the cell population during different fermentation phases could be due to oxidative damage, the activities of scavenger enzymes and oxidation of proteins and total cellular lipids were determined. For

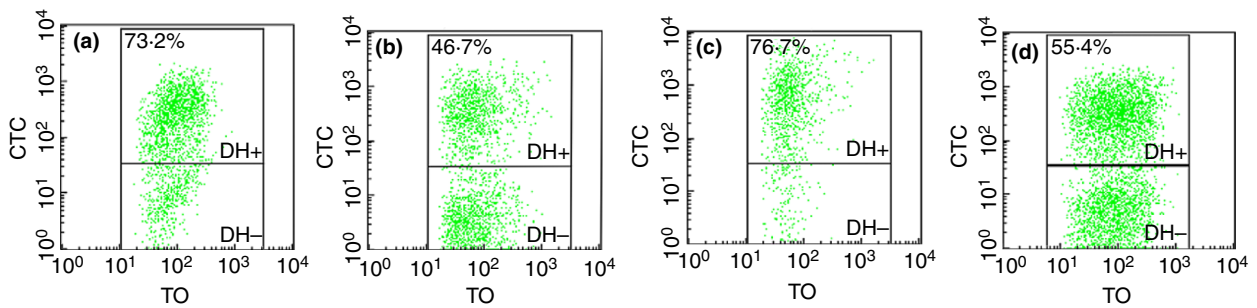

Figure 4 Flow cytometric analysis of total cellular dehydrogenases of *Acetobacter senegalensis* during different fermentation phases. Two different subpopulations were discriminated, based on reduction of CTC by cellular dehydrogenases. The distribution of the two different subpopulations in exponential phase (a), at the end of stationary phase (b), at the end of co-feeding phase (c), and at the end of stationary phase (d) is shown. Sampling points are shown in Fig. 1(b) by arrows. Each analysis was performed at least three times. [Colour figure can be viewed at wile yonlinelibrary.com]

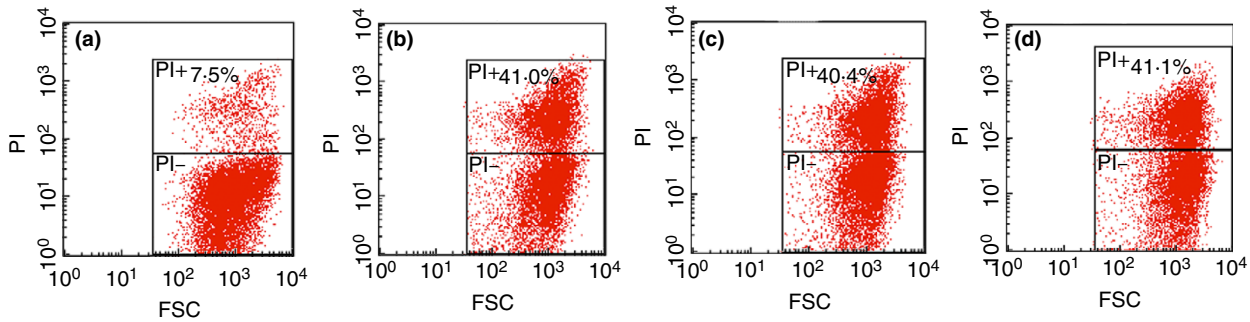

Figure 5 Flow cytometric analysis of cell envelope integrity of *Acetobacter senegalensis* during different fermentation phases. Two different subpopulations were discriminated based on PI uptake. Distribution of the two different subpopulations are shown in exponential phase (a), at the end of stationary phase (b), at the end of co-feeding phase (c), and at the end of stationary phase (d). Sampling points are shown in Fig. 1(b) by arrows. Each analysis was conducted at least three times. [Colour figure can be viewed at wileyonlinelibrary.com]

this, the activities of catalase and SOD of cells during different fermentation phases were measured, then carbonylation of protein and lipid peroxidation as indicators of oxidative damage were determined.

Catalase exhibited minimum and maximum activity during exponential and stationary phases, respectively. As shown in Fig. 7A, catalase activity of co-feeding phase cells was significantly higher than exponential phase cells, which may indicate a cellular response against the stimulus. Similarly, SOD activity was higher in stationary phase cells and co-feeding phase cells than in exponential phase cells. However, unlike catalase activity, there was no significant difference between SOD activity in stationary phase cells and exponential phase cells (Fig. 7B).

Since carbonylation of proteins can indicate oxidative damage, we analysed carbonylation of total cellular proteins by ELISA. As shown in Fig. 8, there was no significant difference in total cellular carbonylated proteins between different growth phases. Analysis of lipid peroxidation by the TBARS method showed that the amounts of MDA were similar and low (concentration close to zero) for the different fermentation phases, indicating that no lipid peroxidation occurred during the process.

Discussion

The physiological data obtained in this study made it possible to design a modified semi-continuous fermentation process that enabled cells to consume glucose during different fermentation cycles. It should be mentioned that although *A. senegalensis* can produce gluconic acid in a batch culture, stationary-phase cells neither grow on glucose nor produce gluconic acid in subsequent fermentation cycles (Fig. 1a, Table 1). These results are in agreement with the results of our previous study (Shafiei *et al.* 2017).

To understand the reasons for this progressive reduction in the rate of glucose oxidation to gluconate during stationary phase, we evaluated cell viability (by CTC and PI), the activity of certain cellular enzymes, and the

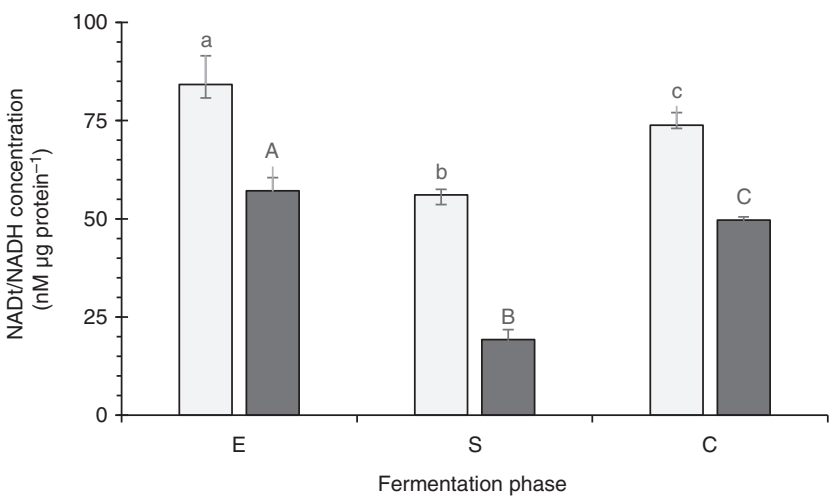

Figure 6 Quantification of total NAD (NADt) and NADH content of *Acetobacter senegalensis* at different fermentation phases. NADt concentration (white columns) or NADH concentration (black columns) (in µmol l^{-1}) were normalized to the protein concentration of the extracts (in µmol l^{-1}). Results represent the average of three independent experiments, each conducted in duplicate. Error bars represent mean ± standard deviation. Mean values followed by the different letters indicate statistically significant ($P < 0{\cdot}05$) differences in NADt or NADH concentrations.

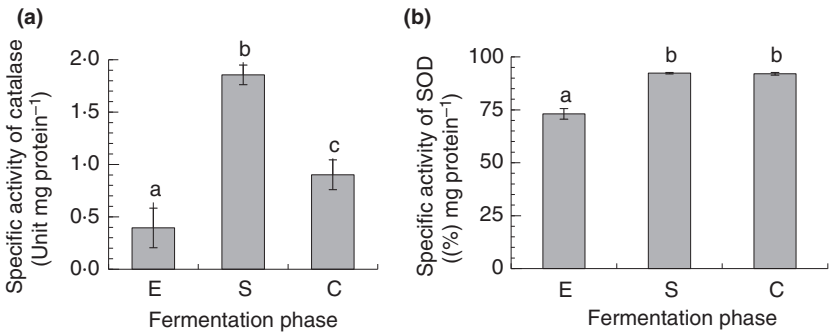

Figure 7 Catalase activity (a) and superoxide dismutase (SOD) activity (b) of *Acetobacter senegalensis* cells during different fermentation phases. E: exponential-phase cells, S: stationary-phase cells, and C: co-feeding-phase cells. One unit of catalase activity was defined as a one-unit decrease in absorbance (240 nm) per minute. Specific activity was expressed as units per milligram of protein. Specific activity of SOD was expressed as percent inhibition of WST reduction per minute per milligram of protein. Sampling points are shown in Fig. 1(b) by arrows. Error bars represent mean ± standard deviation. Mean values followed by the different letters indicate statistically significant ($P < 0{\cdot}05$) differences.

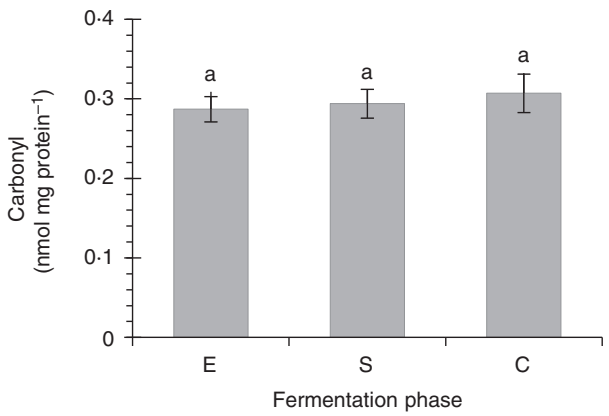

Figure 8 Quantification of carbonylated proteins of *Acetobacter senegalensis* cells during different fermentation phases. E: exponential-phase cells, S: stationary-phase cells, and C: co-feeding-phase cells. Sampling points are shown in Fig. 1(b) by arrows. Error bars represent mean ± standard deviation. Mean values followed by the different letters indicate statistically significant ($P < 0{\cdot}05$) differences.

potential occurrence of lipid peroxidation and protein carbonylation during semi-continuous fermentation.

The cell membrane of Gram-negative bacteria contains a large number of proteins and is the location of several important functions, such as nutrient acquisition, signalling and protection from the environment (Kulp and Kuehn 2010). The integrity of the cell envelope depends on environmental conditions and also on cell physiological status (Bonomo *et al.* 2013). Disruption of any of the cell envelope layers by stressors results in the collapse of cellular energetics and active transport that can be lethal to cells (Grégori *et al.* 2001). In the present study, we observed that the oxygen requirement of cells increased during exponential phase due to rapid growth of the cells. Since the oxygen flow rate was held constant at 1 vvm, agitation continuously increased to compensate for increased oxygen demand. To decrease shear stress, A340 impellers were used that impart lower stress on bacteria. We assessed bacterial damage during exponential phase by checking cell envelope integrity. In exponential phase, cell membrane integrity of the majority of the cells (>90%) was not compromised, indicating that mechanical stress did not affect the process.

In addition, an increase in membrane permeability during stationary phase is hypothesized to be a consequence of oxidative damage to cell structures, which causes loss of dehydrogenases positive cells and a lower production rate of gluconic acid. The biological targets of oxidative damage mainly resulting from the highly reactive hydroxyl radical (·OH) (Cabiscol *et al.* 2000; Landolfo *et al.* 2008) are DNA, RNA, proteins and lipids. However, despite the observed higher activity of antioxidant defence enzymes (Fig. 7) during stationary phase, the results of protein carbonylation (Fig. 8) and lipid peroxidation did not support the hypothesis of cellular damage by reactive oxygen species (ROS). Carbonylated protein content is the most general indicator and most commonly used marker of protein oxidation (Dalle-Donne *et al.* 2003a; Dalle-Donne *et al.* 2003b; Dalle-Donne *et al.* 2006; Suzuki *et al.* 2010), thus it can be deduced that the production of ROS did not prevail over the cellular defence systems.

During stationary phase, although the total number of cells was nearly constant, a gradual decrease of CTC-reducing cells was observed and, subsequently, the number of culturable cells and the amount of released CO_2 also decreased. Based on previous studies, the AAB respiratory chain consists of several periplasmic dehydrogenases, some cytoplasmic dehydrogenases, ubiquinone, and at least two sets of ubiquinol oxidases. Periplasmic dehydrogenases (pyrroloquinoline quinone-dependent dehydrogenase) catalyse the oxidation of

various substrates to provide the primary source of energy, whereas cytosolic enzymes (NAD(P) dependent-dehydrogenases) play a role in the assimilation of substrates that is related to biomass formation (Saichana *et al.*, 2015). Second, CO_2 is produced and released by cytosolic enzymes (Mas *et al.* 2007). Third, in many Gram-negative bacteria, including AAB, the synthesis and secretion of gluconic acid are dependent on PQQ-dependant membrane-associated glucose dehydrogenases (PQQ-GDHs) (de Werra *et al.* 2009). In addition, CTC has been shown to be an efficient fluorescent marker for studying electron transport chain activity or respiration. It was shown that CTC is reduced prior to ubiquinone in *E. coli* K-12 respiratory chain by the primary aerobic [succinate and NAD(P)H] dehydrogenases (Smith and McFeters 1997; Créach *et al.* 2003). Thus, it can be deduced that periplasmic dehydrogenases and cytoplasmic dehydrogenases are both involved in the reduction of CTC. Additionally, the activity of cytosolic dehydrogenases in stationary phase was low (because CO_2 production was also low). In contrast, PQQ-dependent glucose dehydrogenase activity was high in stationary phase because the majority of gluconate was produced in this phase (Fig. 2), which was confirmed by an enzymatic assay (Fig. 3).

Co-feeding stationary phase cells with a mixture of ethanol, glucose and acetic acid caused the cells to resume growth. In addition, the number of CTC-positive cells and the amount of CO_2 released increased during the co-feeding phase. Since the concentrations of glucose and acetic acid did not change significantly during co-feeding phase, it can be deuced that ethanol may serve as a substrate that enhances metabolic pathways to regenerate redox cofactors that most likely lead to NADH. The strategy of increasing the NADH regeneration rate through co-feeding and co-metabolism has also been reported for well-established industrial microbes such as *S. cerevisiae* and *E. coli*. For example, it was shown that the addition of formate leads to a significant increase in the NADH regeneration rate, resulting in a very high biomass yield (Zobel *et al.* 2017). In another study, co-metabolism of formate with glucose increased glycerol production in anaerobic cultures of engineered strains of *S. cerevisiae* (Geertman *et al.* 2006). Finally, the use of ethanol as an energy source regenerated mainly NADH through alcohol oxidation in baker's yeast-mediated bioreduction (Kometani *et al.* 1994). Since AAB contains ALDH and ADH-dependent dehydrogenases in the cytoplasm, feeding with ethanol may increase NADH regeneration and acetic acid production in the cytoplasm, which in turn flux into TCA. The addition of ethanol as a carbon source between consecutive fermentation cycles repaired minimally damaged cells, presumably through changes in metabolic pathways. This enabled cells to restore their multiplicative ability and to further oxidize glucose. Transcriptome analysis of *Acetobacter aceti* also revealed that cells growing on glucose-induced oxidative stress, and different metabolic pathways are induced by changing the carbon source (Sakurai *et al.* 2011).

In the present work, two different feeding strategies were examined for optimizing the production of sodium gluconate by *A. senegalensis* at 38°C. Conventional semi-continuous fermentation and modified semi-continuous fermentation were compared. It was observed that co-feeding *A. senegalensis* with a mixture of ethanol, glucose and acetic acid as compared with glucose alone not only resuscitated nongrowing stationary phase cells but also enabled cells to grow on glucose and produce gluconic acid. Our approach allows consideration of recycling biomass through different fermentation cycles for the production of gluconic acid. No direct oxidation products were observed, whereas disturbance of the NADH pool was identified as the main source of perturbations. Additional studies are needed for uncovering the molecular mechanisms behind the generation of VBNC cells, as well as their resuscitation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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