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# Reducing agents assisted fed-batch fermentation to enhance ABE yields

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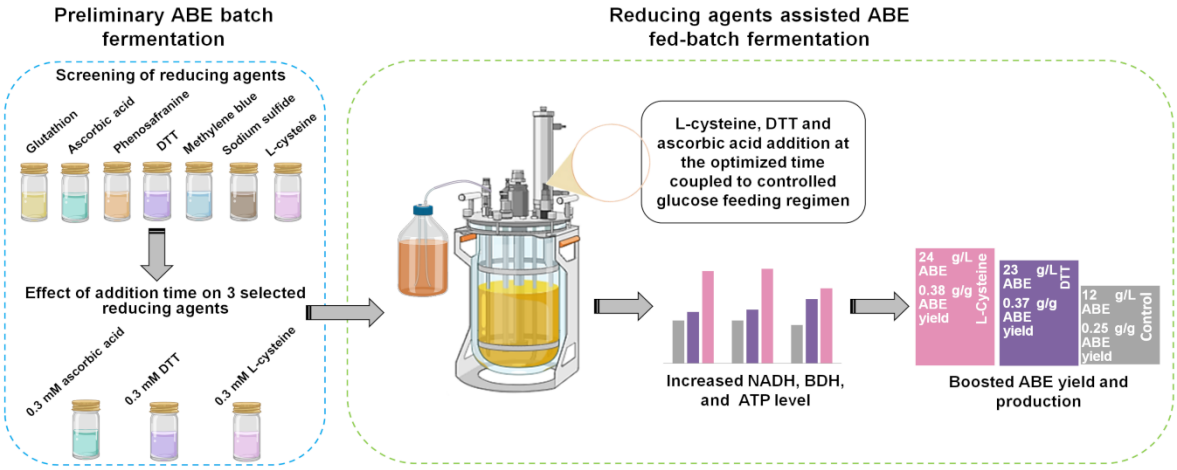
ABSTRACT

Acetone-butanol-ethanol (ABE) fermentation process is a promising bioenergy option amid rising concerns over the environmental impact of fossil fuel usage. However, the commercialization of the ABE process has been marred by challenges of low product yield and titer, thereby non-competitive process economics. Here, we coupled low-cost reducing agents with a controlled feeding strategy to improve both product titer and yield. Reducing agents promote cofactor dependent butanol production while fed-batch operation enhances glucose consumption, final ABE titer, and partly mitigates product toxicity. We investigated the effects of ascorbic acid, L-cysteine, and dithiothreitol (DTT) on fed-batch ABE production using *Clostridium acetobutylicum*. Moreover, to study the metabolic modifications triggered by these reducing agents, we performed NADH, ATP, extracellular amino acid secretion, and NADH-dependent butanol dehydrogenase (BDH) assays. L-cysteine and DTT improved ABE solvent titer by 2-fold, producing 24.33 and 22.98 g/L ABE with solvent yields of 0.38 and 0.37 g/g, respectively. NADH, BDH, and ATP levels increased significantly which also reflected in elevated ABE titer and yield. Furthermore, histidine secretion emerged as an important factor in *Clostridial* acid stress in this study. The results demonstrate that reducing agents and the fed-batch combination enables efficient utilization of glucose and remarkably enhances ABE production.

***Keywords***:

ABE fermentation, Butanol dehydrogenase, *Clostridium acetobutylicum*, Fed-batch, NADH, Reducing agents

***Graphical abstract***




***Highlights***

- ABE solventogenesis with reducing agents and controlled feeding regimen.
- L-cysteine and DTT fed-batches achieved solvent yield of 0.38 and 0.37 g/g.
- 24.33 and 22.98 g/L of ABE produced by L-cysteine and DTT fed-batches.
- Reducing agents boosted NADH, BDH, and ATP levels.
- Histidine secretion is important in *Clostridial* acid stress.

## 1. Introduction

Anthropogenic activities that emit atmospheric greenhouse gases (GHG) have been linked to climate change [1]. The climate change impact warrants reduction in carbon emission, and the scale-up of renewable energy supplies [2]. Among biofuels, biobutanol has attracted considerable attention because of its superior properties (such as high octane number, energy density) which are comparable to gasoline [3]. Moreover, it performs well with Jet A and diesel blends and produces lower emissions of nitrogen oxides (NO*x*) and carbon monoxide (CO) [4,5].

Traditionally biobutanol has been generated via the process of acetone–butanol–ethanol (ABE) fermentation of starch feedstocks using *Clostridia* [6]. In the ABE pathway (Fig. 1), the carbon flow from acetyl Co-A leads to the formation of acids and solvents via major branches of the ABE pathway. ABE fermentation is a biphasic process. In the first phase of acidogenesis, acetic and butyric acids are produced from sugars. These acids are then secreted into the medium. Acids lower the external pH and serve as inducers for the biosynthesis of solvent generating enzymes. In the second phase, acids assimilate back into *Clostridia* and act as co-substrates to generate neutral solvents (ABE) accompanied by further sugar consumption [7]. However, substantial practical problems of low product titer and yield, unwanted by-products, and butanol toxicity make the ABE process challenging for industrial-scale production of biobutanol [8]. To render the ABE process commercially viable, several process and genetic engineering approaches are being studied, including strain improvement [9], use of lignocellulosic substrates [10,11], and overexpression of targeted functional genes [12].

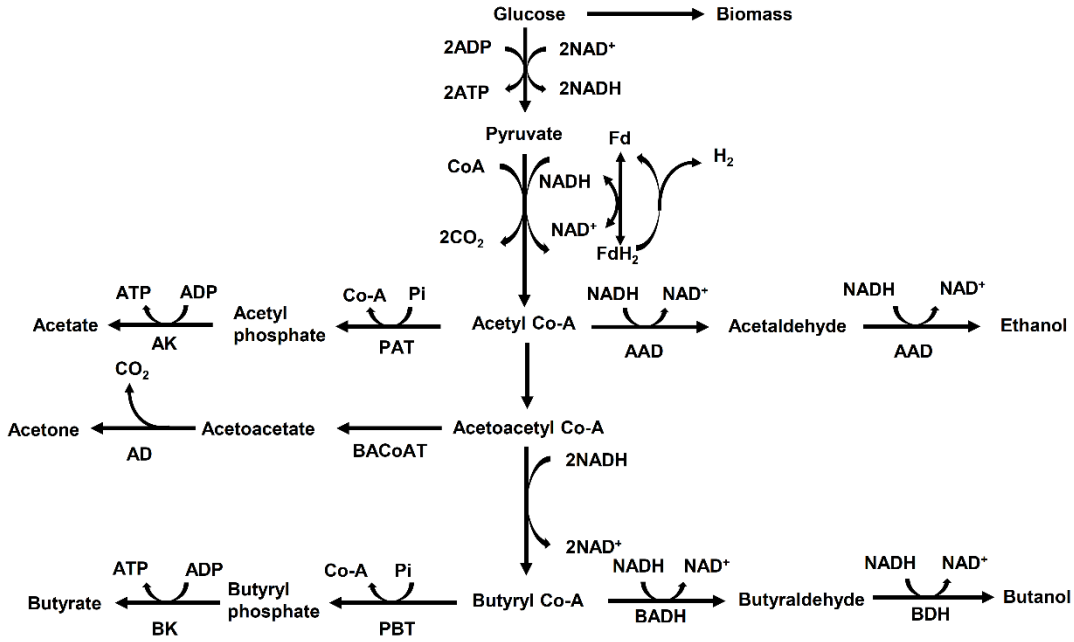



**Fig. 1.** The ABE production pathway in *Clostridium acetobutylicum* showing the formation of products and the role of reducing power NADH (AK: acetate kinase PAT: phosphate acetyltransferase, AAD: aldehyde alcohol dehydrogenase, AD: acetoacetate decarboxylase, BACoAT: butyrate-acetoacetate CoA-transferase, BADH: butyraldehyde dehydrogenase, BDH: butanol dehydrogenase, BK: butyryl kinase, PBT: phosphate butyryl transferase, Pi: inorganic phosphate) [7,13].

Production of butanol involves a sequence of reduction steps which utilize intracellular reducing equivalent, NADH (Fig. 1). Electron supply is essential to regenerate consumed reducing equivalents and thereby balance redox metabolism [14]. Redox balance can be manipulated to improve butanol production via electron receptor addition [15], and bioelectrolysis (BEC) [16]. As the majority of *Clostridia* lack electroactivity, bioelectrical production of butanol needs a redox mediator [14]. Another option is the use of low-cost reducing agents to enhance ABE fermentation.

Addition of reducing agents improve end-product formation in both aerobic and anaerobic fermentation processes. In heterotrophic microbes, most enzymes preferentially utilize NADH as their cofactor [17,18]. These agents affect the parameters of redox/oxidation or redox potential (ORP), ATP, and cofactors, such as NADH. In turn, they influence the expression of certain genes and activities of specific enzymes. They donate electrons to regenerate NADH from $NAD^+$ and facilitate the metabolic flux towards the desired metabolite [19]. The addition of neutral red in ABE fermentation resulted in enhanced butanol production with decreased acetone production [20]. Similarly, viologen dyes altered the electron flow towards NADH formation, thereby promoted alcohol formation [21]. However, considering the costs of viologens and neutral red, alternate less expensive reducing agents are essential to make this approach more feasible.

The threshold of substrate level is necessary for sustained solventogenesis [22]. Low substrate levels after the exponential phase (typically after 96 h) in *Clostridial* batch

fermentation lead to cessation of ABE production despite the presence of residual substrate, resulting in low yields [23]. Hence, fed-batch operation, which maximizes substrate utilization [24] was considered in the current study. We hypothesized that channeling reducing equivalent towards butanol generation with the aid of reducing agents and maintaining a threshold of substrate level necessary for solvent generation throughout the fermentation would increase the solvent titer, yield, and consequently the efficacy of substrate utilization. Besides, intermittent feeding partially mitigates ABE solvent toxicity [25]. Hence, the current work involves a comprehensive study of the role of reducing agents in conjunction with the fed-batch operation to enhance ABE production. It provides extensive insights into metabolism dynamics of energy and reducing equivalents, extracellular amino acid secretion under stress, and butanol dehydrogenase in presence of L-cysteine, DTT, and ascorbic acid in fed-batch operation mode. The study sheds light on the possible mechanisms of action of these reducing agents in ABE pathway. The findings demonstrate an efficient strategy to enhance ABE production in a simple set-up using reducing agents. Furthermore, findings of stress-responsive amino acid secretion can be useful in the development of acid-tolerant *Clostridial* strain for industrial applications.

This study aimed to establish an efficient ABE production system by employing inexpensive reducing agents with *Clostridium acetobutylicum* NRRL B-527 strain. We investigated the effect of L-cysteine, DTT, and ascorbic acid on ABE solvent production in the fed-batch regimen. To study metabolic modifications induced by reducing agents, we analyzed NADH, ATP, extracellular amino acid secretion, and NADH dependent BDH assays.

## 2. Material and methods

### 2.1 Bacterial culture and medium

*Clostridium acetobutylicum* strain NRRL B-527 culture was a gift for research work from ARS (Agricultural Research Services) Culture Collection, USA. It was germinated by inoculating in RCM (reinforced Clostridium medium) containing (g/L): glucose (30), meat extract (10), peptone (5.0), sodium chloride (5.0), yeast extract (3.0), sodium acetate (3.0), starch (1.0), L-cysteine (0.50), and 5 M potassium hydroxide was used to adjust pH to 6.8 ± 0.2. Spore suspension for future use was prepared by inoculating germinated *Clostridial* cells (10 % v/v) into starch solution (6% w/v) and stored at room temperature. Spores were activated by adding 2.5% v/v spore suspension into RCM and heat-shocked at 80 $^{o}$C for 10 minutes [26]. The pre-culture was grown for 20-24 h until OD 600 reached 1.5. Then ABE fermentation batches were inoculated with 5% v/v pre-culture in production (P2) medium with the following composition (g/L) : glucose (60), ammonium acetate (2.2), potassium dihydrogen phosphate (0.5), di-potassium hydrogen phosphate (0.5), magnesium sulfate (0.2), p-aminobenzoic acid (0.1), thiamin (0.1), sodium chloride (0.01), manganese sulfate (0.01), ferrous sulfate (0.01), and biotin (0.01). The medium pH was adjusted to 6.5 with 5 M potassium hydroxide and bubbled with nitrogen for 10 minutes before autoclaving at 120 $^{o}$C for 20 min. Analytical grade chemicals were used in this study and were procured from Sigma Aldrich, Finland.

### 2.2 Batch study of the effect of reducing agents on ABE fermentation

Preliminary experiments were conducted to determine which reducing agents impact ABE fermentation the most and to find their optimum time of addition in the fermentation medium. It was performed in 125 mL airtight serum bottles having a 95 mL P2 medium. Serum bottles inoculated with a 5% v/v actively growing pre-culture of *Clostridium acetobutylicum* NRRL B-527. Seven reducing agents which are relatively economical than conventionally used viologens: glutathione (GLUT), sodium sulfide (SS), methylene blue (MB), phenosafranine

(PS), ascorbic acid (AA), L-cysteine (CYS), and dithiothreitol (DTT) were selected. These reducing agents were dissolved in oxygen-free deionized water and were filter-sterilized (0.2-μm membrane filter). Reducing agents were added at the beginning of fermentation (0 h). The optimum concentration of reducing agents was identified by testing 0.1, 0.3, 0.5, and 0.7 mM concentrations. Furthermore, reducing agents were supplemented at 9, 12.5, and 17 h of fermentation to determine their optimal performance. These time intervals represent the initial, the mid, and the end of the exponential phase of fermentation. Batches were cultivated at 37 ± 2 °C and terminated after 96 h. Each experiment was carried at least in triplicate and the presented results report average ± standard deviation.

2.3 Reducing agent-assisted fed-batch experiments

Fed-batches were conducted in 3 L bioreactors (BIOSTAT B plus, Sartorius, Germany). Experiments were started with a 1000 mL P2 medium. A pH-breakpoint is linked to the shift from acidogenesis to solventogenesis. Hence, pH control was not used [27]. The three best reducing agents from the preliminary experiments were selected and added at an optimized time of 12.5 h from the onset of fermentation. Glucose feeding was initiated when the residual glucose in the bioreactor decreased beneath 40 g/L. The required volume of each feed pulse was determined based on the amount of glucose necessary to replace the utilized glucose in the bioreactor. A total of 5 feedings were performed at 30 (36 for ascorbic acid), 48, 72, 96, and 120 h of fermentation. The fermentation terminated after 168 h. In the end, the medium volume was 1144.29, 1454.83, and 1539.31 mL for ascorbic acid, DTT, and L-cysteine batch, respectively. The feed medium had a similar composition as the P2 medium except for the glucose concentration (120 g/L). Concentrated substrate feed helps to reduce the required fermentation volume and minimizes the waste disposal streams [27]. The control batch experiment was conducted in a bioreactor to compare its results with the fed-batches assisted by the reducing agents.

2.4 Analytical techniques

Solvents (acetone, butanol, and ethanol), acids (acetic, butyric, lactic), glucose, and acetoin were measured by high-performance liquid chromatography (HPLC) on Waters Alliance 2695 equipped with Rezex H (7.8 x 300 mm) column and Security Guard $H^+$ pre-column. The refractive index detector was set at 30 °C, and 14 mM sulphuric acid was used as an eluent in isocratic mode with a 0.6 mL/min flow rate. *Clostridial* growth was estimated by measuring optical density at 600 nm (Eppendorf BioPhotometer plus, Germany).

ATP (Roche 11 699 709 001, Germany) and NADH (Sigma-Aldrich MAK037, USA) were determined by following the methods supplied with the kits. The ATP was assayed by measuring luminescence produced by the reaction between ATP, added D-luciferin, and luciferase. Luminescence was measured with BioTek Cytation3 multimode reader (BioTek Instruments, USA). NADH was estimated after adding the NAD cycling enzyme mix by measuring absorbance at 450 nm.

NADH-BDH activity was analyzed by applying the method reported by Rajagopalan et al. [28]. The activity determined in the forward direction at 25 °C with 0.5 mM NADH in MES buffer (50 mM, pH 6) and 50 mM butyraldehyde. The reaction was started by adding 10% crude enzyme extract and absorbance was determined at 340 nm by Eon microplate reader (BioTek Instruments, USA). One unit of activity (U) is equivalent to the amount of BDH needed for oxidation of 1 μmol of NADH/min.

Protein concentration was estimated by applying the Bradford assay and bovine serum albumin as a standard [29].

Amino acids in ABE fermentation broth were quantified by S433 automatic amino acid analyzer (Sykam Chromatography, Germany) with the following conditions: amino acids separated with cation separation column LCA K06/Na 4.6 x 150 mm; column temperature 80 $^{o}$C; flow rate 0.45 mL/min; eluent was a mixture of buffer A ( sodium citrate, pH 3.45) and buffer B (sodium citrate/borate, pH 10.85); colorimetric detection at 570 nm and 440 nm (proline) after ninhydrin reaction.

## 3. Results and discussion

### 3.1 Screening of reducing agents in the batch study

The reducing agents were supplemented at 0$^{th}$ h (beginning of the batch) of ABE fermentation. Their effect was studied on butanol and total solvent production. Fig. 2 summarizes the effect of reducing agents at four different concentrations in the batch operation. 0.3 mM DTT emerged as the best reducing agent producing 9.19 ± 0.23 g/L butanol against the control with 6.27 ± 0.02 g/L. 0.3 mM L-cysteine and 0.3 mM ascorbic acid generated 8.33 ± 0.23 and 8.19 ± 0.19 g/L butanol, respectively. Total solvent production followed a similar trend, a corresponding increase of 62.53, 59.56, and 44 % when supplemented with 0.3 mM of DTT, L-cysteine, and ascorbic acid, respectively. Raising DTT concentration from 0.3 mM to 0.7 mM resulted in a steep reduction in butanol and ABE titer. DTT in high concentration (3mM) has proven to be toxic to bacterial cells [30]. Furthermore, higher DTT concentration impacts disulfide bond formation and intracellular protein folding in *E. coli* [31,32]. The addition of 0.1-0.5 mM phenosafranine, sodium sulfide, glutathione, and methylene blue also showed an increase in ABE titer compared to the control.

The ABE pathway (Fig. 1) consists of a sequence of reduction reactions where NADH acts as a crucial coenzyme. Hence, the improvement in ABE titer evident in the preliminary study has probably stemmed from the elevation of both, the regeneration of NADH and the activity of key enzymes such as butanol dehydrogenase (BDH).

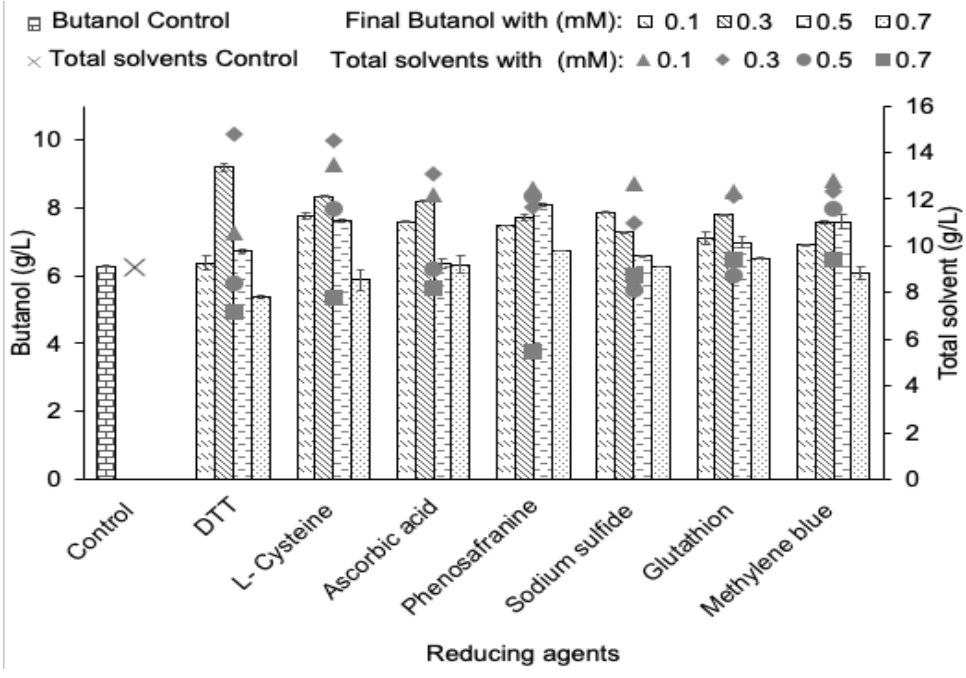



**Fig. 2.** Effect of reducing agents on total solvent and butanol production by using *C. acetobutylicum* NRRL B-527 batch study after 96 h.

Although the present study was conducted in the anaerobic milieu, an improved oxygen-scavenging or aero-tolerance attributed by reducing agents (Ascorbic acid, DTT, and L-cysteine) could prove very beneficial since it is difficult to maintain strict anaerobic conditions on the industrial scale [33]. L-cysteine is reported to be beneficial for *Clostridial* growth in ABE fermentation under stress [34]. Other reducing agents such as glutathione also improved *Clostridial* aero-tolerance and enhanced butanol production [33].

We selected ascorbic acid, DTT, and L-cysteine at 0.3 mM concentration to further explore their effect on ABE production.

3.2. Adding reducing agents at different time intervals in batch mode

The physiological state of microbial cultures varies at different fermentation times. Therefore, we assumed that the supplementation of reducing agents at different fermentation times would affect butanol and total solvent titers to different extents. Ascorbic acid, DTT, and L-cysteine were added at 9, 12.5, and 17 h of fermentation, which represents the initial, the mid, and the end of the *Clostridial* exponential (high metabolic activity) phase.

All the reducing agents showed the best results when added at 12.5 h (Fig. 3). DTT produced the highest butanol and solvent titer of 10.48 ± 0.22 and 17.23 ± 0.23 g/L against 7.00 ± 0.26 and 10.56 ± 0.41 g/L of butanol and total solvents in the control (without reducing agent) experiment. L-cysteine and ascorbic acid generated 9.85 ± 0.11 and 9.64 ± 0.08 g/L butanol along with 15.53 ± 0.14 and 13.11 ± 0.01 g/L of total solvents respectively. This translates into a 14-18 % boost in the butanol titer compared to the addition of reducing agents at $0^{th}$ h (Fig. 2 and 3).

In the mid-exponential phase (12.5 h), the NADH conversion circulation is high [35]. Thus, higher NADH generation possibly improved butanol production. The addition of reducing agents at the end of the exponential phase (17 h) declined solvent generation due to decreasing metabolic activity of pyruvate oxidation to acetyl-CoA. Reducing agent supplementation moderately improved the butanol and the total solvent titer in the batch study. Their positive effect was studied further in fed-batch operation.

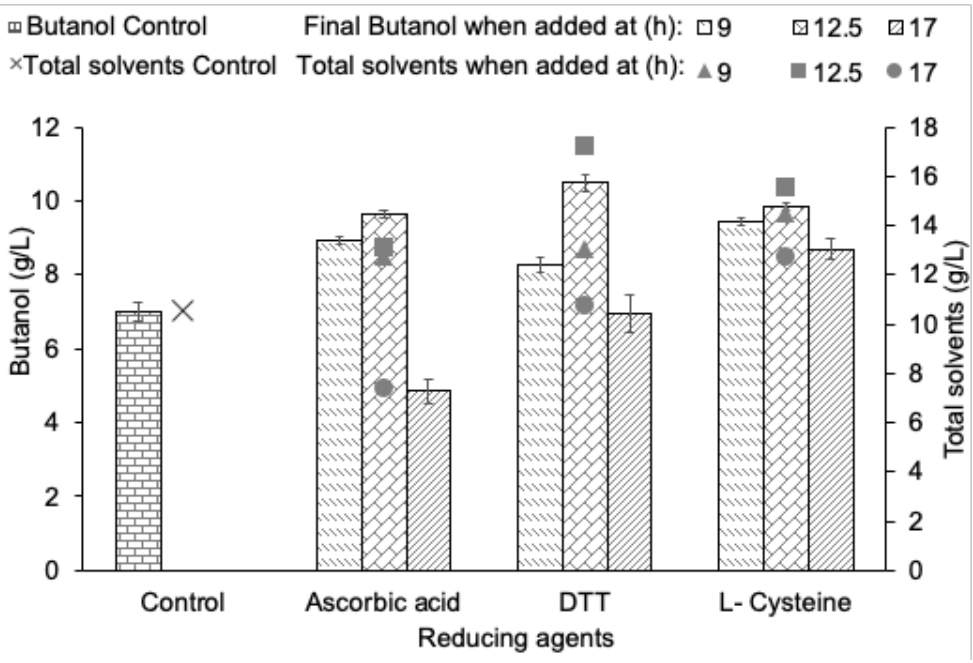



**Fig. 3.** Effect of time of addition of reducing agents on total solvent and butanol production using *C. acetobutylicum* NRRL B-527 batch study after 96 h.

3.3 Reducing agent-assisted fed-batch operation to enhance ABE production

3.3.1 Effect of reducing agents on ABE production

The threshold of glucose level necessary for solventogenesis can limit the efficiency of ABE production. ABE generation is very low when the medium glucose concentration is below 20 g/L [22]. The current study followed a similar trend. Butanol titer in the batch fermentation stagnated in the late stationary phase, especially due to substrate limitation. Table 1 shows the residual glucose and ABE yields in batch fermentation experiments. Therefore, reducing agent addition after 12.5 h was coupled to fed-batch fermentation with controlled feeding to extend the solventogenic phase (thereby improving glucose consumption, ABE titer, and yield).

**Table 1** Residual glucose and solvent yield after 96 h of batch fermentation in serum bottle.

| Sample | Residual glucose (g/L) | % Residual glucose | ABE yield (g/g) |
|---|---|---|---|
| Control | 14.64 ± 0.51 | 24.4 | 0.233 |
| 0.3 mM DTT | 7.5 ± 0.73 | 12.5 | 0.328 |
| 0.3 mM L-Cysteine | 9.77 ± 0.11 | 16.28 | 0.309 |
| 0.3 mM Ascorbic acid | 9.93 ± 0.09 | 16.55 | 0.262 |

Preliminary fed-batches were conducted to determine the optimum glucose level necessary to achieve efficient ABE production in the solventogenic phase. Both continuous and pulse feeding strategies were tested to select a suitable substrate feeding mode. Among the tested medium glucose concentrations, 40 g/L glucose and pulse feeding strategy proved suitable for efficient ABE production (data not shown).

In ABE fed-batch operation, L-cysteine (0.3 mM) and DTT (0.3 mM) significantly improved solvent production. L-cysteine and DTT boosted the total ABE solvents up 98 % (24.33 ± 0.30 g/L) and 87 % (22.98 ± 0.09 g/L) compared to the control batch production (12.28 ± 0.1 g/L) (Fig. 4a). Ascorbic acid (0.3 mM) supplemented fed-batch showed a modest increase of 38 % by producing 16.95 ± 0.21 g/L total solvents.

From 24 h onward, the DTT batch displayed relatively lower butanol and solvent production than the L-cysteine batch due to slower reassimilation of acids especially acetic acid (Fig. 4 and 5). Lower quantities of acids in the culture medium reflect their higher reassimilation in *Clostridia* and their effective conversion in solvents [36]. A study conducted with $C^{14}$ labeled acids showed that a major quantity of reassimilated butyrate and more than half of the acetate could be both reduced to butanol [7]. Likely, the slower reassimilation of acids into *Clostridia* can be due to the modulation of the membrane proteins and the transport systems by DTT during acid reuptake. In fact, DTT is a membrane permeating reducing agent. It affects the thiol group of proteins in the bacterial membrane, which in turn influence the affinity and the activity of both transport systems and membrane-associated enzymes [37,38]. Despite the slower reassimilation at the beginning, DTT showed a significant gain in solvent production during late solventogenesis.

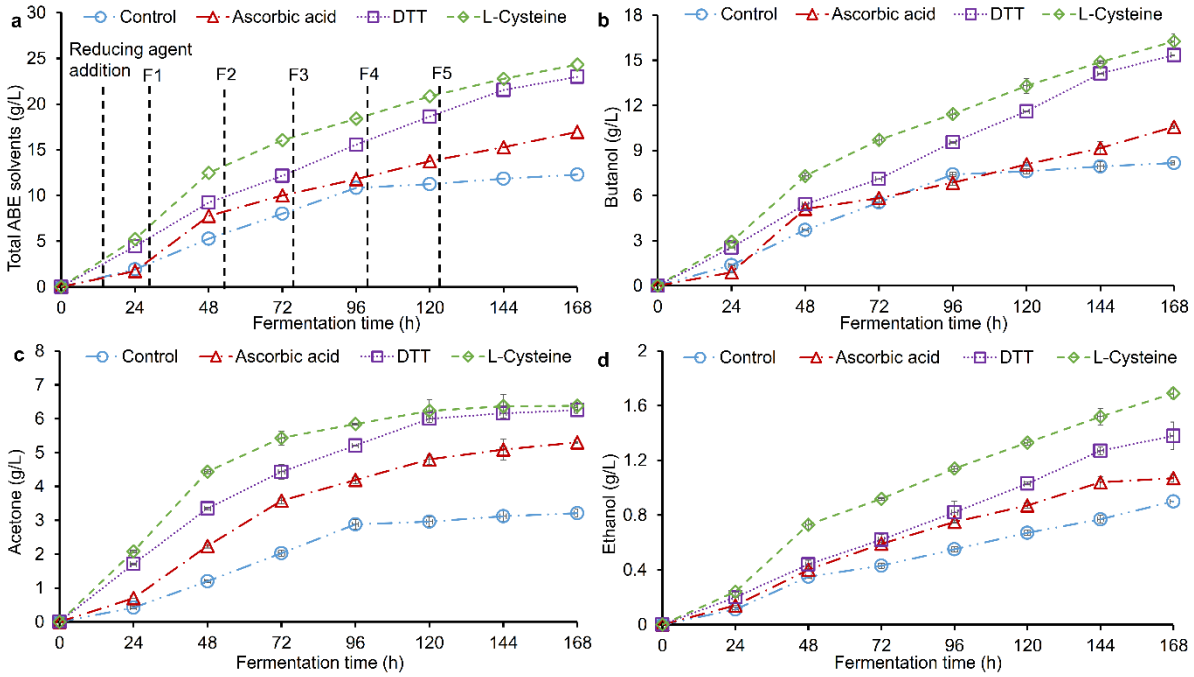



**Fig. 4.** Effect of reducing agents on ABE production using *C. acetobutylicum* NRRL B-527 fed-batch operation (a) total ABE solvents, (b) butanol production, (c) acetone production, (d) ethanol production. F1, F2, F3, F4, and F5 represent feeding points at 30 (36 for ascorbic acid), 48, 72, 96, and 120 h of fermentation.

The addition of L-cysteine demonstrated a significant effect on solvent production and ABE total solvent yield in fed-batch operation. The L-cysteine batch manifested higher acid reuptake than DTT and ascorbic acid batches. Butyric acid reassimilation was faster in the L-cysteine batch than other batches including the control batch. Fed-batch operation re-assimilated even lower quantities of organic acids in bacteria from the fermentation medium (Fig. 5). This is also evident in Fig. 6, where L-cysteine showed the highest solvents to acids ratio of 27.97 followed by DTT with 25.25, and ascorbic acid with 14.49 against 8.9 in the control.

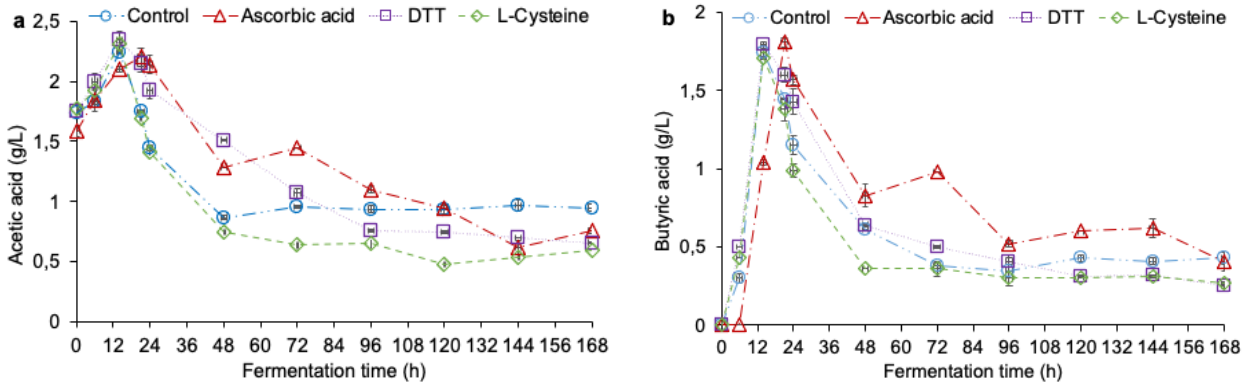



**Fig. 5.** Effect of reducing agents on (a) acetic acid and (b) butyric acid production in the fed-batch study using *C. acetobutylicum* NRRL B-527.

Ascorbic acid showed only a moderate impact on ABE production compared to L-cysteine and DTT in fed-batch experiments. It is reported that ascorbic acid forms a chelate complex with transition metal ions [39]. Hence, ascorbic acid might have formed a chelate complex with transition metal ions, iron, and manganese present in the production medium and feeding medium resulted in delayed *Clostridial* growth (Supplementary figure). The acid production profile reflected this delay where it reached the maximum level at 21$^{st}$ h contrary to 14$^{th}$ h in presence of other reducing agents (Fig. 5). Consequently, the ascorbic acid batch indicated slower acid re-assimilation and thereby lower ABE production (Fig. 4).

In fed-batch operation, the butanol level passed from 8.17 ± 0.12 g/L in the control to a maximum of 16.26 ± 0.51 g/L in the L-cysteine batch. DTT and ascorbic acid batches measured 15.35 ± 0.03 and 10.58 ± 0.09 g/L butanol, respectively. Addition of DTT and L-cysteine decrease the redox potential of fermentation medium [38,40,41]. Negative redox potential is linked to the amplification of reduction processes which is a peculiar feature of anaerobic fermentation [42], and it enhances intracellular NADH [42–44]. Reducing agents reduce $NAD^+$ back to NADH by donating electrons. This occurs if their redox potential is below -316 mV ($NAD^+$ redox potential) [45] and thus assists in NADH regeneration. DTT and L-cysteine have redox potential of -332 mV [46] and -340 mV [47] respectively. Therefore, DTT and L-cysteine increased NADH regeneration and consequently improved butanol production. Furthermore, elevated NADH levels might have contributed in enhancing activities of crucial enzymes of the butanol production pathway, such as butanol dehydrogenase (BDH) and butyraldehyde dehydrogenase (BADH) which are NADH dependent [48,49].

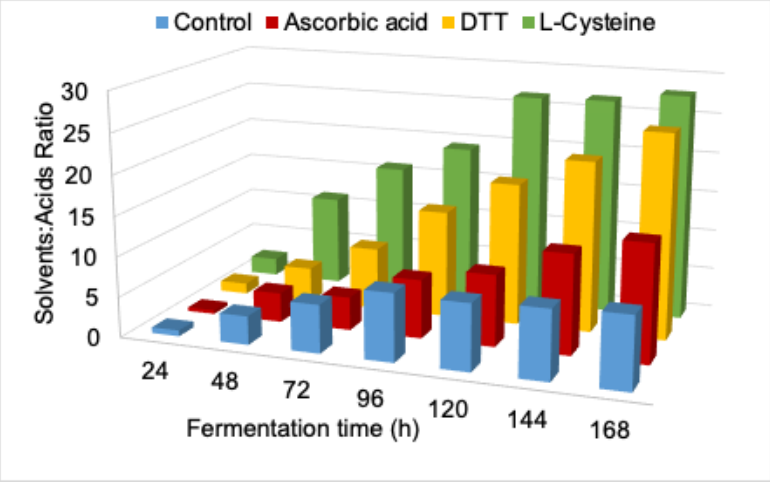



**Fig. 6.** Solvents to acids ratio in reducing agent-assisted fed-batches

Wild-type *C. acetobutylicum* typically produces maximal butanol titer of only 13 g/L [7,50]. Butanol accumulation deranges physiological functions of bacterial cell membrane including membrane ATPase, pH gradient, membrane potential, and transportation [51–53]. The disruption of the membrane functions may impede the proteins involved in oxidation-reduction reactions, promoting the accumulation of reactive oxygen species (ROS) [54]. *C. acetobutylicum* is armed with several pathways for detoxification of ROS including glutathione peroxidase-like proteins [55] and superoxide reductase (SOR) such as desulfoferrodoxin [56].

The enhanced fermentation performance achieved in the present study shows improved survival of *Clostridia* to solvent, acid, and osmotic stress reflected in conventional ABE solvent production (Fig. 4), glucose consumption (Fig. 7), and growth profile (Supplementary figure). Experimental data validate that the fermentation did not falter even in the presence of 24 g/L total solvents and 16 g/L butanol. These results are in alignment with Burphan et al. [57] study where cysteine derivative N-acetyl-L-cysteine (NAC) mitigated the oxidative stress in yeast and thereby enhanced the fermentation performance. NAC also enhanced the activity of cytosolic Cu-Zn supraoxide dismutase (Cu-Zn SOD), which is important in protection from

ROS in the yeast [57]. Disulfoferrodoxin enzyme in *Clostridium acetobutylicum* functions as a superoxide reductase in ROS detoxification, and its active center consists of [Fe(NHis)$_4$(SCys)] cysteine residue [56]. Considering this, the present study raises the possibility that L-cysteine may have exerted a positive impact on reductases and peroxidases such as disulfoferridoxin involved in ROS scavenging in ABE fermentation.

Interestingly DTT is also reported to decrease the level of ROS by 65.7% in *Pseudomonas chlororaphis* GP72AN and enhanced generation of biocontrol antibiotic phenazine-1-carboxylic acid (PCA) by 1.8- fold [58]. Therefore, DTT as well might have protected the *Clostridia* against ROS in ABE fermentation.

The protective effect of ascorbic acid is well documented in aerobic fermentation. However, the literature on its impact on anaerobic fermentation is scarce. Ascorbic acid exerted a positive effect on bacterial and yeast cells through modulating medium redox potential, prolonging the mean and maximal life span in Cu-Zn superoxide dismutase mutant yeast [59,60]. Nonetheless, in the present study ascorbic acid supplemented experiments could not cross the lethal butanol level. Hence, the effect of ascorbic acid in mitigating solvent stress is not clear from this study.

Other reducing agents, neutral red and methyl viologen have been frequently used in ABE fermentation to divert metabolic flux towards butanol production [20,21]. Interestingly, the mechanism to improve butanol titer seems dissimilar in the current study, especially because of the prominent acetone production (Fig. 4 and Table 2) unlike in production processes supplemented with methyl viologen or neutral red. In fact, all reducing agents improved acetone yield compared to the control (Table 2). The improvement in acetone yield can be attributed to improved *Clostridial* survival and intermittent glucose feeding. Reducing agents also exerted a beneficial effect on ethanol production and yield (Fig. 4 and Table 2). Since both ethanol and butanol production pathways require NADH (Fig. 1), their order of effect on production and yield is also similar (L-cysteine>DTT>Ascorbic acid). Butanol yield increased noticeably than ethanol in the present study (Fig. 4 and Table 2). The huge difference in ethanol and butanol yield is explained by the fact that, when *Clostridia* in ABE fermentation transit from the acidogenic phase to the solventogenic phase amidst the adverse environment of low pH, butanol is the primary product, while ethanol is only the marginal one.

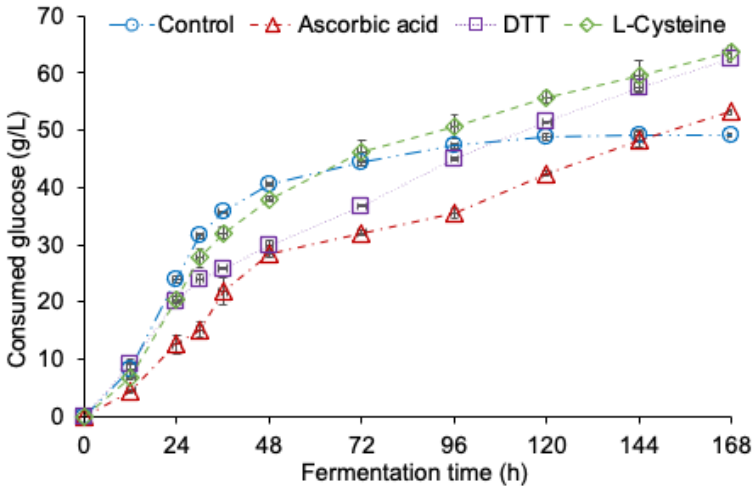



**Fig. 7.** Effect of reducing agents on *C. acetobutylicum* NRRL B-527 glucose consumption in fed-batch mode.

The butanol level and the solvent yield benefitted from the application of both controlled glucose feeding and reducing agents. Although experiments with reducing agents consumed more glucose (Fig. 7), they produced less biomass as evident from the OD profile (Supplementary figure). Thus, reducing agents apparently diverted glucose more towards solvent production. Therefore, at the end of the fermentation (168 h), the L-cysteine batch exhibited a 54 % increase in butanol yield followed by DTT with 48 %, and ascorbic acid with

a 20 % increase compared to the control. The total ABE solvent yield followed a similar trend since butanol is the major product (Fig. 8 and Table 2). From the very beginning, the L-cysteine batch exhibited better acid reassimilation and its effective conversion in solvents, thereby displayed the highest solvents to acids ratio (Fig. 6) throughout the fermentation period. Hence, L-cysteine was found to be most active in terms of total ABE solvents yield. This system also enhanced solvent productivity compared to the control. The maximum productivity of 0.26 g/L.h in L-cysteine, 0.19 g/L.h in DTT, and 0.16 g/L.h in ascorbic acid versus 0.11 g/L.h in the control batch were observed at 48 h of fermentation.

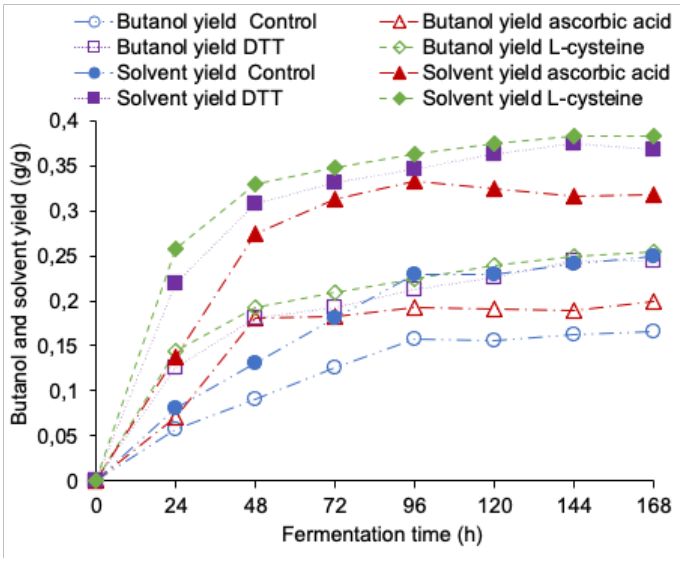



**Fig. 8.** Effect of reducing agents on butanol yield and solvent yield in *C. acetobutylicum* NRRL B-527 fed-batch study

**Table 2** Effect of reducing agents on ABE fermentation after 168 h.

| Parameter | Control | L-Cysteine | DTT | Ascorbic acid |
|---|---|---|---|---|
| Butanol (g/L) | 8.17 ± 0.12 | 16.26 ± 0.51 | 15.35 ± 0.03 | 10.58 ± 0.09 |
| Acetone (g/L) | 3.21 ± 0.1 | 6.38 ± 0.1 | 6.25 ± 0.09 | 5.3 ± 0.2 |
| Ethanol (g/L) | 0.9 ± 0.01 | 1.69 ± 0.04 | 1.38 ± 0.01 | 1.07 ± 0.03 |
| Acetic acid (g/L) | 0.95 ± 0.03 | 0.6 ± 0.03 | 0.66 ± 0.01 | 0.76 ± 0.02 |
| Butyric acid (g/L) | 0.43 ± 0.04 | 0.27 ± 0.01 | 0.25 ± 0.03 | 0.41 ± 0.06 |
| Total ABE (g/L) | 12.28 ± 0.1 | 24.33 ± 0.3 | 22.98 ± 0.09 | 16.95 ± 0.21 |
| Total acids (g/L) | 1.38 ± 0.04 | 0.87 ± 0.03 | 0.91 ± 0.03 | 1.17 ± 0.06 |
| ABE productivity(g/L.h)[a] | 0.11 ± 0.02 | 0.26 ± 0.05 | 0.19 ± 0.02 | 0.16 ± 0.01 |
| Butanol yield (g/g) | 0.166 | 0.25 | 0.245 | 0.199 |
| Acetone yield (g/g) | 0.065 | 0.1 | 0.099 | 0.099 |
| Ethanol yield (g/g) | 0.018 | 0.026 | 0.022 | 0.02 |
| ABE yield (g/g) | 0.25 | 0.382 | 0.367 | 0.318 |
| Solvents : acids ratio (g/g) | 8.9 | 27.97 | 25.25 | 14.49 |

[a] value measured at 48 h of fermentation

Table 3 shows a comparison of ABE fermentation performance with commonly used reducing agents and the current study. The present study (with L-cysteine and DTT) is competitive with the other studies in terms of butanol and ABE titer. However, ABE productivity still needs to be improved for economic large-scale production. The productivity can be improved by implementing advanced bioprocess technologies such as two-stage packed bed column reactor operation with simultaneous product recovery module system [26].

Likewise, the employment of engineered *Clostridia* [61,73] can further enhance both butanol yields and productivities. Hence, initial findings in this study indicate the applicability of reducing agents in fed-batch combination to further improve ABE fermentation production. The use of ascorbic acid is not as promising as L-cysteine and DTT.

**Table 3** Comparison of performance indicators of ABE fermentation with different reducing agents.

| Reducing agent | Strain | Operation mode, carbon source | Butanol (g/L) | Improvement to control (%) (Butanol titer) | Butanol yield (g/g) | ABE (g/L) | ABE yield (g/g) | ABE productivity (g/L.h) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| 0.1 mM Neutral red | *Clostridium beijerinckii* IB4 | Batch in micro-electrolysis cell (MEC), glucose | 10.5 | 12 | 0.250 | 14.68 | 0.349 | 0.52 | [20] |
| 5.1 mM Sodium sulfide | *Clostridium acetobutylicum* CICC8012 | Batch, wheat straw hydrolysate | 7.63 | 48 | 0.192 | 13.85 | 0.350 | 0.19 | [62] |
| 1.0 mM Methyl viologen | *Clostridium acetobutylicum* ATCC 824 | Batch, glucose | 11.93 | 23 | - | 13.98 | - | - | [63] |
| 0.5 mM Methyl viologen | *Clostridium tyrobutyricum CtΔack-adhE2* | Batch, glucose | 13.00 | 29 | 0.34 | - | - | - | [61] |
| 0.025 mM Benzyl viologen | *Clostridium tyrobutyricum CtΔack-adhE2* | Batch, glucose | 13.70 | 50 | 0.31 | - | - | - | [61] |
| 0.5 mM Methyl viologen and 0.025 mM Benzyl viologen | *Clostridium tyrobutyricum CtΔack-adhE2* | Repeated batch with immobilized cells in a fi-brous-bed bioreactor (FBB), first batch with glucose, subsequent batches with cassava bagasse hydrolysate | 15.00 | 63 | 0.30 | - | - | - | [61] |
| 0.3 mM L-cysteine | *Clostridium acetobutylicum* NRRL B-527 | Fed-batch, glucose | 16.26 | 99 | 0.250 | 24.33 | 0.382 | 0.26 | This study |
| 0.3 mM DTT | *Clostridium acetobutylicum* NRRL B-527 | Fed-batch, glucose | 15.35 | 88 | 0.245 | 22.98 | 0.367 | 0.19 | This study |
| 0.3 mM Ascorbic acid | *Clostridium acetobutylicum* NRRL B-527 | Fed-batch, glucose | 10.58 | 29 | 0.199 | 16.95 | 0.318 | 0.16 | This study |

3.3.2 Effect of reducing agents on NADH

After verifying the effect of reducing agents on the butanol production, the effect of the same agents was evaluated on the NADH levels in *Clostridia* during the solventogenic phase. NADH accumulates during acidogenesis and then decreases to the basic level during the solvent production phase [64]. As visible in Table 4, L-cysteine, DTT, and ascorbic acid increased NADH concentration by 36 %, 29 %, and 7 % compared to the control.

**Table 4** Effect of reducing agents on NADH after 48 h *C. acetobutylicum* NRRL B-527 fed-batch operation.

| Fermentation sample | NADH (μmole/g dry cell weight) |
|---|---|
| Control | 0.809 (± 0.01) |
| Ascorbic acid | 0.862 (± 0.03) |
| DTT | 1.04 (± 0.01) |
| L-Cysteine | 1.101 (± 0.02) |

Enhanced solvent production and yield reflected the beneficial effect of elevated NADH. Nevertheless, it is conceivable that increased NADH levels achieved after the addition of reducing agents is not the sole factor responsible for higher butanol production. Presumably, the reducing agents (especially L-cysteine and DTT) must have exerted their favorable effect on the ABE pathway through the multiple mechanisms discussed in section 3.3.1, culminating in the high butanol yield and titer.

3.3.3 Effect of reducing agents on NADH-dependent butanol dehydrogenase activity

*Clostridia* has multiple aldehyde/alcohol dehydrogenases. NADH-dependent butanol dehydrogenase (NADH-BDH) is the major enzyme accountable for butanol synthesis in ABE fermentation as it exhibits the highest activity during solventogenesis [28].

**Table 5** Effect of reducing agents on NADH-BDH activity after 168 h *C. acetobutylicum* NRRL B-527 fed-batch cultivation.

| Fermentation sample | NADH-BDH (U/mg) |
|---|---|
| Control | 0.021 (± 0.002) |
| Ascorbic acid | 0.025 (± 0.001) |
| DTT | 0.047 (± 0.002) |
| L-Cysteine | 0.07 (± 0.003) |

Since a reduced environment is conducive for catalytical activity and stability of alcohol dehydrogenases including BDH [28], the reducing agent supplemented fed-batches displayed higher NADH-BDH activities (Table 5). L-cysteine batch showed the highest NADH-BDH activity (3-fold higher than control), followed by DTT (2-fold higher than control) and ascorbic acid (a slight increase over control). The butanol levels achieved in the present study highlight the quintessential role of NADH-BDH in ABE fermentation. Besides, enhanced NADH-BDH activities led to elevated solvents to acids ratio of 27.97 and 25.25 in L-cysteine and DTT batches, relative to the ratio of 8.9 in the control (Fig. 6).

3.3.4 Effect of reducing agents on ATP

The promotion of solvent formation reduces ATP demand [64]. Here, solvent production improved in the presence of reducing agents. Hence ATP analysis was performed to check their impact on ATP levels during fermentation (Fig. 9). Before the addition of reducing agents (12 h), ATP levels were high due to the exponential phase conversion of glucose into acetic and butyric acid (acid formation generate ATP) [65]. After 24 h, ATP concentration decreased in all the batches, especially in the ascorbic acid batch. This decrease can be explained by higher ATP demand (utilization) to enhance membrane-active (ATPase) permeability to maintain physiological intracellular pH in the presence of high acid concentrations [66]. Interestingly, the steep ATP decrease in the ascorbic acid batch coincides with the delayed acid peak and the prolonged acidogenic phase (Fig. 5 and Fig. 9). ATP increased till 48 h accompanied by acid reuptake and biomass increase (Supplementary figure). A decrease in biomass from 48-120 h led to a decrease in the ATP level in *Clostridia*. In the late solventogenic phase (72-120 h), the ATP levels in the DTT and L-cysteine batches were still high compared to the control.

As discussed in section 3.3.1, DTT and L-cysteine might have protected *Clostridia*, from fermentation stress while the same cannot be stated for ascorbic acid. ATP is crucial in cell maintenance and repair. Hence high ATP levels detected in DTT and L-cysteine batches can be attributed to lower ATP demand due to their protective actions. On the other hand, the protective effect was not evident in the ascorbic acid batch. Thus, it might have led to higher ATP consumption for cell repair and maintenance and therefore lower levels of ATP detected in the ascorbic acid batch. The high ATP levels obtained in DTT and L-cysteine batches reflected in increased butanol and acetone production as decreased ATP demand promotes solventogenesis [64].

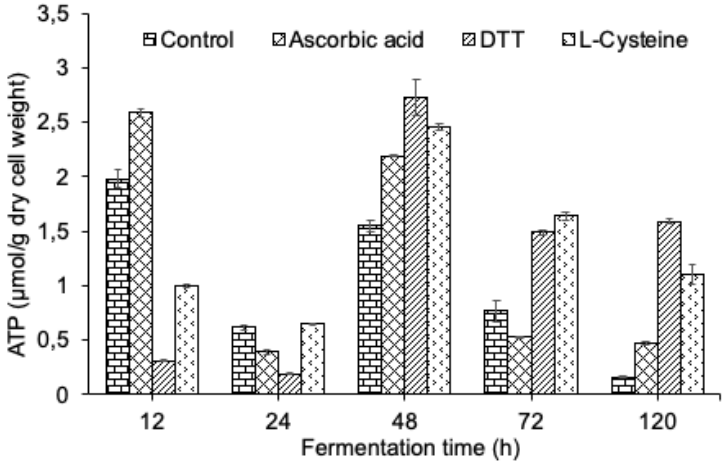



**Fig. 9.** Effect of reducing agents on ATP level in *C. acetobutylicum* NRRL B-527 fed-batch cultivation.

3.3.5 Effect of reducing agents on protective amino acids secretion in ABE culture

During ABE fermentation, *Clostridia* are subjected to multiple stresses, including acid and solvent stress. Cells respond, adapt, and develop tolerance to multiple stressors during cultivation. However, the mechanisms involved in encountering the overlapped stresses are only partially investigated and remain poorly understood [67]. Metabolomic analysis of *Clostridia* has revealed that increased amino acid levels, such as isoleucine and valine, confer butanol tolerance to bacteria [68]. Several studies found that to better survive under the stress caused by ABE fermentation, *Clostridia* secrete amino acids such as phenylalanine, methionine, leucine, valine, and tyrosine in culture medium [15,69]. Given this, we verified whether elevated butanol levels achieved in the current study increased the secretion of protective amino acids in the *Clostridia*. Thus, after the supplementation of reducing agents, we analyzed the secretion of stress-responsive amino acids in the ABE culture medium.

The majority of amino acids were quantified after 30 h of fermentation. Interestingly, a significant increase in proline, methionine, isoleucine, alanine, and histidine levels were detected (Fig. 10). DTT, L-cysteine, and ascorbic acid batch demonstrated an approximately 2 to 3-fold increase in amino acid release in the medium. Branched-chain amino acids (BCAAs) including isoleucine help in the adaptation of membrane fluidity in response to butanol stress in *Clostridia* [68,70]. Proline protects the cells from alcohol stress [71].

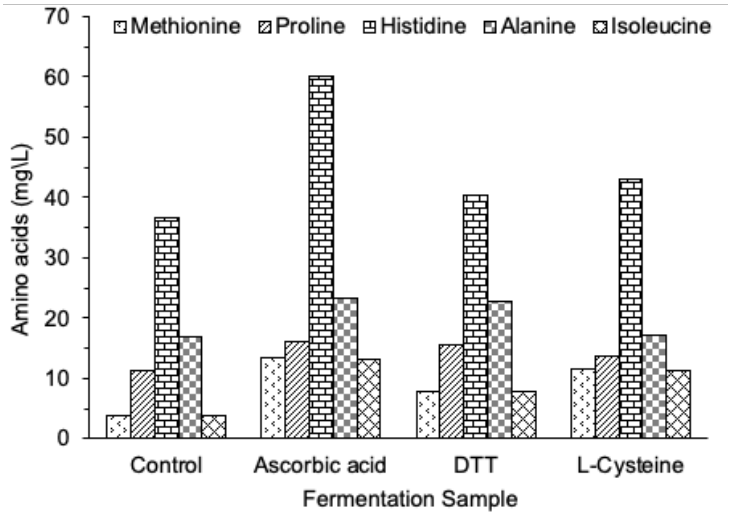



**Fig. 10.** Effect of reducing agents on protective amino acid secretion in *C. acetobutylicum* NRRL B-527 culture broth after 30 h.

Although ascorbic acid batch only moderately improved butanol production, histidine level manifested a 64 % increase over the control (Fig. 4b and Fig. 10). This led us to believe that, besides the butanol stress, some other stress might have promoted histidine secretion in the ascorbic acid culture broth. Some protective amino acids are not only involved in countering solvent stress but also carboxylic acid stress in bacteria [67]. Broadbent et al. [72] proved that the induction of histidine biosynthesis genes improved acid tolerance in *Lactobacillus casei*. In the present study, ascorbic acid experiments showed decreased reuptake of acids. It led to their high residual levels in the fermentation medium (Fig. 5). Therefore, higher secretion of histidine in ascorbic acid batch, confirms its direct protective role in acid tolerance in *C. acetobutylicum*. This finding is interesting since Wang et al. [70] found that histidine biosynthesis genes (CAC0935-43) were upregulated under both butyrate and butanol stress in *C. acetobutylicum*. Hence, the current study proposes the protection of *Clostridia* by histidine from butyrate as well as acetate stress. The experimental results also validate the importance of protective amino acids in *Clostridial* stress. These amino acids may influence other aspects of *Clostridial* physiology in addition to those discussed here. Therefore, to gain insights, we recommend further study such as transcriptional analysis of amino acid synthesis in the presence of these reducing agents.

## 4 Cost comparison of reducing agents

The current study demonstrates the positive impact of L-cysteine, DTT, and ascorbic acid on solvent yield and titer in ABE fermentation. However, to apply them to industrial-scale cost-effectiveness is crucial. The reducing agents used in this study are noticeably more affordable than commonly used reducing agents in ABE fermentation, neutral red, and methyl viologen (Fig. 11 and Fig. 12). Moreover, the use of smaller amounts (0.3 mM) would further facilitate their application. Although both L-cysteine and DTT significantly improved butanol and ABE titer, in terms of reducing agent cost per unit butanol and ABE production, L-cysteine is a more attractive option compared to DTT. Hence, more apt for large scale application. The cost can be further cut down by employing food wastes rich in these reductants, for instance, ascorbic acid from citrus fruits juice and L-cysteine from chicken broth. We recommend detailed techno-economic analysis to establish the economic feasibility of the current process.

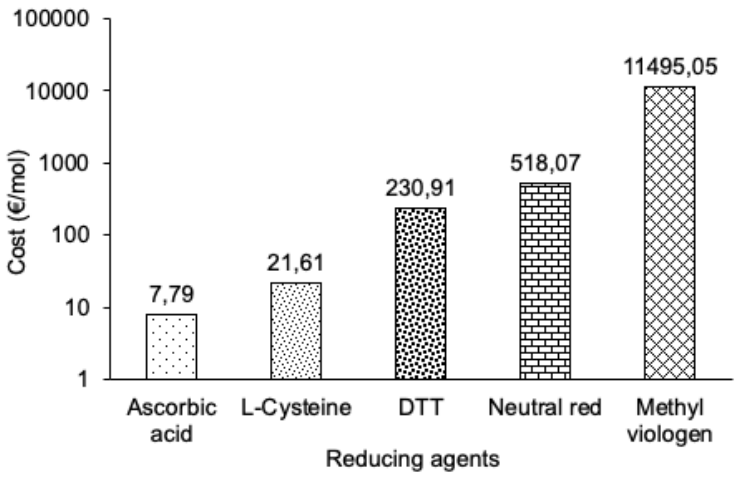



**Fig. 11.** Cost comparison of reducing agents used in the present study versus commonly used reducing agents in ABE fermentation. Costs are based on data from Molekula Group and Acros Organics (Methyl viologen) on 22/04/2020

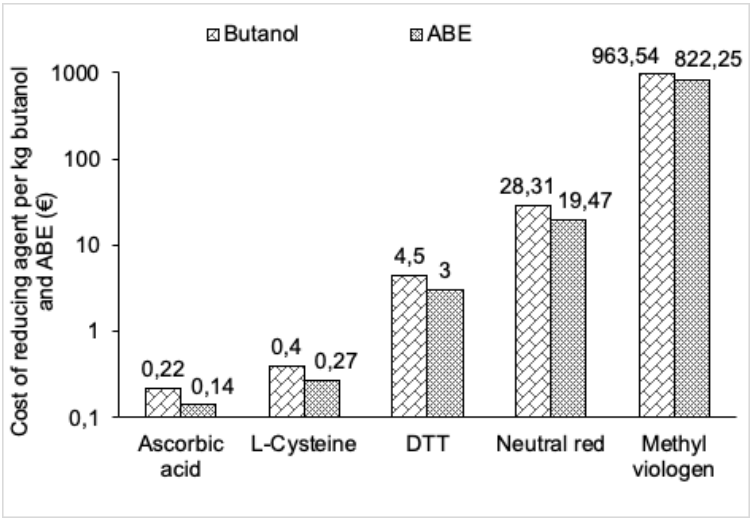



**Fig. 12**. Comparison of reducing agents cost per unit butanol and ABE production. Ascorbic acid, DTT, and L-cysteine: present study, neutral red: [73], methyl viologen: [63].

## 5 Conclusions

An efficient ABE production system was developed by employing cost-competitive reducing agents by using controlled glucose feeding regimen in fed-batch operation. ABE titer, yield, and consequently the efficacy of substrate utilization were significantly increased in this study. Substantial increase in NADH, BDH, and ATP levels were observed in L-cysteine and DTT batches which reflected in improved ABE production. Reducing agents used in the present study did not impact acetone production. Hence, it is proposed that L-cysteine, ascorbic acid, and DTT have a different mechanism of action than conventional reducing agents such as viologens and neutral red. Furthermore, histidine secretion emerged as an important factor in *Clostridial* acid stress in this study. This factor will be useful in developing acid-tolerant *Clostridial* strain for industrial applications. The ABE productivity of the current system can further be enhanced by integrating it with the *in-situ* ABE solvent recovery module. To gain a deeper understanding of stress-responsive amino acid secretion in ABE fermentation further studies such as transcriptional analysis of amino acid synthesis in the presence of reducing agents is recommended. The results from the current study will be useful in developing an advanced cultivation system and separation technology by carefully considering the feedstock applicability in large scale operation.

**Conflict of interest**

There are no conflicts to declare.

## CRediT authorship contribution statement

**Vijaya Chandgude:** Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft, Visualization, Writing - Review & Editing. **Teemu Välisalmi**: Formal analysis. **Juha Linnekoski:** Formal analysis. **Tom Granström:** Conceptualization. **Bruna Pratto:** Visualization, Writing - Review & Editing. **Tero Eerikäinen:** Conceptualization. **German Jurgens:** Conceptualization. **Sandip Bankar:** Funding acquisition, Supervision, Writing - Review & Editing, Conceptualization.

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