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Role of Trace Elements as Cofactor: An Efficient Strategy toward Enhanced Biobutanol Production

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Supporting Information

ABSTRACT: Metabolic engineering has the potential to steadily enhance product titers by inducing changes in metabolism. Especially, availability of cofactors plays a crucial role in improving efficacy of product conversion. Hence, the effect of certain trace elements was studied individually or in combinations, to enhance butanol flux during its biological production. Interestingly, nickel chloride (100 mg L\(^{-1}\)) and sodium selenite (1 mg L\(^{-1}\)) showed a nearly 2-fold increase in solvent titer, achieving 16.13 ± 0.24 and 12.88 ± 0.36 g L\(^{-1}\) total solvents with yields of 0.30 and 0.33 g g\(^{-1}\), respectively. Subsequently, the addition time (screened entities) was optimized (8 h) to further increase solvent production up to 18.17 ± 0.19 and 15.5 ± 0.13 g L\(^{-1}\) by using nickel and selenite, respectively. A significant upsurge in butanol dehydrogenase (BDH) levels was observed, which reflected in improved solvent productions. Additionally, a three-dimensional structure of BDH was also constructed using homology modeling and subsequently docked with substrate, cofactor, and metal ion to investigate proper orientation and molecular interactions.

KEYWORDS: Biobutanol, Butanol dehydrogenase, Homology modeling, Molecular docking, Trace elements

INTRODUCTION

Butanol is an imperative industrial chemical, possessing excellent fuel properties, and thus can be thought to have potential to replace/supplement fossil gasoline. Especially, the Asia-Pacific region is known to cover the biggest market of n-butanol, which accounted for 51.3% consumption by volume in 2014. Moreover, the global n-butanol market is expected to reach USD 9.9 billion by 2020. Due to such an eye-catching worldwide market, the historical biobutanol production, usually referred to as acetone-butanol-ethanol (ABE) fermentation by solventogenic Clostridia, has reasserted its importance as a green alternate renewable fuel.

Conventional ABE fermentation process observed major challenges viz. low butanol concentration, yield, productivity, and solvent intolerance resulting in a high overall production cost, thus impeding its commercialization. To alleviate these concerns, increasing butanol concentration and the B:A (butanol:acetone) ratio without sacrificing the total solvent productivity have been considered to be key points by many research groups. In this view, a couple of techniques including strain mutagenesis, genetic engineering, and metabolic regulation have been implemented. Additionally, overexpression of targeted functional genes in the engineered host has also been practiced to overcome butanol toxicity obstruction in microorganisms. However, unstable butanol production shows difficulty and complexity in transferring related pathways to host bacteria, due to the inherent instability and inactive expression in contrast to a wild-type strain.

In relation to the aforesaid approaches, studies pertaining to alteration in metabolic flux with the help of microbial electro-synthesis and electro-fermentation have demonstrated potential and feasibility in enhancing microbial production. Additionally, cofactors involved in biosynthetic pathways would be considered to be possible targets to induce changes in metabolism. In the case of solventogenic Clostridia, butanol
dehydrogenase (BDH) is a key enzyme that catalyzes conversion of butyraldehyde to butanol, and the reaction is cofactor dependent (Figure 1). Thus, constant availability of cofactor (NADH and NADPH) in the solventogenic phase is essential to achieve a redox balance so as to improve butanol titer.\textsuperscript{10,14} Recently, numerous electron carriers/pigments are studied to overproduce NADH which ultimately accelerates butanol flux.\textsuperscript{15} On the other hand, literature reports also explain the role of trace elements as cofactors for enzymes involved in the metabolic pathway.\textsuperscript{16} Rajagopalan et al.\textsuperscript{14} discussed that BDH from \textit{C. acetobutylicum} ATCC 824 requires a metal ion and a reduced condition for its activity. However, BDH from \textit{Clostridium} sp. BOH3 requires neither any metal ion nor reduced conditions thus inferring that enzyme requirements differ from species to species. Several in silico approaches have been usually performed to reveal the interactions involved in enzyme–substrate/inhibitor binding.\textsuperscript{17−19} Further, the protein sequences of BDH from different Clostridia are also available at the National Center for Biotechnology Information (NCBI). However, the 3D structure of BDH from \textit{C. acetobutylicum} ATCC 824 is neither determined experimentally (X-ray and/or NMR) nor predicted by computational techniques, to date. Hence, the crystal structure of BDH from Clostridia is not available in the Protein Databank (PDB). Indeed, to understand the biophysical properties of enzymes, it is essential to have 3D structures of the target molecule. At the same time, obtaining X-ray diffraction quality crystals of a protein is quite difficult.\textsuperscript{20} Thus, homology modeling is thought to be reliable and an efficient method for 3D structure prediction.\textsuperscript{21} Besides, active sites in crystal structures can be resolved using molecular docking protocols.\textsuperscript{22}

The present study attempted to enhance butanol titer in fermentation broth by using \textit{Clostridium acetobutylicum} NRRL B-527 (ATCC 824). Therefore, trace elements (act as enzyme cofactors) were screened to investigate their effect for improved production. Additionally, this study also highlights physiological changes occurring during ABE fermentation. Furthermore, the 3D model was constructed with the help of homology modeling and assessed using different assessment tools to reveal the catalytic potential of BDH from \textit{C. acetobutylicum} ATCC 824. The current study also enlightens the mode of possible interactions of the substrate and/or inhibitor with BDH, using molecular docking studies. This work proves the significance of trace elements in enhancing butanol production, and in silico studies confirm that BDH is a metalloenzyme possessing a Rossmann fold in its structural domain.

\section*{Materials and Methods}

\textbf{Cell Culture and Fermentation Experiments.} The bacterial strain of \textit{C. acetobutylicum} NRRL B-527 was a kind gift from ARS Culture Collection, U.S.A. The cells were stored as spores in 6\% (w/v) starch solution. These spores were activated in reinforced clostridial medium (RCM) as mentioned by Harde et al.\textsuperscript{23} and further used as seed inoculum for fermentation batches. The production medium used in this study consisted of the following (g L\textsuperscript{−1}): glucose (60), magnesium sulfate (0.2), sodium chloride (0.01), manganese sulfate (0.01), iron sulfate (0.01), dipotassium hydrogen phosphate (0.5), potassium dihydrogen phosphate (0.5), ammonium acetate (2.2), biotin (0.01), thiamin (0.1), and p-aminobenzoic acid (0.1), at pH 6.5. Fermentation experiments were performed in 100 mL airtight glass bottles with 80 mL of production medium. The production medium was purged with nitrogen to maintain anaerobic environment and sterilized at 121 °C for 20 min.

Trace elements investigated in this study were: sodium selenite (Na\textsubscript{2}SeO\textsubscript{3}·5H\textsubscript{2}O), sodium tungstate (Na\textsubscript{2}WO\textsubscript{4}·2H\textsubscript{2}O), nickel chloride (NiCl\textsubscript{2}·6H\textsubscript{2}O), zinc sulfate (ZnSO\textsubscript{4}), and iron(II) chloride (FeCl\textsubscript{2}·4H\textsubscript{2}O). Each element was prepared in a varied concentration range (1−100 mg L\textsuperscript{−1}) and added by filter sterilization (0.22 μm), before inoculation. Subsequently, 5\% (v/v) (OD\textsubscript{600} = 1.56) of actively growing cells (from seed culture) were inoculated and fermentation was continued until 120 h at 37 ± 2 °C. All of the chemicals used in this study were of analytical grade. All experiments were carried out at least in triplicate, and the results mentioned are average ± standard deviation.

\textbf{Analytical Methods.} Fermentation samples were withdrawn at regular time intervals and centrifuged at 20 000g for 10 min. The resulting supernatant was analyzed for total solvents (acetone, butanol, and ethanol) and total acids (acetic and butyric acid) by gas chromatography (Agilent Technologies 7890B) equipped with a DB-WAXetr column (30 m × 0.32 mm × 1 μm) and a flame ionization detector. The oven temperature was programmed as 80 (1 min hold) to 200 °C at 30 °C/min (1 min hold), and the injector and detector were set at 200 and 250 °C, respectively. A 0.5 mL sample was injected with a split ratio of 20:1. Clostridial growth was also monitored by measuring optical density (OD) at 600 nm using UV−visible spectrophotometer (3000i, LabIndia). In addition, a medium pH was observed throughout the fermentation process by using a laboratory pH meter (Global, India). The glucose concentration was determined by phenol-sulfuric acid method.\textsuperscript{24} Besides, BDH activity was also assayed at certain times of interest according to the method reported by Rajagopalan et al.\textsuperscript{14}

\textbf{Homology Modeling and Structural Assessment.} Amino acid sequence of targeted protein, BDH (accession no. AAA23206) was retrieved from NCBI (https://www.ncbi.nlm.nih.gov/). The online BLAST (Basic Local Alignment Search Tool) search algorithm was used in order to find out homologous template. Afterward, the pairwise sequence alignment between target and template sequences was carried out using CLUSTALW to discover sequence similarity.\textsuperscript{25} Further, MODELLER 9.19 software was employed to build a 3D structure of the target protein.\textsuperscript{21} The best model was opted among 50 generated structures, which was based on certain scoring parameters such as MODELLER objective function, DOPE (discrete optimized protein energy) pseudoenergy value, and GA341 score.\textsuperscript{25} The predicted model was evaluated using ERRAT, PROCHECK, and ProSA which was then visualized with UCSF Chimera.\textsuperscript{26,29} Moreover, unfavorable nonbonded contacts were removed by energy minimization using the steepest decent algorithm in UCSF Chimera.

\textbf{Molecular Docking Studies.} Molecular docking is a simulation process in which a receptor−ligand conformation can be predicted. The receptor can either be a protein or nucleic acid, whereas the ligand is quite a tiny molecule which can be any organic compound.\textsuperscript{22} In the present study, the stabilized 3D structure of BDH was used to dock ligand (NADH) and substrate (butyraldehyde) by binding-site using PATCHDOCK online program.\textsuperscript{26} On the other hand, experimentally known fermentation inhibitors such as furan derivatives and weak acids were also docked with BDH protein to investigate the binding mode between them. Particularly, 3D structures of the ligand, substrate, and inhibitor were retrieved from the PubChem database in SDF (Structure data file) format. Furthermore, these structures were converted to PDB format using Openbabel.\textsuperscript{31} Finally, they were individually sent along with the

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{reaction_catalyzed_by_BDH.png}
\caption{Reaction catalyzed by BDH from \textit{C. acetobutylicum} ATCC 824.}
\end{figure}
RESULTS AND DISCUSSION

Effect of Varying Trace Element Concentration on Biobutanol Production. The impact of different trace elements namely sodium selenite, nickel chloride, zinc sulfate, iron chloride, and sodium tungstate was studied with respect to butanol production and overall solvent yield by using C. acetobutylicum NRRL B-527. These elements were selected based on their active role in different biochemical reactions. Various concentration ranges for each element were individually supplemented to fermentation medium in order to find the optimal concentration responsible for the increment in butanol level. Figure 2 highlights butanol and total solvent production under varied trace elements concentration. It was observed that the addition of almost all trace elements had significantly improved biobutanol production when compared with the control experiment.

Regular P2 medium (control) produced butanol up to 5.34 ± 0.10 g L⁻¹ with a total ABE of 7.88 ± 0.25 g L⁻¹ after 120 h fermentation. On the other hand, selenite addition (1 mg L⁻¹) enhanced butanol production up to 8.21 ± 0.13 g L⁻¹ with total solvents of 12.88 ± 0.36 g L⁻¹. Further increase in selenite concentration up to 100 mg L⁻¹ drastically reduced solvent production, because of restricted Clostridial growth. Kousha et al.32 observed a similar finding, and they concluded that higher selenium concentration (>1 mg L⁻¹) activates the detoxification processes, which transforms selenium to elemental selenium which gets deposited near the periphery of bacterial cells thus affecting microbial growth.

Supplementation of iron chloride also showed significant increment in butanol concentration, irrespective of its concentration addition (Figure 2). A similar trend was observed when the fermentation medium was supplemented with tungstate with butanol accumulation up to 7.02 ± 0.29 g L⁻¹. Interestingly, zinc sulfate also showed a positive effect on butanol production accounting to have 9.09 ± 0.12 g L⁻¹ butanol together with 14.08 ± 0.48 g L⁻¹ total ABE. The highest butanol (10.81 ± 0.15 g L⁻¹) and total solvent (16.13 ± 0.24 g L⁻¹) production were achieved in a medium supplemented with 100 mg L⁻¹ nickel chloride, which is around 50% higher than in the control experiment (without trace element). The butanol concentration remained unchanged with further addition (>100 mg L⁻¹) of nickel chloride.

Interestingly, an exogenous inclusion of trace elements in this study have led to step up in ABE and butanol concentrations which thought to be due to enrichment of BDH activity.10 Several researchers have also studied the effect of reducing agents and/or precursors for improved butanol titer.10,33 Isar and Rangaswamy34 showed moderate increase in butanol production by using Clostridium beijerinckii when the medium has been supplemented with calcium ions. Furthermore, Saxena and Tanner35 also demonstrated that ethanol production by C. ragdai was improved 4-fold by optimizing the trace metal concentrations because of enhanced metalloenzyme activities. The improved performance by nickel, selenite, and zinc propelled us to evaluate their performance with a more detailed study such as time of addition during the fermentation experiment.

Time of Trace Element Addition for Enhanced Butanol Production. Nickel chloride, sodium selenite, and zinc sulfate with optimal concentrations of 100, 1, and 100 mg L⁻¹, respectively, were used to study their effect on “addition time” in fermentation medium. These elements were added at different fermentation time intervals of 0, 4, 8, 18, and 24 h. Since, Clostridia tend to enter into the stationary phase after 36 h, to produce solvents, this study was not extended after 24 h of fermentation.

From Figure 3A, it was found that the highest butanol concentration (12.22 ± 0.09 g L⁻¹) was achieved when nickel chloride was added after 8 h of fermentation. Initial supplementation (0 h) of nickel chloride resulted in comparatively lower butanol production (9.32 ± 0.19 g L⁻¹). Interestingly, the growth profiles of C. acetobutylicum B-527 (data not shown) with and without nickel chloride did not show any substantial difference. Furthermore, the ethanol production profile was also unaffected without change in concentration. Incidentally, acetone production was slightly fluctuated with a different time of addition.

Sodium selenite was also effective in enhancing the butanol concentration when included after 8 h of fermentation. The maximum amount of butanol achieved was 10.69 ± 0.52 g L⁻¹ along with 4.67 ± 0.21 g L⁻¹ acetone and 1.37 ± 0.12 g L⁻¹ ethanol. Considering the time profile of addition, selenite did not show remarkable variations in individual solvent production (Figure 3B). Conversely, it significantly affected growing Clostridia, which was indicated by the growth profile (data not shown). This was evident from the observation that selenite slowed down the growth (OD₆₀₀ = 1.55) when added...
However, results obtained by zinc sulfate addition in this study attributed this increase to rapid acids reassimilation to solvents. Concentration of around 77% with zinc supplementation, and microbial growth. On the other hand, zinc sulfate showed physiological environment, causing oxidative stress to reduce incorporation of selenite during this period may alter the growth curve corresponds to adaptation of microbial cells, and after intermittent additions. Usually, the initial time of the beginning (0 h) and supported the growth (OD₆₀₀ = 2.12) after intermittent additions. Usually, the initial time of the growth curve corresponds to adaptation of microbial cells, and incorporation of selenite during this period may alter the physiological environment, causing oxidative stress to reduce microbial growth. On the other hand, zinc sulfate showed improvement in butanol production when added after 4 h of fermentation. Wu et al. also reported an increase in butanol concentration of around 77% with zinc supplementation, and attributed this increase to rapid acids reassimilation to solvents. However, results obtained by zinc sulfate addition in this study were not significantly higher than other trace elements used (Figure 3C).

Many researchers reported that the addition time of stimulators and/or activators influences the solvent production. Ding et al. added 2 g L⁻¹ sodium sulfate (electron receptor) after 24 h fermentation and reported 12.96 g L⁻¹ butanol, which was 34.8% higher than in the control. Furthermore, Nasser Al-Shorgani et al. also showed the highest butanol concentration (18.05 g L⁻¹) when benzyl viologen was incorporated after 4 h of fermentation. Moreover, in order to enhance the butanol/acetone ratio, Li et al. added neutral red at 60 h when the butanol production rate was relatively higher.

Therefore, it was concluded that the addition of nickel chloride and sodium selenite to the fermentation medium was of vital importance which ultimately resulted in better solvent production. Hence, nickel chloride and sodium selenite were critically investigated further in order to study their effect on growth, pH, glucose consumption, and total solvent production.

**ABE Fermentation Profile in the Presence of Nickel and Selenite.** According to earlier results, nickel chloride (100 mg L⁻¹) and sodium selenite (1 mg L⁻¹) were individually added to the fermentation medium at 8 h, and their effects were evaluated by analyzing the samples at particular time intervals. The obtained results were then compared with control experiment in order to figure out the changes during fermentation operation. As can be seen in Figure 4a, the cell growth was comparatively increased in the presence of trace elements. Supplementation of nickel and selenite resulted in higher biomass formation at 72 h compared to that in the control, although its behavior was quite aligned until 24 h. A lag period of ~4 h was observed (with or without trace element) wherein Clostridial cells adapted themselves to growth conditions. Thereafter, a gradual increase in cell density was recorded indicating exponential behavior of cells. Trace element incorporation positively affected cell behavior without being lethal to budding Clostridia. This outcome is in agreement with Li et al., who found improved cell growth due to a large quantity of reduced equivalents (NADH and NADPH) with the aid of a precursor (nicotinic acid) in the fermentation medium.

Furthermore, medium pH plays a crucial role during ABE fermentation, thus being responsible for shifting microbial acidogenic phase toward solventogenesis. However, addition of trace elements during fermentation did not severely affect the pH profile (Figure 4b). A classical pH trend was observed both in the control and trace element supplemented experiments.

The sugar consumption profile was also studied to see the effect of trace elements on sugar uptake and solvent production. Residual glucose concentrations in the control and in selenite were nearly similar (Figure 4c). However, a suitable amount of selenium in the fermentation medium may elevate the content of essential elements and total amino acids which in turn enhances bacterial growth followed by better solvent production. On the other hand, nickel supplementation aided almost complete sugar utilization which is thought to be because of the regulatory effect on sugar utilization and metabolism. However, in-depth transcriptional analysis should be essential to elucidate the detailed mechanism underlying complete utilization. Xue et al. explained that micronutrients have a regulatory effect on sugar utilization and showed significant improvement in butanol production and fructose utilization with the addition of zinc in a culture medium.

Solvent production profiles were also studied with the addition of trace elements in order to get detailed insight on its effectiveness for biobutanol production (Figure 4d–f). The highest butanol and ABE as 10.08 ± 0.14 and 18.17 ± 0.19 g L⁻¹, respectively, were achieved by nickel supplementation (Figure 4e). Nickel supplementation improved biobutanol production. Moreover, in order to enhance the butanol/acetone ratio, Li et al. added neutral red at 60 h when the butanol production rate was relatively higher.

**Figure 3. Time course of trace elements addition: (A) nickel chloride, (B) sodium selenite, and (C) zinc sulfate.**
production up to 68% higher than in the control (Figure 4e).

Looking into Figure 4d, f, acetone and ethanol production were started late (18–24 h) while butanol production was initiated at 8 h (Figure 4e) in the control as well as in the trace element supplemented medium. Nair and Papoutsakis also demonstrated that butanol production gets initiated in priority than...
acetone and ethanol when cells sense a hostile environment (reduced pH), which is mainly due to the active role of aldehyde-alcohol dehydrogenase (AAD).

Acetic and butyric acid are main metabolic precursors for solvent formation. Figure 4g,h shows the acid formation profiles. A time course revealed that the first acidogenic phase was supplemented at 36 h with a second acidogenic phase with

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**Figure 5.** (A) Overlay similarity between BDH (cyan) and template 1VLJ (magenta). (B) Ramachandran plot of BDH model. (C) ERRAT analysis of refined BDH model.
rapid reassimilation of acids into solvents thereafter. This indicates acidogenesis and solventogenesis took place twice during the entire fermentation process. The dual acidogenesis in the current study is also supported by Pang et al.\textsuperscript{41} who carried out fed-batch fermentation for butanol production using sugar cane bagasse by \textit{Clostridium acetobutylicum} GX01. They observed second acidogenesis after 40 h fermentation, mainly due to rapid assimilation of produced acids into solvents during early stages. Acetic acid levels were also comparatively elevated during the current fermentation experiments with trace element addition, thereby observed an increase in acetone concentration and thus unimproved B:A ratio.

Of interest, both solvent yield and productivity were notably higher with trace element addition suggesting their consequent effect on aforesaid parameters. Certainly, selenite was found to be more effective in enhancing solvent yield (0.33 g g\textsuperscript{−1}) than nickel (0.30 g g\textsuperscript{−1}) with solvent productivities to be 0.12 g L\textsuperscript{−1} h\textsuperscript{−1} and 0.15 g L\textsuperscript{−1} h\textsuperscript{−1}, respectively. Therefore, the synergistic effect of selenite (1 mg L\textsuperscript{−1}) and nickel (100 mg L\textsuperscript{−1}) was investigated by adding them after 8 h fermentation. The resulting total solvents (16.78 ± 0.21 g L\textsuperscript{−1}) were fairly less as compared to individual addition of nickel (data not shown), thus proving the fact that higher metal ions in medium could be detrimental to microorganisms.\textsuperscript{32,42} Overall, nickel was found to be potent cofactor which significantly improved the solvent titer.

The improved butanol concentration in the presence of nickel was attributed to BDH activity at particular instances viz. in acidogenic and solventogenic phases. As expected, NADH-dependent BDH exhibited reasonably higher activities of about 0.41–0.44 (acidogenic phase) and 0.63–0.69 U mg\textsuperscript{−1} protein (solventogenic phase) with trace element addition. Similarly, Li et al.\textsuperscript{10} reported NADH-dependent BDH activity in the range of 0.40–0.60 U mg\textsuperscript{−1} when nicotinic acid was used as a precursor in culture medium. On the other hand, Rajagopalan et al.\textsuperscript{14} detected comparatively lower BDH activity (0.03 U mg\textsuperscript{−1}) in cell extract of \textit{Clostridium} sp. BOH3 after 24 h of fermentation. Overall, the activity of NADH-dependent BDH was improved by 42% with the addition of trace elements as compared to control. Hence, it was thought desirable to characterize BDH by developing a three-dimensional structure and subsequently molecular docking studies to elucidate interactions involved.

**Homology Modeling and Structural Assessment.** The retrieved target sequence of BDH enzyme from \textit{C. acetobutylicum} ATCC 824 (accession no. AAA23206) comprises 389 amino acids. The template of NADH-dependent BDH from \textit{Thermotoga maritima} was identified using the BLASTp program which showed 41% identity and 99% query coverage with target BDH sequence. In addition, sequence alignment which was carried out using CLUSTALW also showed homology between the target and template sequences with conserved regions (Figure S1).\textsuperscript{25} A three-dimensional structure of target BDH was constructed by homology modeling based on the crystal structure of chain A of \textit{Thermotoga maritima} BDH (resolution = 1.78 Å, PDB: 1VLJ, chain A). The model was built with the help of MODELLER 9.19 software. A total of 50 models were generated, out of which the best model with the lowest DOPE score and highest GA341 value was selected for further processing. Additionally, the initial selected model was refined by 5000 steps of energy minimization using the steepest descent with the help of UCSF Chimera to eliminate nonbonded interactions.
The final refined model was superimposed with the template structure which showed a root-mean-square deviation (RMSD) value of 0.265 Å and thus implies a close relationship between these structures (Figure SA). Usually, RMSD is calculated between C-alpha atoms of matched residues in 3D superposition of the target and template. The RMSD values indicate a closeness among superimposed structures. The greater the RMSD value, the more distant the matched structures. Further, the Ramachandran plot shows the relationship between phi and psi angles of a protein which can be helpful for determining the role of the amino acid in the secondary structure. It is derived through the PROCHECK online server and depicted the backbone dihedral angle distributions of all amino acid residues. The Ramachandran plot showed that 94.4% of residues were to be in the core region while 5% were in the allowed region and only 0.3% in the disallowed region, thereby showing that the backbone dihedral angles of the model are reasonably perfect (Figure SB). Besides, ERRAT analyzes statistics of nonbonded interactions between different atom types, and its score was found to be 96.54% signifying the constructed structure is of good quality with high resolution (Figure SC). On the other hand, the ProSA web server compares the Z-score of the predicted model with all protein chains present in protein database which have already been determined experimentally through X-ray diffraction and NMR techniques. In present study, the Z-scores estimated by ProSA were −9.38 and −11.51 for target and template, respectively, which also supported the quality of the model (Figure S2). All of these findings indicate that the 3D structure of BDH obtained by homology modeling is acceptable and can be used for subsequent docking studies.

Molecular Docking Studies. The validated model was further used for the docking study to examine the interactions between ligand–receptor bindings. Interestingly, the structure of BDH represents a typical α/β fold particularly dominated by helical bundles that are linked by unordered loops. Like other NADH/NADPH dependent dehydrogenases, BDH features an extended β-sheet domain, which contains the Rossmann fold and is crucial for cofactor (NADH) binding (Figure 6). A similar motif was reported by Sommer et al. during characterization of β-hydroxybutyryl CoA dehydrogenase. Additionally, Sulzenbacher et al. identified the glycine-rich cofactor (NADH and/or NADPH) binding site in alcohol dehydrogenase (ADH). However, such region was not found in NADH-docked BDH from the current study which is in line with the report by Walter et al. Since, BDH is involved in conversion of butyraldehyde to butanol, their docking was carried out using the PATCHDOCK server. Figure 6 shows the binding pose of butyraldehyde in BDH. The substrate is situated in front of the cofactor binding domain, near to the catalytic site. Similar conformations have been reported by other researchers. The interactions of butyraldehyde in active cleft are shown in the “substrate-inhibitor pocket” callout (Figure 6). Furthermore, the substrate docked structure exhibited proper intermolecular hydrogen bonding, and possible interacting residues are TYR276, TYR277, GLU272, and PHE385.

Our previous studies reported that numerous inhibitors hamper the solvent production during ABE fermentation. Hence, it was decided to investigate the effect of few inhibitors on BDH by incorporating in silico techniques. Two experimentally known inhibitors namely acetic acid and hydroxymethyl furfural were docked with BDH protein using the PATCHDOCK server. Surprisingly, these inhibitors were found to have similar binding domain as like substrate (butyraldehyde) with consistent interacting residues (Figure 6). This resemblance may result in competitive inhibition which in turn affects BDH activity resulting in lowered solvent titer.

BDH is a metalloenzyme and thus requires a metal ion for its effective activity. Figure 6 callout “catalytic triad” revealed that three histidine residues along with aspartate formed a perfect metal binding groove, and residues involved are conserved with template BDH protein for ferrous ion, depicted by sequence alignment (Figure S1). This perfect metal binding groove formed is mainly due to the hydrophobic nature of BDH which is evaluated through amino acid composition and hydrophobicity profile (Figure S3). Furthermore, an analogous metal binding groove within modeled BDH is expected to form when nickel is present in culture medium. Hence, all together (BDH + cofactor + metal ion) drives the reduction of butyraldehyde, and proper possible interactions confirmed the major role of BDH in enhancement of butanol concentration with trace element incorporation. Schwarzenbacher et al. demonstrated the same interacting residues in catalytic cleft with square pyramidal coordination for iron in 1,3-propanediol dehydrogenase (TM0920) from Thermotoga maritima.

CONCLUSIONS

The purpose of the present research was to improve butanol concentration in order to make it a future alternate liquid biofuel. Hence, supplementation of cofactors in the fermentation medium would be considered as a potential approach to increase solvent titers in C. acetobutylicum NRRL B-527. The addition of trace elements viz. nickel chloride, and sodium selenite have led to significant improvement in butanol concentrations which is thought to be due to redirection of metabolic flux toward more reduced products. This study also showed the remarkable impact of varying the addition time on solvent production (10–20% increment in solvent titer). Furthermore, fermentation profiling revealed that the solvent production was positively triggered as soon as cells entered into the stationary phase and achieved maximum butanol concentration of 8–10 g L⁻¹ which is higher than that of the control. Additionally, the 3D structure of the crucial BDH enzyme was also developed. The subsequent molecular docking experiments helped to understand the possible substrate–inhibitor interactions in the BDH protein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b01611.

Figure S1: Pairwise sequence alignment using CLUSTALW: metal ion binding residues are marked in red;

Figure S2: ProSA Z-score plot of crystal structure (1VLJ) and predicted model (BDH);

Figure S3: Amino acid composition (A) and mean hydrophobicity profile (B) of BDH from C. acetobutylicum ATCC 824 (PDF)
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Notes
The authors declare no competing financial interest.

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