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Strategic intensification in butanol production by exogenous amino acid supplementation: Fermentation kinetics and thermodynamic studies

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Highlights

- Exogenous addition of tryptophan:phenylalanine triggered butanol production
- Amino acid addition after 8 h in batch cultivation resulted in 12.43 ± 0.1 g/L butanol
- Fed-batch with *in-situ* product recovery was promising for improved butanol titer
- ABE production follow first order kinetics in respect to intermediate concentration
- Thermodynamics of ABE fermentation was studied to evaluate reaction feasibility

Abstract

Amino acids are vital precursors in many biochemical production pathways in addition to efficient nitrogen source which could enhance microbial growth yields. Therefore, in present study, the effect of amino acids from aliphatic and aromatic family was comprehensively evaluated in batch and integrated fed batch fermentation system. *Clostridium acetobutylicum* NRRL B-527 was able to utilize 54.15 ± 1.0 g/L glucose to produce 12.43 ± 0.10 g/L butanol under batch cultivation. Interestingly, a significant step up in butanol titer (20.82 ± 0.33 g/L) was achieved by using fed-batch fermentation process integrated with liquid–liquid extraction module. Besides, mathematical modeling studies demonstrated the best fitting of experimental data with first order reaction kinetics. Overall, an enhancement in solvent titer by induction of essential cellular components coupled with advance bioprocess strategy was successfully utilized in this study for its further applications.

Keywords

Amino acid; Biobutanol; Clostridia; Fermentation kinetics; Integrated fed-batch and separation system

Graphical abstract

1. Introduction

Energy is an indispensable component of modern society and thus has a prominent role in country's development (Khanal and Lamsal, 2010). India's energy demand is projected to soar over the coming years as energy consumption grows fastest among all major economies by 2040 (BP Energy Outlook, 2018). More importantly, transport is the second largest energy end-use sector and remains largely reliant on fossil fuels (IRENA, IEA and REN21, 2018). At the same time, several concerns *viz.* security of supply and instability in crude oil prices together with rising green house gases in the atmosphere have led to put forth new energy policies (Maiti et al., 2016; Khedkar et al., 2017). Additionally, renewable energy deployment needs to be integrated into the daily life to develop green alternative solutions for gasoline. Butanol, being a potential renewable liquid biofuel is established globally, due to its excellent fuel properties (Maiti et al., 2016). However, its commercial usage as a fuel is still obstructed because of several technoeconomic concerns (Khedkar et al., 2018).

Conventionally, biobutanol is produced *via* anaerobic conversion of carbohydrates into acetone-butanol-ethanol (ABE) using Clostridial strains (Ibrahim et al., 2017). However, several hurdles such as low product titer/yield, end product inhibition, and insufficient productivities in the industrial butanol production must still be overcome to compete with petroleum based fuels (Bankar et al., 2013a,b). Therefore, numerous approaches such as random chemical mutagenesis, genome shuffling, strain development and ribosome engineering technology have been greatly explored recently, to enhance biobutanol titer (Isar and Rangaswamy, 2012; Xue et al., 2017). Improving the overall bioprocess by incorporating cofactors, stimulators and/or developing advanced fermentation technology would be more suitable way outs for its successful large-scale operation (Nasser Al-Shorgani et al., 2015; Ibrahim et al., 2018).

Recently, the butanol production was considerably enhanced by using methylimidazolium based ionic liquids (Chen and Li, 2018). The ionic liquids could interfere with enzymes in biosynthetic pathway thereby influencing butanol production. On the other hand, *xylo*-oligosaccharides act as a prebiotic and stimulate fermentative metabolism that resulted in 17% gain in butanol titer (Grassi et al., 2018). Interestingly, exogenous gene of ferredoxin-NAD(P)⁺ oxidoreductase (FdNR) was introduced in *C. acetobutylicum* and it was observed that FdNR can divert redox to NAD(P)H that can be used for higher alcohol production (Qi et al., 2018). Moreover, researchers are also trying to improve cellular robustness by using different approaches such as, by induction of heat shock proteins in *C. acetobutylicum* which overexpresses DnaK and GroESL to retain the enzymes of EMP pathway in more active state for improved butanol tolerance (Liao et al., 2017).

Another study revealed that strengthening proline biosynthesis could be an efficient strategy to boost cell tolerance since it sweeps away the intracellular ROS and maintain membrane stability (Liao et al., 2018).

Amino acid plays an important role in regulating several intracellular functionalities. Amino acids are also secreted under microbial stressed conditions (Luo et al., 2015). Therefore, accumulation/assimilation of these compounds might be beneficial in raising solvent titer and strain robustness. The *meta*-analysis of transcriptional response of *C. acetobutylicum* demonstrated that amino acids (aspartic acids family) could up-regulate butanol synthesis (Heluane et al., 2011). Therefore, it was thought desirable to improve the Biobutanol titer by supplementing amino acids during cultivation. The use of amino acid in biobutanol production at fed-batch fermentation mode is expected to improve the solvent productivity with reduced end product inhibition. The butanol toxicity can further be reduced by incorporating an *in-situ* separation module.

The butanol re-commercialization largely depends on biomass utilization, microbial strain development, advanced bioprocess development and others. Therefore, strategic development considering all aforesaid parameters is necessary to propose butanol as a viable green fuel. Hence, in current study amino acid pairs were incorporated and their effect on ABE fermentation was systematically investigated at batch scale. Further, fed-batch operational mode was also employed to overcome substrate deficiency to improve the process yields. In addition, an integrated liquid-liquid extraction system was introduced to mitigate butanol toxicity to growing *Clostridia*. This study also sheds light on thermodynamic aspects explaining reaction feasibility and fermentation kinetics.

2. Materials and methods

2.1. Bacterial strain and media

Clostridium acetobutylicum NRRL B 527, used in all fermentation experiments was generously gifted by ARS Culture Collection, U.S.A. The cells were maintained in 6% (w/v) starch solution in the form of spores. Further, the seed culture for fermentation was prepared in 80 mL sterile reinforced Clostridial medium as explained earlier (Nimbalkar et al., 2017). Standard P2 medium was used as a fermentation medium, with 60 g/L glucose and other essential components (g/L) as follows: 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. The production medium was sparged with nitrogen to maintain anaerobic condition. Medium was sterilized at 121 °C for 20 min.

2.2. Batch fermentation

Amino acids screened in this study were: valine, arginine, phenylalanine and tryptophan which were purchased from Sigma Aldrich. These amino acids were specifically studied because of their active role in multiple biological processes to support Clostridial growth (Fairbairn et al., 2017). Therefore, a pair of valine:arginine and tryptophan:phenylalanine at specified ratio (80:20, 50:50, 20:80) with different concentration (1 and 5 g/L) were incorporated prior to inoculation by using filter sterilization. Furthermore, the fermentation was carried out in 100 mL air tight glass bottles by inoculating 5% (v/v) seed culture and cultivation was continued till 120 h at 37 ± 2 °C. A controlled bioreactor cultivation (3 L bioreactor, Dhruv Biotech, India) containing 1500 mL production medium with 60 g/L total sugar concentration was also performed. Fermentation was initiated with inoculation of 5% active cells. Selected amino acid solution (1 g/L of tryptophan:phenylalanine) was introduced in a bioreactor at optimized time period by using filter sterilization. The fermentation was continued at 37 ± 2 °C until 120 h with intermittent stirring (120 rpm) and pH monitoring by using pH sensor.

2.3. Fed-batch fermentation coupled with in-situ extraction

Generally, the maximum solvent titer and yield are restricted with batch cultivation, mainly due to fixed substrate availability. Fed-batch fermentation although considered suitable for improved butanol production, often observes end product inhibition at higher titers. These concerns can effectively be resolved by adopting an appropriate fed-batch system coupled with *in-situ* recovery. Liquid-liquid extraction was used in this study for selective butanol removal with high efficiency. An overall outline of integrated bioreactor set up is shown in Fig. 1. Fed-batch fermentation was carried out in a bioreactor with an initial volume of 1500 mL. Our previous studies showed that immobilized cell reactor could preferably be used to attain higher reactor productivities (Bankar et al., 2013a; Survase et al., 2012). Hence, 6% cationic resins were used as supporting matrix to

adhere actively growing Clostridial cells in the bioreactor. These resins and their loading ratio were previously optimized for maximum cell growth (data not shown). Amino acid pair (1 g/L of tryptophan:phenylalanine) was exogenously added at optimized time period by filter sterilization. Fermentation was permitted to carry out at batch mode until glucose concentration was decreased to ~15 g/L (72 h). Further drop in glucose concentration may possibly accumulate acids which ultimately halt fermentation process (Nimbalkar et al., 2017; Survase et al., 2011). Therefore, sugar concentration was re-initialized and kept in between 20 and 30 g/L by pulse feeding sterile stock solution, whenever required. This feeding was occurred two to three times and the fermentation was ended after further glucose decrease.

In-situ butanol recovery- The liquid-liquid extraction module was used to remove the produced solvents. *In-situ* product recovery was initiated when butanol production was reached to 10 g/L. Furthermore, around one tenth of the total volume of fermentation broth was pumped into extraction apparatus. The extractant volume (20% decanol in oleyl alcohol) was 1.5 times higher than that of fermentation broth with distribution coefficient of 5.8 ± 0.24 for butanol.

The liquid-liquid extraction parameters were optimized in our previous work (Bankar et al., 2013a) and were directly used in this study. The aqueous phase from separator vessel containing unconsumed nutrients was re-circulated back in bioreactor for its further utilization. Organic phase from separator vessel containing extracted solvents was also re-circulated back into extraction vessel in order to concentrate the solvents in extractant medium.

2.4. Mathematical modeling of ABE production

Literature reports, fermentation kinetics wherein Michaelis-Menten model has been used to estimate the reaction rate terms related to glucose, xylose, acetate and butyrate consumption (Diaz and Willis, 2018). It is essential to propose fermentation kinetics that encompasses rate of utilization of glucose and rate of production of ABE, simultaneously. ABE fermentation is an anaerobic process wherein sugars are consumed by Clostridia to form intermediates product, preferably acids (acetic and butyric acid). These acids are further re-assimilated by Clostridia to produce solvents namely acetone, butanol, and ethanol.

This production pathway can be represented by following reaction scheme:

where A, B, C, D, and E represent glucose, intermediate product, acetone, butanol, and ethanol compounds, respectively. k_1 , k_2 , k_3 , and k_4 are rate constants for rate of formation of intermediate, acetone, butanol, and ethanol, respectively.

The rate of disappearance of glucose can be expressed by following expression:

$$\frac{-dC_A}{dt} = k_1 C_A \quad (1)$$

Integrating Eq. (1) within limits $C_A = C_{A0}$ and $C_A = C_A$ for $t = 0$ and $t = t$, respectively, to get:

$$C_A = C_{A0} e^{-k_1 t} \quad (2)$$

The rate of formation of intermediate product can be expressed by following equation:

$$\frac{dC_B}{dt} = k_1 C_{A_0} - (k_2 + k_3 + k_4) C_B \quad (3)$$

Eq. (3) can be rewritten using Eq. (2) and with $k_5 = k_2 + k_3 + k_4$

$$\frac{dC_B}{dt} = k_1 C_{A_0} e^{-k_1 t} - k_5 C_B \quad (4)$$

Integrating Eq. (4) for limits $C_B = 0$ when $t = 0$ and $C_B = C_B$ when $t = t$, to get:

$$C_B = \frac{C_{A_0} k_1}{k_5 - k_1} \left(e^{-k_1 t} - e^{-k_5 t} \right) \quad (5)$$

The rate expression of formation of acetone can be written as follows:

$$\frac{dC_C}{dt} = k_2 C_B \quad (6)$$

Using Eq. (5), Eq. (6) can be re-written as follows:

$$\frac{dC_C}{dt} = k_2 \frac{C_{A_0} k_1}{k_5 - k_1} \left(e^{-k_1 t} - e^{-k_5 t} \right) \quad (7)$$

Integrating Eq. (7) between the limits $C_C = 0$ and $C_C = C_C$ for the corresponding time limits $t = 0$ and $t = t$, we get:

$$C_C = \frac{k_2 C_{A_0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_2 C_{A_0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right) \quad (8)$$

Similarly, following mathematical equations can be obtained for butanol (Eq. (9)) and ethanol (Eq. (10)) that represent change of concentration of butanol and ethanol with respect to time during fermentation process, respectively.

$$C_D = \frac{k_3 C_{A_0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_3 C_{A_0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right) \quad (9)$$

$$C_E = \frac{k_4 C_{A_0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_4 C_{A_0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right) \quad (10)$$

2.5. Thermodynamics of ABE fermentation

ABE fermentation essentially involves sequential acidogenic and solventogenic phases. The former takes place in an exponential growth phase, wherein the substrate is converted to acetic acid, butyric acid, H_2 , and CO_2 while the latter takes place in a stationary phase, wherein the formed acids are reassimilated into acetone, butanol, and ethanol (Nimbalkar et al., 2017).

The general chemical reaction for ABE production can be written as follows:

The thermodynamic feasibility of fermentation process represented by Eq. (11) can be demonstrated by estimating Gibbs free energy change. This change is equal to the change in enthalpy minus change in product of temperature times the entropy during fermentation process. Since the fermentation process was carried at constant temperature (310.15 K), the change in Gibbs free energy at standard state can be written as follows:

$$\Delta G^{\circ} = \Delta H^{\circ} - T \Delta S^{\circ} \quad (12)$$

The change in enthalpy and entropy at standard state can be estimated using following equations, respectively:

$$\Delta H^{\circ} = \Delta H_0^{\circ} + R \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} dT \quad (13)$$

$$\Delta S^{\circ} = \Delta S_0^{\circ} + R \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} \frac{dT}{T} \quad (14)$$

where ΔH° and ΔS° are standard enthalpy and entropy changes of reaction at fermentation temperature T (310.15 K) while ΔH_0° and ΔS_0° are standard enthalpy and entropy changes at reference temperature T_0 (298.15 K). The standard change in specific heat (ΔC_p°) is given by following expression:

$$\Delta C_p = \sum v_i C_{pi} \quad (15)$$

where C_{pi} and v_i are specific heat capacity and stoichiometric number of a given chemical species i , respectively. The sign convention adopted for stoichiometric number is positive for product and negative for reactant.

Eq. (12) can be re-written using Eqs. (13) and (14) as follows:

$$\Delta G^{\circ} = \Delta H_0^{\circ} + R \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} dT - T \Delta S_0^{\circ} - RT \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} \frac{dT}{T} \quad (16)$$

The standard entropy changes for a reference temperature can be represented as follows:

$$\Delta S_0^{\circ} = \frac{\Delta H_0^{\circ} - \Delta G_0^{\circ}}{T_0} \quad (17)$$

Using Eqs. (16) and (17), we get

$$\frac{\Delta G^{\circ}}{RT} = \frac{\Delta G_0^{\circ} - \Delta H_0^{\circ}}{RT_0} + \frac{\Delta H_0^{\circ}}{RT} + \frac{1}{T} \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} dT - \int_{T_0}^T \frac{\Delta C_p^{\circ}}{R} \frac{dT}{T} \quad (18)$$

Eq. (18) can be used to estimate standard free energy change for a given temperature.

2.6. Analytical methods

Clostridial growth profile was monitored by estimating optical density (OD) at 600 nm using UV-visible spectrophotometer (3000+, LabIndia). A medium pH during fermentation was also measured using a laboratory pH meter (Global, India). Residual sugar was quantified by dinitrosalicylic acid (DNS) method (Nimbalkar et al., 2018). Total solvents (acetone, butanol, and ethanol) and acids (acetic and butyric acid) were analyzed by gas chromatography as per previous study (Nimbalkar et al., 2018).

3. Results and discussion

3.1. Effect of exogenous amino acid supplementation on butanol titer

Generally, microorganisms secrete specific amino acids which help them to sustain in harsh environmental conditions. Besides, it has been observed that some microorganisms preferentially

and sequentially degrade amino acids to use them as carbon and energy source (Fonknechten et al., 2010). *Clostridium* is able to utilize several amino acids *via* Stickland reaction (Sangavai and Chellapandi, 2017). Therefore, it was thought desirable to evaluate the effect of exogenous aromatic and aliphatic amino acids on Clostridial behaviour and ABE fermentation. Valine, arginine, tryptophan and phenylalanine were specifically assessed in the current investigation. Valine and arginine are also good nitrogen sources and thus support bacterial growth. On the other hand, tryptophan and phenylalanine are mainly secreted in defense mechanism and might be useful to elevate cell survival rate. Moreover, amino acids are also used to produce various 2-keto acids which can be diverted to higher alcohol synthesis (Atsumi et al., 2008). Interestingly, amino acid assimilation in Clostridial genus is mainly characterized by oxidation of one amino acid coupled with reduction of another (Fonknechten et al., 2010). Therefore, the amino acids were studied in combination to observe their synergistic influence during butanol production. Initially, the pairs (valine:arginine and tryptophan: phenylalanine) were tested at two different concentrations of total 1 and 5 g/L with their ratio as 80:20, 50:50, and 20:80. Fig. 2 depicts ABE production in presence of 1 g/L total amino acids.

A

b

Fig. 2. (A) ABE production by using *C. acetobutylicum* NRRL B-527 in presence of valine:arginine (V:A): a-80:20; b-50:50; c-20:80; C- Control (B) ABE production by using *C. acetobutylicum* NRRL B-527 in presence of tryptophan:phenylalanine (T:P): a-80:20; b-50:50; c-20:80; C- Control.

Control experiment without amino acid was able to produce butanol up to 6.92 ± 0.01 g/L with total ABE of 10.03 ± 0.11 g/L. Interestingly, both amino acid pairs significantly improved butanol production thus resulted in enhanced solvent titer. Fig. 2A shows ABE production profile when valine:arginine (1 g/L) was added in the fermentation medium. All combinations of valine:arginine ratios were effective in increasing butanol level as compared to control. The addition of valine:arginine at ratio of 20:80 resulted in highest butanol production of 10.11 ± 0.23 g/L with total ABE to be 15.70 ± 0.31 g/L. This outcome clearly suggest that arginine has a huge effect on solvent production in Clostridia. It has been observed that arginine is rapidly metabolises into ornithine *via* citrulline which results into energy (ATP) build up (Fonknechten et al., 2010; Sangavai and Chellapandi, 2017). Besides, butyric acid production is also known to be up-regulated in presence of valine:arginine, which directly supports the butanol production in Clostridia (Fig. 2A-c). Valine, mostly influence the production of branched chain alcohols and hence perhaps played a minor role in combination with arginine (Atsumi et al., 2008).

Another pair namely tryptophan:phenylalanine showed nearly 18% increase in butanol concentration as compared to control, when added at ratio of 80:20 (Fig. 2B-a). This improvement is because of tryptophan which is a precursor in de novo synthesis of NADH and NADPH that derived more metabolic flux toward reduced product (butanol). Moreover, slight fluctuation in production patterns were observed for other varied ratios of this combination (Fig. 2B-b,c). The higher amount (5 g/L) of amino acid incorporation was not effective and resulted in lowered product titer (although more than control) compared to 1 g/L addition (Fig. S1). Therefore, 5 g/L of amino acid addition was not considered in further experimental trials. The higher concentration of metabolic precursors are inhibitory to the *Clostridium* butanol production. Chen and Li (2018) observed that higher concentration of ionic liquids could inhibit the cell growth mainly because of their hydrophobic nature and thus decelerated the butanol production. Furthermore, amino acid production in yeast can result in the accumulation of precursors, thereby providing more substrate pool for successive conversion which ultimately improves final product output (Su et al., 2016). Clostridial cells secrete certain amino acids only under stress conditions which could then elevate butanol titer as well as strain tolerance (Luo et al., 2016). The molecular basis of this phenomenon is still unknown. The presence of amino acids effectively triggered solvent production in current

study. Hence, it was decided to evaluate their impact more thoroughly. Therefore, tryptophan:phenylalanine and valine:arginine at optimal ratio of 80:20 and 20:80, respectively, were added at different fermentation time intervals.

Fig. S1. ABE production by using *C. acetobutylicum* NRRL B-527 in presence of valine:arginine (A) and tryptophan:phenylalanine (B) at 5 g/L concentration

3.2. Time of amino acid addition during fermentation study

Amino acids were added at different fermentation time intervals to study their effect on triggering the biobutanol pathway at different stages of Clostridial growth. Both the amino acid pairs were separately added in fermentation medium at time interval of 0, 4, 8, 18, and 24 h. The Clostridia tend to enter in solventogenic phase after 24 h cultivation (Nimbalkar et al., 2018). Hence, addition of amino acids after 24 h was not considered in this study.

Fig. S2 shows solvent production profiles when amino acids were added at different fermentation times. The varying time of addition positively influenced ABE fermentation, thereby resulted in additional increment in solvent titer. Tryptophan (80):phenylalanine (20) at 1 g/L concentration produced maximum butanol and ABE of 11.41 ± 0.02 and 18.70 ± 0.11 g/L respectively, when added after 8 h fermentation. This reflects 26% improvement in solvent production when compared with initial (0 h) amino acid supplementation (Fig. S2). Amino acid addition after 8 h fermentation did not substantially improve the solvent production. Amino acids added during lag phase (0-8 h) are likely to be utilized as a nutritional component instead of metabolic precursor during biosynthetic pathway. It is evident that amino acids generally assimilates in the exponential

growth phase (Sangavai and Chellapandi, 2017). Thus, delayed amino acid addition may not trigger Clostridial behaviour. This fact is in-line with a report wherein amino acids were supplemented after 24 h fermentation and very limited improvement in ABE titer was observed (Luo et al., 2015). Further, the acetone and ethanol productions were slightly lowered when amino acids were added after 18 h. Therefore, it was concluded that addition of tryptophan: phenylalanine at 8 h fermentation was optimum for enhanced solvent production.

Fig. S2. Time-course of amino acid supplementation in ABE fermentation process by using *C. acetobutylicum* NRRL B-527: A- tryptophan:phenylalanine (80:20); B- valine:arginine (20:80) Valine (20):arginine (80) with optimal concentration of 1 g/L did not show considerable difference in butanol production when added at different fermentation time intervals (Fig. S2). Moreover, slight increment in acetone production was observed thus reaching total ABE to be 17.13 ± 0.12 g/L along with butanol titer of 10.81 ± 0.03 g/L after 8 h addition. Grassi et al. (2018) examined high acetone production either because of an earlier activation of solventogenic phase or as a result of changes in cellular metabolism. Another reason could be the presence of arginine which facilitated acetate formation thereby leading to increase in acetone level.

Gutierrez et al. (2015) concluded that timing of nitrogenous compound addition, determines the fermentation performance and aroma characteristics of wines by using three different yeast strains. Incidentally, butanol titer was improved in current study with early supplementation of amino acids. This finding is in-line with our previous report, wherein trace elements showed a profound effect when added after 8 h of fermentation (Nimbalkar et al., 2018). Interestingly, amino acids added at different fermentation times greatly altered butanol to acetone (B:A) ratio, especially during delayed additions (Fig. S3). This alteration in B:A ratio is evident because of increased acetone

level which is in agreement with previous reported study that utilized benzyl viologen for reduced B:A ratios (Nasser Al-Shorgani et al., 2015).

Fig. S3. Impact of varying time of amino acid addition on butanol:acetone (B:A) ratio: valine:arginine (V:A); tryptophan:phenylalanine (T:P)

3.3. Batch fermentation kinetics

Based on initial screening data, it was found that tryptophan:phenylalanine pair was most favorable for butanol synthesis. Therefore, it was critically evaluated further to observe their effect on Clostridial growth, pH shift during fermentation, and glucose consumption. Tryptophan:phenylalanine pair significantly enhanced Clostridial growth in comparison with control (maximum O.D600=1.75) that can be seen in Fig. S4.

Initially, a gradual increase in cell density was observed till 36 h of fermentation which corresponds to exponential growth phase. Subsequently, cells entered in the stationary phase wherein maximum solvent formation was recorded (19.85 ± 0.18 g/L). The pH trend of culture with added amino acid was quite similar to the control, indicating that this addition did not alter the physiology of Clostridia. Therefore, the switch from acidogenic phase to solventogenesis took place at around pH 4.5 that remained constant in between 4.5 and 5 during rest of fermentation.

The glucose consumption by *C. acetobutylicum* B-527 was rapid in a medium supplemented with amino acids. Thus, about 90% glucose was effectively utilized within 120 h of fermentation. An increased glucose utilization followed by improved solvent production ultimately resulted in enhanced solvent yield. The highest butanol yield and productivity of 0.22 g/g and 0.10 g/L.h respectively were achieved with batch operational mode. Control experiment (absence of amino acid) demonstrated butanol yield of 0.15 g/g along with productivity of 0.05 g/L.h.

Fig.S4. Effect of tryptophan:phenylalanine (1g/L; after 8 h addition) on growth, fermentation pH and substrate utilization by using *C. acetobutylicum* NRRL B-527

Fig. 3 essentially shows the change in concentration of glucose and fermentation products viz. acetone, butanol, and ethanol with respect to time. All the symbols represent experimental values while continuous solid lines represent predicted values from corresponding mathematical models. The kinetic parameters were estimated by fitting mathematical models to experimental data as summarized in Table 1. A good agreement was observed between experimental and predicted values with standard deviation of $\pm 5\%$. It can be inferred that the rate of disappearance of glucose follows first order kinetics with respect to its concentration. The rate of ABE production also follows first order kinetics with respect to intermediate product concentrations. This implies that the rate of formation of ABE is linearly proportional to the concentration of intermediate products viz. acetic acid and butyric acid. However, it is well known that the rate of ABE formation is adversely affected when acid concentration exceeds critical value (4.4 g/L) (Behera et al., 2014; Napoli et al., 2011). It has been reported that critical value of acid concentration is mainly a function of type of strain and the medium composition (Napoli et al., 2011). Accordingly, Ezeji et al. (2007) reported the critical acid concentration equal to 1.98 g/L for *C. beijerinckii* BA101 whereas Kudahettige-Nilsson et al. (2015) found out threshold limit of 3.3 g/L for *C. acetobutylicum* ATCC 824. Eqs. (8)-(10) indicates that concentration of acetone, butanol and ethanol, respectively, is a strong function of initial glucose concentration at a given time.

Batch fermentation under controlled condition was also conducted in 3 L bioreactor. The Clostridial behaviour was similar to the bottle culture cultivation with some improvement in total solvent titer. The acetone production was unexpectedly increased which resulted in total ABE to be 21.77 ± 0.19 g/L. This specific upsurge in acetone titer was may be due to moderately lower NADH regeneration rate during ABE fermentation. Based on key results, it was concluded that the exogenous tryptophan:phenylalanine addition positively supported Clostridial growth as well as ABE production. The butyrate and acetate addition also promotes intracellular accumulation of favourable amino acids (methionine, tyrosine and phenylalanine) which in turn enhance solvent

titers (Luo et al., 2015, 2016). Another report indicated that the wort amino acids showed positive effect on higher alcohol fermentation performance and flavour profile (Yin et al., 2017).

Fig. 3. ABE production profile in presence of tryptophan:phenylalanine (1 g/L) by using *C. acetobutylicum* NRRL B-527.

Table 1 Batch model parameter values

Compounds	Kinetic expression (g/L.h)	Concentration expression (g/L)	Rate constants (1/h)	
			Notation	Values
Glucose	$\frac{dC_A}{dt} = k_1 C_A$	$C_A = C_{A0} e^{-k_1 t}$	k_1	0.0196
Acetone	$\frac{dC_C}{dt} = k_2 C_B$	$C_C = \frac{k_2 C_{A0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_2 C_{A0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right)$	k_2	0.0020
Butanol	$\frac{dC_D}{dt} = k_3 C_B$	$C_D = \frac{k_3 C_{A0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_3 C_{A0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right)$	k_3	0.0036
Ethanol	$\frac{dC_E}{dt} = k_4 C_B$	$C_E = \frac{k_4 C_{A0} k_1}{k_5 - k_1} \left(\frac{e^{-k_1 t}}{-k_1} - \frac{e^{-k_5 t}}{-k_5} \right) + \frac{k_4 C_{A0} k_1}{k_5 - k_1} \left(\frac{1}{k_1} - \frac{1}{k_5} \right)$	k_4	0.0006

$$k_5 = k_2 + k_3 + k_4$$

Overall, increments in butanol concentration could mainly be limited due to substrate deficiency, during batch cultivation. Low productivities and product inhibition are major bottlenecks in ABE batch fermentation (Lipovsky et al., 2016). Hence, fed-batch fermentation strategy was considered and discussed in subsequent section.

3.4. Fed-batch fermentation coupled with in situ extraction

During fed-batch operation, fermentation was initially allowed to run in batch mode until glucose level was declined up to 15 ± 1.0 g/L after 72 h from its initial value of 60 g/L (Fig. 4). Therefore, first feeding was carried out at 72 h using sterile P2 medium containing 12% glucose. The pulse feeding resulted into glucose re-initialization to 25 ± 1.0 g/L and fermentation was continued

thereafter. The rapid glucose consumption in this phase was due to high cell density, which was achieved using supporting matrix (cationic resins). As a result, a steep increase in solvent production was observed thereby reaching ABE titer to 18.69 ± 0.09 g/L after 96 h cultivation. Simultaneously, the glucose concentration was further dropped below threshold limit (< 20 g/L) which halt the acid re-assimilation (Ibrahim et al., 2015). Hence second feeding (P2 medium containing 12% glucose) was initiated in order to mitigate the substrate limitation. The feeding strategy adopted was helpful in preventing excessive product dilution as feeding was performed in pulse-feed mode, which is a hurdle in further separation step. Also, it is worthwhile to mention that the fermentation medium pH was not severely affected throughout cultivation and remained constant at around pH 4.8. Furthermore, butanol concentration was consistently higher in the later stage of fermentation (Fig. 4A). As a consequence, an inhibitory effect of butanol was observed that restricted its titer to 13.44 ± 0.16 g/L with total ABE of 24.49 ± 0.29 g/L in fed-batch cultivation. Overall, ABE productivity in fed-batch fermentation (0.14 g/L.h) was quite comparable with batch fermentation mode which eventually supports its further up scaled operational behaviour. These findings indicated that the positive effect of tryptophan:phenylalanine (1 g/L) was consistent in fed-batch fermentation as well. The other research reports also demonstrated that fed-batch fermentation could be a better strategy to improve final solvent titer (Chang et al., 2012; Gao and Tan, 2003; Pang et al., 2016). In order to overcome severe product inhibition, it is essential to remove butanol soon before it gets detrimental. Among other byproducts, butanol is known to have major inhibitory effect on growing Clostridia which then reduces glucose utilization affecting fermentation performance (Mayank et al., 2012; Rochon et al., 2017). Hence, it was highly desirable to incorporate a recovery module during fed-batch fermentation. A liquid-liquid extraction module which was optimized for higher ABE extraction was used based on previous experience (Bankar et al., 2012, 2013a). Fig. 4B depicts the performance of integrated fed-batch system with respect to sugar consumption and ABE production. It was observed that, with depleting glucose concentration, the ABE production was linearly increased. The respective glucose feeding was carried out to ensure sufficient substrate concentration during fermentation. It should be noted that extraction was performed only when butanol concentration rose above 10 ± 1.0 g/L. The first cycle of extraction was performed after 96 h cultivation, during which butanol titer was about 11.37 ± 0.11 g/L. An efficient and selective butanol recovery ($\sim 77\%$ removal efficiency) was helpful in relieving butanol toxicity. The overall growth in a bioreactor was consistent even at higher ABE titer (33.16 ± 0.63 g/L) especially because of lower butanol toxicity executed by *in-situ* product recovery module. Table 2 compares the performance of batch and fed-batch fermentation. The fed-batch mode of operation clearly showed an edge over batch fermentation in terms of solvent titer and yield. However, the batch mode showed better performance in terms of productivity than fed-batch mode. The lower solvent productivity in fed-batch fermentation is because of longer operational time. However, the fed-batch system was continuously operated until 240 h without any system failures. The successful longer operation can be advantageous for large scale operations. The accumulation of inactive and dead cells in a bioreactor is one of limitations in this system.

Fig. 4. Fed-batch fermentation performance in presence of tryptophan:phenylalanine by using *C. acetobutylicum* NRRL B-527: A- without solvent recovery integration; B- integrated solvent recovery system; (red arrows) - glucose feeding; (blue arrow) - solvent extraction

Interestingly, significant step up in butanol concentration (20.82 ± 0.33 g/L) was achieved by incorporating integrated recovery system (Table 2). Indeed, improvement in solvent yield and productivity is expected to get through continuous production system. Thus, attempts to explore the continuous ABE production are ongoing, which will be followed in next report.

Table 2 Comparison of butanol production in different modes of fermentation

Parameters	Batch fermentation		Fed-batch fermentation*	
	Serum bottle (100 mL)	Bioreactor (3 L)	Without <i>in situ</i> recovery	<i>In situ</i> recovery

Total initial sugars (g/L)	60±1.0	60±1.0	60 (+25 [#])±1.0	60 (+41 [#])±1.0
Fermentation time (h)	120	120	168	240
Sugar conversion (%)	~90	~90	~94	~98
Acetone (g/L)	5.98±0.05	7.35±0.08	9.09±0.10	10.08±0.21 ^{\$}
Butanol (g/L)	11.88±0.12	12.43±0.10	13.44±0.16	20.82±0.33 ^{\$}
Ethanol (g/L)	1.99±0.01	1.99±0.01	1.95±0.03	2.26±0.09 ^{\$}
Total ABE (g/L)	19.85±0.18	21.77±0.19	24.49±0.29	33.16±0.63
Butanol yield (g/g)	0.21	0.22	0.16	0.21
Butanol productivity (g/L.h)	0.09	0.10	0.08	0.08
Total acids (g/L)	0.30±0.01	0.59±0.01	0.94±0.11	1.39±0.09

*Cationic resins were used to adhered cells; # Total sugar added during fed batch mode;

\$ Calculated by considering recovered titers

3.5. Thermodynamics of ABE fermentation

Thermodynamic analysis of fermentation process has been performed under isothermal and isobaric conditions with special emphasis on microbial growth yield (Teh and Lutz, 2010; VanBriesen, 2002). Thermodynamic analysis of overall fermentation process (Eq. (11)) was performed to determine spontaneity and feasibility of the reaction. The spontaneity and feasibility of fermentation process can be predicted by estimating thermodynamic property, standard Gibbs free energy change (ΔG°) under isothermal and isobaric conditions. The ΔG° determines whether a reaction is spontaneous in the forward direction, backward direction or is at equilibrium. When $\Delta G^\circ < 0$, the process is exergonic and will proceed spontaneously in the forward direction to form products. When $\Delta G^\circ > 0$, the process is endergonic and not spontaneous in the forward direction. Instead, it proceeds in the backward direction spontaneously. When $\Delta G^\circ = 0$, the system is in equilibrium and the concentrations of products and reactants remain constant.

In present work, ABE fermentation was carried out at 310.15 K under atmospheric pressure. The values of ΔH° , ΔS° , and ΔG° were estimated using Eqs. (13), (14) and (18) to be -179.57×10^{13} kJ/mol, 3.94 kJ/mol/K and -1401.55 kJ/mol, respectively. It can be inferred that fermentation reaction is exothermic ($\Delta H^\circ < 0$) and subsequently release -4830.03×10^{-11} kJ/mol of glucose consumed. Further, change in entropy during fermentation process is positive, indicating that entropy of the fermentation system increases as fermentation reaction proceeds in forward direction. The exothermic nature of fermentation process and positive change in entropy during fermentation process point out that, the change in ΔG° is negative. The negative change in ΔG° signifies that fermentation process is always spontaneous in forward direction to produce ABE.

4. Conclusions

This work investigated the effect of amino acid on butanol production during batch and fed batch operation. It also confirmed that tryptophan and phenylalanine can essentially up-regulate ABE production by using *Clostridium*. Besides, the time course of amino acid inclusion revealed that the carbon flux gets diverted to acetone during delayed solventogenic phase additions. Further, fed-batch fermentation substantiates the elevated solvent levels with longer operation. According

to reaction engineering, it was concluded that ABE fermentation is spontaneous process wherein solvent formation took place by first order kinetics. This means that the rate of solvent formation is linearly dependent on substrate concentration.

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