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# A green process for the production of butanol from butyraldehyde using alcohol dehydrogenase: process details†

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Depletion of energy sources has drawn attention towards production of bio-butanol by fermentation. However, the process is constrained by product inhibition which results in low product yield. Hence, a new strategy wherein butanol was produced from butyraldehyde using alcohol dehydrogenase and NADH as a cofactor was developed. Butyraldehyde can be synthesized chemically or through fermentation. The problem of cofactor regeneration during the reaction for butanol production was solved using substrate coupled and enzyme coupled reactions. The conventional reaction produced 35% of butanol without regeneration of cofactor using 300  $\mu\text{M}$  NADH. The process of substrate coupled reaction was optimized to get maximum conversion. NADH (30  $\mu\text{M}$ ) and 100  $\mu\text{g}$  per ml of alcohol dehydrogenase (320  $\text{U mg}^{-1}$ ) could convert 17.39 mM of butyraldehyde to butanol using ethanol (ratio of butyraldehyde to ethanol 1 : 4) giving a maximum conversion of 75%. The enzyme coupled reaction under the same conditions showed only 24% conversion of butyraldehyde to butanol using the glutamate dehydrogenase-L-glutamate enzyme system for the regeneration of cofactor. Hence, substrate coupled reaction is suggested as a better method over the enzyme coupled reaction for the cost effective production of butanol.

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## 1. Introduction

High prices of conventional oil in the global market coupled with depletion of resources have been the driving force for researchers all over the world to work on renewable energy sources. This has made bio-fuels commercially important and efforts to produce it economically are being made worldwide. Butanol is an alternative source of energy and is better than ethanol. Butanol is less volatile, less hygroscopic and less corrosive.<sup>1</sup> Production of bio-butanol by fermentation process is well reported by several researchers. Chemical method of synthesis of butanol has been reported using either the oxo process starting from propylene or the aldol process starting from acetaldehyde.<sup>2</sup> Ethanol is a renewable source which can be derived from biomass. It can be converted to valuable chemicals. Chemical conversion of ethanol to butanol has been previously reported. The yield of butanol production using ethanol with MgO as catalyst was 18.39%.<sup>3</sup> This conversion

needs high temperature with very low yield of 15–21%. The temperature required for liquid phase reaction is 120 °C and for gas phase reaction 300–450 °C.<sup>4,5</sup> Moreover, the process involves multiple steps and many byproducts. Hence attempts were made successfully to selectively synthesize butanol from ethanol over strontium phosphate hydroxyapatite catalysts.<sup>6</sup>

Biological synthesis of butanol is by the fermentation using *Clostridium* spp. It can be obtained from renewable sources by acetone–butanol–ethanol (ABE) fermentation.<sup>7,8</sup> The traditional ABE fermentation gives low yield which is due to an inhibitory effect of the produced butanol on the growth of the organism.<sup>9</sup> Recently, many molecular and fermentation techniques have been used to increase the production of butanol. The development of hyper-butanol producing strain and integrated ABE fermentation for the simultaneous production and removal of butanol from the fermentation broth has been reported.<sup>10</sup> The need for inexpensive feedstocks, improved fermentation performance, sustainable solvent recovery, and water recycle are the major challenges for ABE fermentation.<sup>11</sup> ABE fermentation has a drawback of low yield due to butanol toxicity for the organism which makes it necessary to explore new strategies for the production of butanol. In *Clostridium* spp., the last step for butanol production is the conversion of butyraldehyde to butanol using butanol dehydrogenase. Based on this *in vivo* reaction occurring in the cell, we report a new strategy where butyraldehyde (which itself can be obtained from variety of

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chemical and microbial sources) can be biocatalytically converted to butanol *in vitro*.

Butyraldehyde has been produced from 1,2-butanediol by catalytic dehydration in supercritical water.<sup>12</sup> It can also be synthesized by hydroformylation of propylene.<sup>13</sup> Butyraldehyde can also be obtained by fermentation to overcome the limitations of chemical processes and raw materials. A spontaneous mutant of *Clostridium acetobutylicum* has been shown to produce significant amounts of butyraldehyde.<sup>14</sup> *Streptomyces cinnamonensis* has been shown to excrete butyraldehyde, isobutyraldehyde and valeraldehyde.<sup>15</sup> Butyraldehyde obtained by the above processes can be used for the butanol production.

Recent concerns on the environmental quality and energy resources have made biocatalysis attractive for industrial applications. Biocatalytic reactions are environmentally friendly, selective and energy efficient.<sup>16</sup> Butanol dehydrogenase can be used to obtain butanol from butyraldehyde. However, this enzyme is not easily available and literature on the production and purification of butanol dehydrogenase is scant.<sup>17,18</sup> Here, we have used alcohol dehydrogenase for the production of butanol from butyraldehyde using NADH as a cofactor. Alcohol dehydrogenase is easily available from many sources including *Saccharomyces cerevisiae*. It is an important catalyst for organic synthesis especially for chiral alcohols.<sup>19</sup>

The cofactors are usually expensive. This necessitates finding a way to regenerate and reuse them efficiently.<sup>20</sup> Different cofactor regeneration strategies including biological, enzymatic (substrate coupled and enzyme coupled), electrochemical, chemical and photochemical have evolved over a period of time.<sup>21</sup> Substrate coupled reaction contains one enzyme which use both oxidized and reduced form of cofactor. It synthesizes the product in one reaction using one substrate and regenerates the cofactor in another reaction using second substrate. Enzyme coupled reaction uses two enzymes to carry out two different reactions. Each enzyme needs separate substrate to convert into product and regenerate the cofactor.<sup>16</sup>

Here, we have attempted a biocatalytic conversion of butyraldehyde to butanol using alcohol dehydrogenase as the catalyst and NADH as the cofactor. The cofactor regeneration was attempted using ethanol as substrate for the substrate coupled reaction and glutamate dehydrogenase-L-glutamate system for the enzyme coupled reaction. We compared two methods of cofactor regeneration to suggest a possible cost effective method for the butanol production from butyraldehyde.

## 2. Experimental

### 2.1 Materials

Alcohol dehydrogenase (320 U mg<sup>-1</sup>) from *Saccharomyces cerevisiae*, butyraldehyde, NADH and butanol was purchased from Sigma Aldrich, USA. Ethanol, L-glutamic acid and glutamate dehydrogenase were purchased from Altia Oy, Finland, M.P. Biomedicals Inc., Germany and Roche Diagnostics, Germany, respectively.

### 2.2 Production of butanol from butyraldehyde by conventional reaction

The reaction mixture was prepared by adding butyraldehyde (17.39 mM), alcohol dehydrogenase (100 µg ml<sup>-1</sup>) and NADH (50–300 µM). Control was prepared similarly but with the enzyme being replaced by water. The reaction mixture was continuously mixed by keeping it on the shaker for 3 h. The reaction was stopped by adding oxidized pectin (0.5 mg ml<sup>-1</sup>) which inhibits the alcohol dehydrogenase. In our previous work, oxidized pectin had shown complete inhibition of alcohol dehydrogenase (data not shown). An aliquot of the mixture was then checked for the production of butanol using gas chromatography (GC) (Hewlett Packard series 6890) equipped with a flame ionization detector and DB-WAXetr capillary column (30 m × 0.32 mm × 1 µm). The injector temperature was 200 °C and the detector temperature was 250 °C. The heating profile was 10 °C min<sup>-1</sup> starting from the initial value of 40 °C. Internal standard (1-propanol) was used for all samples. The retention times for acetaldehyde, butyraldehyde, ethanol and butanol were 1.2, 2.2, 2.8 and 5.6 minutes, respectively. The injector volume was 10 µl.

### 2.3 Production of butanol from butyraldehyde by substrate coupled reaction

**2.3.1 Optimization of concentration of NADH for the production of butanol.** Reaction mixture was prepared by adding butyraldehyde (17.39 mM), ethanol (69.56 mM), alcohol dehydrogenase (100 µg ml<sup>-1</sup>) and NADH (2.5–50 µM). The reaction mixture was continuously mixed by keeping it on the shaker for 3 h. Control was prepared similarly but with enzyme replaced by water. The reaction was stopped by adding oxidized pectin (0.5 mg ml<sup>-1</sup>) which inhibited the alcohol dehydrogenase. An aliquot of mixture was then checked for the production of butanol using GC.

**2.3.2 Optimization of ratio of butyraldehyde to ethanol for the production of butanol.** The reaction mixture was prepared as explained in Section 2.3.1. The ratio of butyraldehyde : ethanol was varied from 4 : 1–1 : 8 using 17.39 mM butyraldehyde and ethanol varying from 4.34–139.13 mM. NADH (30 µM) and alcohol dehydrogenase (100 µg ml<sup>-1</sup>) were kept constant. All other conditions were maintained as explained above and checked for the production of butanol after 3 h.

**2.3.3 Optimization of concentration of enzyme for the production of butanol.** The reaction mixture was prepared as explained in Section 2.3.1. Enzyme concentration was varied from 10–120 µg ml<sup>-1</sup>. Ratio of butyraldehyde : ethanol (1 : 4) and NADH (30 µM) were kept constant. Other conditions were maintained as explained above and checked for the production of butanol after 3 h.

**2.3.4 Optimization of concentration of butyraldehyde for the production of butanol.** The reaction mixture was prepared as explained in Section 2.3.1. The concentration of butyraldehyde was varied from 4.34–43.47 mM, while keeping the ratio of butyraldehyde : ethanol at 1 : 4. Enzyme concentration (100 µg ml<sup>-1</sup>) and NADH (30 µM) were kept constant. Other conditions

were maintained as explained above and checked for production of butanol after 3 h.

**2.3.5 Optimization of time for the production of butanol using 17.39 mM butyraldehyde.** The reaction mixture was prepared similarly as explained in Section 2.3.1. Butyraldehyde (17.39 mM), ethanol (69.56 mM), alcohol dehydrogenase (100  $\mu\text{g ml}^{-1}$ ) and NADH (30  $\mu\text{M}$ ) were used in the reaction. The reaction mixture was continuously mixed by keeping it on the shaker till 3 h. Samples were withdrawn every 30 min and checked for production of butanol.

**2.3.6 Optimization of time for the production of butanol using 34.78 mM butyraldehyde.** The reaction was carried out as explained in Section 2.3.1. Butyraldehyde (34.78 mM), ethanol (155.78 mM), alcohol dehydrogenase (100  $\mu\text{g ml}^{-1}$ ) and NADH (30  $\mu\text{M}$ ) were used in the reaction. The reaction mixture was continuously mixed by keeping it on the shaker till 9 h. Samples were withdrawn every 60 min and checked for production of butanol.

## 2.4 Production of butanol from butyraldehyde using enzyme coupled reaction

Reaction mixture was prepared by adding butyraldehyde (17.39 mM), alcohol dehydrogenase (100  $\mu\text{g ml}^{-1}$ ), glutamate dehydrogenase (100  $\mu\text{g ml}^{-1}$ ), L-glutamate (69.56 mM) and NADH (30  $\mu\text{M}$ ). The reaction mixture was continuously mixed by keeping it on the shaker for 3 h. Control was prepared by using same reaction mixture except that the enzyme was replaced with water. The reaction was stopped by adding oxidized pectin (0.5  $\text{mg ml}^{-1}$ ) which inhibits the alcohol dehydrogenase. An aliquot of the mixture was then checked for the production of butanol using GC.

## 3. Results and discussion

### 3.1 Production of butanol from butyraldehyde by conventional reaction

*Clostridium* spp. produces butanol by the fermentation process in which the last step is the conversion of butyraldehyde to butanol by butanol dehydrogenase.<sup>22</sup> The same reaction can be mimicked *in vitro* using alcohol dehydrogenase. Alcohol dehydrogenase is easily available, less expensive as compared to butanol dehydrogenase and it catalyses the reduction of aldehyde to alcohol.<sup>23</sup> Conventionally, butyraldehyde can be enzymatically converted to butanol using NADH, where NADH is the limiting factor in the reaction. The reaction stops after all the NADH is consumed. Hence there is a need to provide large amount of NADH in the reaction to convert all the available butyraldehyde to butanol. An increase in the NADH supply from 50 to 300  $\mu\text{M}$  increased the butanol production from 0.4 to 1.2 mM (Fig. 1). The conversion of butanol production varied from 25 to 35%. The conventional reaction is not cost effective as there is a continuous need to supply large amount of NADH. This is a drawback of the reaction to proceed further. Hence the cofactor should be regenerated to make it continuously available in the reaction.

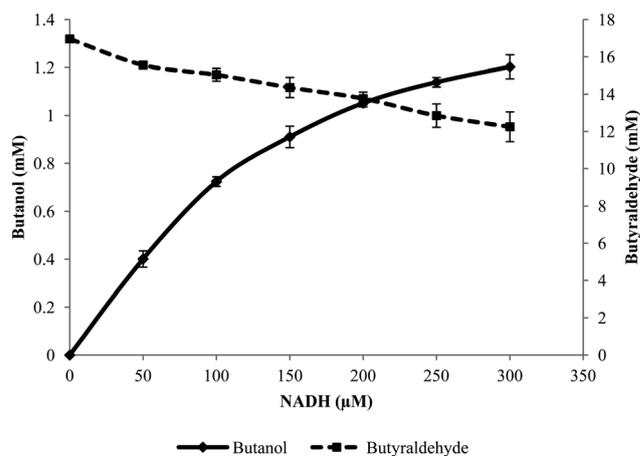


Fig. 1 The production of butanol from butyraldehyde by conventional reaction.

### 3.2 Production of butanol from butyraldehyde by substrate coupled reaction

**3.2.1 Optimization of concentration of NADH for the production of butanol.** Substrate coupled reaction overcome the drawbacks of conventional reaction by continuous supply of NADH by its regeneration through another reaction occurring in the process. Butyraldehyde and ethanol were used as substrates for the enzyme alcohol dehydrogenase. Lower level of NADH was used for the substrate coupled reaction as there is continuous supply of NADH in the reaction. Butanol production was seen to increase with an increase in concentration of NADH (2.5 to 30  $\mu\text{M}$ ). Maximum conversion of butyraldehyde to butanol (75%) was obtained at 30  $\mu\text{M}$  of NADH (Fig. 2). The butanol production remained unaltered above 30  $\mu\text{M}$  of NADH. Butyraldehyde was converted to butanol with simultaneous conversion of NADH to NAD. The NAD so produced was used by the alcohol dehydrogenase to convert the ethanol to acetaldehyde and which regenerated the NADH. There was a continuous supply of NADH due to which the low initial concentration of

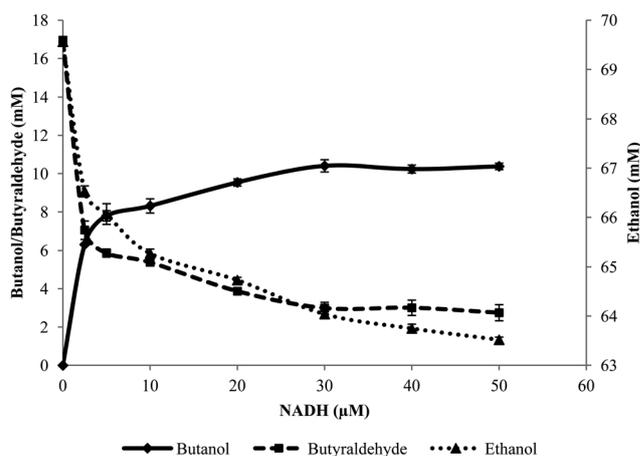


Fig. 2 Optimization of concentration of NADH for the production of butanol by substrate coupled reaction.

NADH could convert all the available butyraldehyde to butanol giving a high conversion. NADH at 30  $\mu\text{M}$  was sufficient to convert 17.39 mM of butyraldehyde to butanol in 3 h.

**3.2.2 Optimization of ratio of butyraldehyde : ethanol for the production of butanol.** Concentration of both the substrates is an important parameter for the substrate coupled reaction because it affects the reaction equilibrium. Concentration of ethanol should be sufficiently high to carry out the forward reaction of conversion of ethanol to acetaldehyde and simultaneously the conversion of NAD to NADH. Different ratios of butyraldehyde : ethanol were used in the reaction. When the ethanol concentration was lower or equal to butyraldehyde (butyraldehyde to ethanol ratio 4 : 1–1 : 1), the production of butanol was 3 to 8 mM (Fig. 3). The production of butanol was found to be maximum (10.63 mM) at butyraldehyde : ethanol ratio of 1 : 4, where the conversion was 75%. The butanol production was found to decrease with increasing amount of ethanol. At ratios of 1 : 6 and 1 : 8, the butanol production was 69% and 68%, respectively. Hence, a 1 : 4 ratio of butyraldehyde : ethanol was selected for the reaction. Higher concentration of ethanol inhibited alcohol dehydrogenase and thereby decreased the production of butanol.<sup>24</sup>

**3.2.3 Optimization of concentration of enzyme for the production of butanol.** The use of minimum amount of enzyme is important in the enzymatic reaction to obtain the product cost effectively. Various concentrations (10–120  $\mu\text{g ml}^{-1}$ ) of alcohol dehydrogenase were used for the reaction of butanol production. At lowest concentration (10  $\mu\text{g ml}^{-1}$ ) of alcohol dehydrogenase, the butanol production was 46% (Fig. 4). The highest butanol production (10.63 mM) and conversion efficiency (75%) were obtained at 100  $\mu\text{g}$  per ml of alcohol dehydrogenase, beyond which there was no further increase in the yield. Hence the optimum concentration of alcohol dehydrogenase for the conversion of 17.39 mM of butyraldehyde to butanol was 100  $\mu\text{g ml}^{-1}$ .

**3.2.4 Optimization of concentration of butyraldehyde for the production of butanol.** The maximum conversion of substrate to the product that an enzyme could carry out was

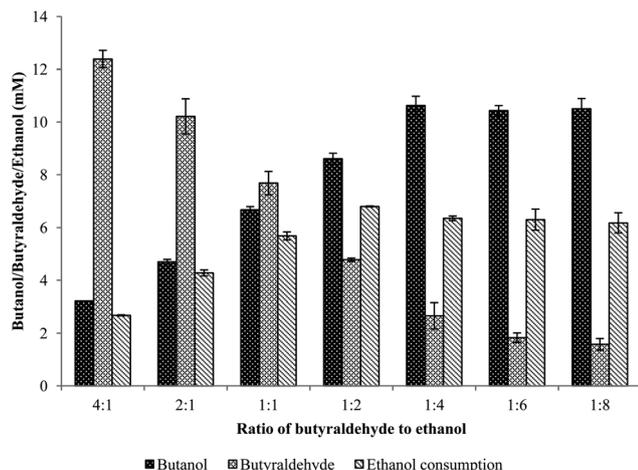


Fig. 3 Optimization of ratio of butyraldehyde to ethanol for the production of butanol by substrate coupled reaction.

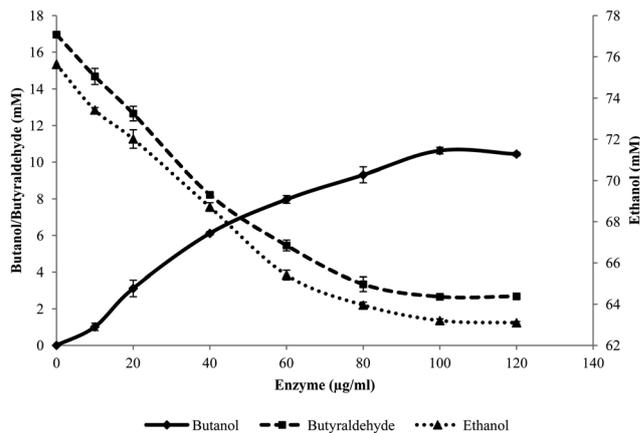


Fig. 4 Optimization of concentration of enzyme for the production of butanol by substrate coupled reaction.

determined using butyraldehyde concentration ranging between 4.34 and 43.47 mM. The production of butanol were 10.63 mM and 15.16 mM from 17.39 mM and 34.78 mM of butyraldehyde, respectively (Fig. 5). The conversion of butyraldehyde to butanol was 74% at butyraldehyde concentration of 17.39 mM. Whereas it was 65% at the butyraldehyde concentration of 34.78 mM. Higher concentration of enzyme would be needed to convert 34.78 mM of butyraldehyde with better conversion.

**3.2.5 Optimization of time for the production of butanol using 17.39 mM and 34.78 mM butyraldehyde.** Time needed for the completion of reaction was determined for 17.39 mM and 34.78 mM of butyraldehyde. The production of butanol was found to increase with progress of time. The maximum production of butanol from 17.39 mM of butyraldehyde was obtained after 150 min (Fig. 6). Initially, the rate of butanol production increased until 60 min and decreased thereafter. The butanol production from 34.78 mM of butyraldehyde was monitored every 60 min (Fig. 7). It was seen that the rate of butanol production increased until 240 min and decreased

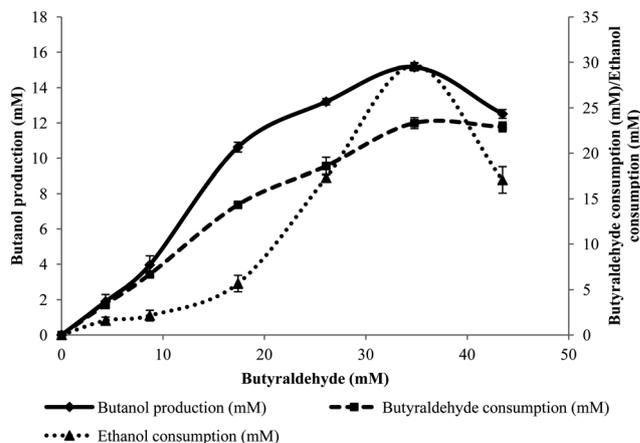


Fig. 5 Optimization of concentration of butyraldehyde for the production of butanol by substrate coupled reaction.

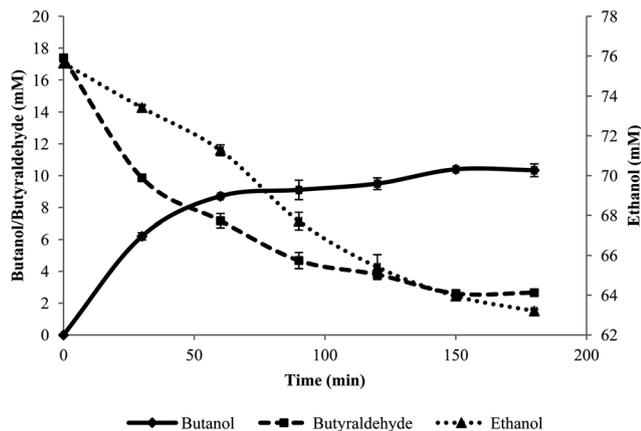


Fig. 6 Optimization of time for the production of butanol using 17.39 mM butyraldehyde by substrate coupled reaction.

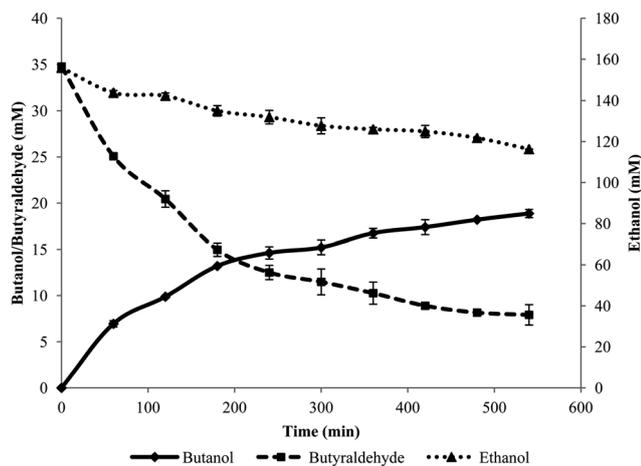


Fig. 7 Optimization of time for the production of butanol using 34.78 mM butyraldehyde by substrate coupled reaction.

thereafter. The production of butanol after 240 min was 14.62 mM and increased to 18.86 mM after 540 min.

### 3.3 Production of butanol from butyraldehyde using enzyme coupled reaction

Two enzymes can be coupled together for continuous regeneration of NADH. An enzyme coupled reaction is an alternative to substrate coupled reaction. Two enzymes, *viz.* alcohol dehydrogenase and glutamate dehydrogenase were used in the reaction with butyraldehyde and L-glutamate as substrates. Glutamate dehydrogenase could convert NAD to NADH by the using L-glutamate as substrate and generating NADH continuously in the reaction. Different ratios of alcohol dehydrogenase : glutamate dehydrogenase (4 : 1–1 : 6) were used for the reaction to determine the effect on the production of butanol. The maximum butanol production was obtained at 1 : 4 ratio of alcohol dehydrogenase : glutamate dehydrogenase 1 : 4 (Fig. 8). The maximum production of butanol was 1.39 mM and conversion of butyraldehyde to butanol was 24% using 30  $\mu$ M of NADH. Lower level of glutamate dehydrogenase (ratio

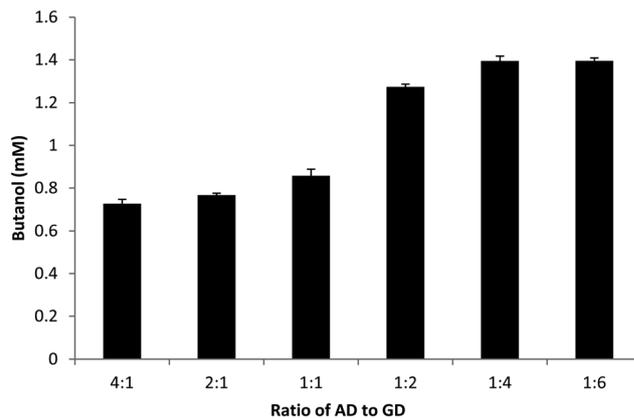


Fig. 8 Production of butanol using enzyme coupled reaction. AD: alcohol dehydrogenase and GD: glutamate dehydrogenase.

of 4 : 1–1 : 1) showed lower butanol production. Glutamate dehydrogenase was needed at higher amount (ratio of 1 : 2–1 : 6) as compared to alcohol dehydrogenase. Higher amount of glutamate dehydrogenase could initiate the conversion of NAD to NADH even at lower concentration of available NAD and make it possible to supply NADH continuously in the reaction.

The substrate coupled reaction where ethanol was used as a second substrate for continuous supply of NADH showed a conversion of 75%. However the enzyme coupled reaction showed only 24% conversion of butyraldehyde to butanol. A comparison between two above mentioned methods suggested substrate coupled reaction to be a better approach than the enzyme coupled reaction. Enzyme coupled reaction is costly as it needs two enzymes and two substrates for the reaction to proceed, whereas substrate coupled reaction needs only one enzyme with two substrates. Besides, ethanol is an inexpensive substrate needed for the substrate coupled reaction and conversion obtained was higher as compared to enzyme coupled reaction. Hence it can be suggested that conversion of butyraldehyde to butanol using alcohol dehydrogenase with substrate coupled reaction for cofactor regeneration can be used as a green, cost effective and rapid method for the production of butanol.

## 4. Conclusions

To overcome the limitation of product inhibition in butanol production, a new strategy was developed for the production of butanol using butyraldehyde as the substrate and NADH as the cofactor. The regeneration of cofactor NADH was comparatively evaluated using substrate coupled and enzyme coupled reactions. The optimized process of butyraldehyde to butanol conversion with substrate coupled cofactor regeneration showed conversion of 75%. However, the enzyme coupled reaction showed 24% conversion of butyraldehyde to butanol. Substrate coupled reaction would also be more cost effective than enzyme coupled reaction with respect to butanol production. The process could be further scaled up and made continuous by packing a column with beads of enzyme and

entrapped NADH. This could leads to continuous production of butanol without any concerns on inhibition of enzyme and regeneration of cofactor.

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