Molecular Characterization and Enzyme Analysis of Butanol Tolerant Bacterium *Paenibacillus* sp AS2 I

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**Abstract**—Due to a limited supply of petroleum oils, microbial production of butanol has gained more attention in recent years. However, major roadblocks of the current butanol fermentation were low yield, low productivity and most importantly low titer due to the toxicity of butanol to their producing strains. In our current research efforts were made to evaluate the potential butanol tolerance bacterial strains for its possible role as a host for butanol production. Among the thirty screened bacterial strains, only few showed tolerance towards butanol in which AS2 I has the capability to tolerate up to 5% butanol at 72 h with 30% of cell growth. Assays for different enzymes involved in butanol production were also carried out. From the present study showed that the best butanol tolerant bacteria was found to be *Paenibacillus* sp. using 16S rDNA sequencing and had enhanced activity of butanol tolerance enzymes. Overall results shows that the strain AS2 I can be engineered as promising host for enhanced butanol production.

**Index Terms**—butanol, *Paenibacillus* sp., butanol tolerance, 16S rDNA amplification

I. INTRODUCTION

A steep rise in the price of petroleum and global warming has directed us to utilize alternative fuels produced from renewable sources. However butanol toxicity, on microorganism is a major concern in the biobutanol production. Butanol can be produced from anaerobic bacteria (*Clostridia*) mediated fermentations through a process in which it also produces acetone, and ethanol which is collection called ABE fermentation. In batch fermentations, the bacteria are sensitive to butanol and are unable to produce anaerobically and have a significantly slow growth rate, leading to low butanol productivity [1]. Further, the relative unknown genetic system and complex physiology of *Clostridium* presents difficulties in engineering the metabolic pathway for efficient butanol production [2]. “Reference [3]” revealed that butanol producing bacteria *Bacillus subtilis* can tolerate up to 2% butanol. Acetoacetyl-CoA: acetate/butyrate: CoA transferase (CoA transferase) is the first enzyme in acetone formation pathway [4]. Several investigations were described in the successful expression of butanol pathway genes and butanol synthesis in *Paenibacillus* which is an industrial platform organism and a convenient host for metabolic engineering [5]. In this study, out of six butanol tolerant microbes a single potent strain, which is a facultative anaerobic, gram positive, rod shaped and endospore-forming with 5% (v/v) butanol-tolerance have been screened. The isolate with relatively high tolerance was further characterized by examining morphological changes of cell surface structures using Scanning Electron Microscopy (SEM). Here, we describe the application to improve butanol tolerance to render it more suitable host for butanol production. Further, selected butanol tolerant strain was identified and characterized as *Paenibacillus* (AS2 I) and cloned in T/A clone vector. Genes associated with butanol-tolerance were also identified and characterized.

II. MATERIALS AND METHODS

A. Isolation of Butanol Tolerant Strains from Sago Industry Waste Samples

One gram of sago industry waste soil sample was serially diluted (10^{-1} to 10^{-7}) for spread plate to screen the bacteria with Minimum Salt Agar (MSA) medium. The plates were incubated for 48 h at 37°C. The purified isolates were inoculated in fresh medium containing 1 to 5% (v/v) butanol. After incubation, the isolated colonies were inoculated into agar plate containing 1 to 5% (v/v) butanol concentration. Several rounds of screening were performed to purify and to isolate strains with high butanol tolerance [1].

B. Butanol Tolerant Assay

Single colonies were inoculated into fresh media and grown to the mid-exponential growth phase at 37°C without shaking and then inoculated into media containing a series of concentrations of butanol in MSA broth supplemented with 1 to 5% butanol. The cultures were maintained for 4 days and observed at 600 nm. The triplicate value were recorded and plotted against time remained in the same culture broth [6].

C. Preparation of Cell Free Extracts and Enzyme Assays

Bacterial cells were collected by centrifugation (5,433 x g, at 4°C for 10 min) and resuspended in 50 mM 3-(N-
morpholino) propanesulfonic acid (MOPS) buffer (pH-7.0), containing 1 mM 1,4-dithiothreitol (DTT). The cells were Ultra sonicated (EN60; Ener Tech., India) and centrifuged at 13,416 X g for 15 min at 4 °C. The supernatant was used as enzyme for further studies. Clostridium acetobutylicum (NRRL-528) served as positive control. The cell free extracts were stored at -20 °C until further use [5]. Total protein was measured by Bradford method [7].

1) Thiolase assay
Thiolase (THL) activity was determined by conversion of acetoacetyl-CoA (AA-CoA) (AA-CoA) as substrate, and decreasing in acetoacetyl-CoA concentration was measured at 303 nm. The enzymatic reaction was initiated by addition of crude extract. The reaction mixture contained 100 mM Tris-HCl (pH-8.0), 10 mM MgCl₂, 200 μM acetoacetyl-CoA, 200 μM CoA. The reaction temperature was maintained at 30 °C. The enzyme activity was determined by calculating the difference in the slope values of the sample and control [3].

2) β-Hydroxy Butyl-CoA Dehydrogenase (HBD) Assay
The HBD activity was measured at 340 nm by the decrease in NADH concentration resulting from β-hydroxy butyryl-CoA dehydrogenase (HBD) formation from acetoacetyl-CoA. The reaction mixture contained 100 mM MOPS, (pH 7.0), 200 μM NADH, 200 μM acetoacetyl-CoA, and crude cell extracts. The reaction was initiated by addition of cell extract. The enzyme activity was calculated the difference in the slope values of the sample and control [3].

3) Crotonase (CRT) assay
CRT activity could be measured as a decrease in optical density at 263 nm due to hydration of crotonyl-CoA and its conversion into β-hydroxy butryl-CoA. The assay mixture contained 100 mM Tris-HCl (pH 7.6) and 200 mM Crotonyl-CoA. The standard curves of crotonyl-CoA and β-hydroxy butyryl-CoA were constructed by measuring the absorbance of the two compounds at 263 nm with different concentrations. The difference in the molar absorbance of the two compounds was used to calculate concentration of crotonyl-CoA [5].

D. Cell Morphology Studies Using SEM
Bacterial cell samples collected from 96 h incubation with and without presence of 5 % (v/v) butanol, cells were collected at late exponential phase by centrifugation. Cell pellets were washed twice with 0.9 % NaCl solution and re-suspended in the same solution. Twenty microliter cell suspension was fixed in 2 % glutaraldehyde at 4 °C for 3 h. Cells were dehydrated by a series of progressive ethanol concentrations at 10, 30, 50, 70, 90 and 100 % each with 15 min incubation and dried at room temperature. Samples were mounted on SEM stubs and coated with platinum for 708 using gold sputter. Observations of samples were carried out under SEM [9].

E. Development of Genetic Transformation of Butanol Tolerant Strain
Butanol tolerance, genetic tractability of the selected bacterium is an essential trait for the development of an alternative host for butanol production. Total genomic DNA was isolated from the bacterial isolate AS2 I using bacterial genomic DNA isolation kit. Amplification of 16S rDNA region by PCR was performed with 27 F (3’AGAGTTTTGATCMGGTCAG 5’) and 1492 R (3’GYYTACCTTGTACGACTT 5’) universal primers. The 16S rDNA amplicons was cloned into the vector pTZ57/R (Fermentas, # K1213). Recombinant clones were detected through blue/white screening [10]. Recombinant plasmid DNA was extracted and cloned 1.5 kb DNA fragments amplified by PCR sequenced by automated DNA sequencing [11]. 16S rRNA gene sequences were edited and sequence similarity determined using the phylogenetic analysis of sequence data of bacteria these studies were aligned with reference sequence homology from NCBI database using multiple sequence alignment of MEGA 5.0 Program [12].

III. RESULTS AND DISCUSSION

A. Butanol Tolerant Microorganism
Butanol tolerant bacterial colonies were screened and identified from sago industrial waste site. One hindered ten colonies grown in 1 % butanol and the number of colonies decreases as the concentration of butanol increases. Hence 30 isolates were found to grow in 5 % of butanol after 4 days. Among the 30 isolates five of them (AS2 I, SD16, SD8, AS35 and SS319) exhibited relatively good tolerance at 5 % (v/v), but due to the limitation of genetic transformation feasibility (as described later), isolate AS2 I was selected for further investigation. Isolate AS2 I is a gram-positive, endospore forming bacterium identified by standard morphological, biochemical characterization and it was confirmed to be Bacillus sp. Data representing the growth rate of the strain AS2 I-30 % cell growth were represented in Fig. 1.

Figure 1. Relative growth rates of butanol tolerant strain AS2 I (Grow in the presence of over 5% butanol) screened from the starch hydrolysing bacteria.
Up to 30% growth was observed in cells grown in 5% butanol when compared to absence of butanol (100%). Our results revealed that, AS2 I have superior tolerance to butanol, when cells were either at late exponential growth phase (or) grown from the initial stage of growth which supported by the reports of [13]. These results were more efficient than previous reports Oksana et al. (2010) [5]. That high butanol tolerance of L. brevis was showed 2% growth at 144 h cultivation. Similar to isolate our strains, the butanol tolerant strain was comparable for its efficiency [14].

It seemed that butanol could disrupt fluidity and function of the cell membrane [15]. Thus, high concentration of butanol destroyed the ability of cell to maintain internal pH, lowered the intracellular level of ATP and inhibited the uptake of carbon source, so as to inhibit growth and multiplication till killing cell [16]. Butanol tolerant as seem in present study might be attributed to the changes in fatty acid profile and fluidity of cell membrane accordingly [17]. As butanol is also a short-chain primary alcohol just like ethanol, similarities exist in the response mode of micro-organisms to them [18]. Only high butanol tolerant Bacillus sp. showed growth within 4 days, suggesting that these isolates have varied fatty acid profile and might possess genetic resistance to butanol. Thus it is not surprising that the growths of the Bacillus sp. strains are highly butanol tolerant. After adaptation, the growth rate was relatively slow and none of the remaining strains exhibited growth in presence of 5% butanol after 4 days. Similar work has been stated Kataoka et al. (2011) [13] in which Bacillus sp. tolerated 1.2% butanol and it can be used as host for the bio production of butanol. Due to the complex mechanisms involved in butanol-induced stress response, such as inhibiting membrane transport systems, enzymes, and disruption of membranes [9]-[19], butanol tolerance phenotype is still difficult to engineer even in microbes with well defined genetic background such as E. coli. The range of our estimate was mainly due to differences in dry mass percentage of the cells of the six bacterial isolates used. The high amylase activity in Bacillus as seen in present study apparently converts available starch to sugar rapidly [20].

### B. Butanol Metabolic Pathway Enzymes

The protein expression in bacterial cell plays an active role in determining the amount of butanol tolerant growth factors and other protein produced necessary for its growth so as to confer inhibitors or non-inhibitory effects [21]. Preparation of cell free extracts from butanol tolerant isolate AS2 I was assayed for THL, HBD and CRT enzymes activities and shown in Table I.

<table>
<thead>
<tr>
<th>Butanol tolerant strains</th>
<th>THL (U/mg)</th>
<th>HBD (U/mg)</th>
<th>CRT (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL-528</td>
<td>1.3 ± 0.054</td>
<td>135.42 ± 0.21</td>
<td>0.837 ± 0.34</td>
</tr>
<tr>
<td>AS2 I</td>
<td>0.057 ± 0.87*</td>
<td>13.321 ± 3.57*</td>
<td>0.033 ± 0.004*</td>
</tr>
<tr>
<td>SD16</td>
<td>0.0651 ± 0.32*</td>
<td>0.056 ± 3.2*</td>
<td>0.019 ± 0.012*</td>
</tr>
<tr>
<td>SD8</td>
<td>0.15 ± 0.17*</td>
<td>Nd</td>
<td>0.015 ± 0.137*</td>
</tr>
<tr>
<td>SS3 21</td>
<td>Nd</td>
<td>0.093 ± 2.97*</td>
<td>0.012 ± 0.008*</td>
</tr>
<tr>
<td>SS3 18</td>
<td>0.014 ± 0.066*</td>
<td>0.521 ± 1.57*</td>
<td>Nd</td>
</tr>
<tr>
<td>AS35</td>
<td>0.023 ± 0.857*</td>
<td>1.1619 ± 1.87</td>
<td>0.0073 ± 1.57</td>
</tr>
</tbody>
</table>

Value are expressed as means ± SD. *P*<0.05 vs NRRL-528 strain. Note: Nd- Not detectable, the minimum specific activity of THL, HBD and CRT is less than 0.0010 U/mg proteins.

The activities of THL, HBD and CRT enzymes in AS2 I was found to be 0.057, 13.321 and 0.033 respectively. The level of these enzymes were found to be significantly (P<0.05) reduced when compared to NRRL-528 strain. Butanol is classified as an extremely toxic chemical to microorganisms; its toxicity becomes the primary problem for its production via microbial fermentation [5].

C. **Cell Morphology Studying of Butanol Tolerant Isolate Using SEM**

In order to examine the morphological changes after incubation with 5% (v/v) butanol, SEM analysis was performed. AS2 I in the presence of butanol, cells showed irregular shape and their cell surface were relatively hard with membrane alterations when compared to control (Fig. 2).
The average cellular size of without butanol was 7.29 ×0.6 μm while that of the AS2 I with butanol was 7.08 ×0.6 μm based on ~50 cells. These results show that cells adapted towards butanol stress through modifying its phenotypic characteristics [22]. To understand the changes of cells SEM analysis of butanol tolerant isolate showed that AS2 I acclimatized to butanol toxicity which was exhibited no change in morphology of cells.

**D. Development of Genetic Transformation of Butanol Tolerant AS2 I**

The AS2 I strain 16S rDNA amplicon was cloned into the vector pTZ57R (Ins T/A clone PCR product cloning kit, Fermentas) and sequenced through M13 forward and reverse primers. The ligated DNA was used for transformation in DH5α E. coli strain. Recombinant plasmids from the white colony were sequenced and compared with sequences in the GenBank (Paenibacillus sp. KC505641). The AS2 I gene sequence was 99% identical with that of other organisms. All ambiguous positions were removed for each sequence pair the rooted using Bacillus sp. strain as out group. The phylogenetic tree shows that Paenibacillus AS2 I was out of group in rooted construction (Fig. 3).

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**REFERENCES**


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