



Isolation and molecular characterization of butanol tolerant bacterial strains for improved biobutanol production

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Abstract

Butanol tolerance is a complex mechanism affecting the ability of microorganisms to generate economically viable quantities of butanol. The objective of this study was to isolate and characterize butanol tolerant bacterial strains which can act as potential alternative hosts for butanol production. The potential bacterial isolates were screened, based on the non toxic effect on cell growth rate and degradation ability of sago waste which was used as a sole carbon source with butanol enrichment. During this study, it was found that a growth barrier existed between 1 to 5 % butanol concentrations and only few selected isolates could tolerate upto 5 % butanol after long term adaptation. Screening of five isolates proved to be more tolerant, which were identified as *Bacillus megaterium*, *B. aryabhatai*, *B. tequilensis*, and *B. circulans* using 16S rDNA sequence. These isolates were markedly attractive to identify butanol tolerance specific stress response genes and further engineered to act as a genetic host for biobutanol production.

Key words

16S rDNA amplification, *Bacillus* sp., Butanol tolerance, Sago starch

Introduction

Increasing crude oil prices and growing awareness of environmental problems, such as global warming, attributed to the use of fossil fuels, has brought significant attention for the production of biofuels. Biobutanol is one of the most promising biofuels due to its gasoline like properties and wide variety of its feed stocks (Lee *et al.*, 2010). Growing acceptance of biobutanol is largely due to its energy content competitive to that of gasoline and low vapour pressure (compared to ethanol), potentially allowing for a complete substitution of gasoline without modifications to the existing vehicle engines or fuel delivery infrastructure (Antoni *et al.*, 2007). Microbes annually hydrolyze 1011 tons of plant biomass which contain energy equivalent to 640 billion barrels of crude oil (Wackett, 2008). Butanol is naturally synthesized by *Clostridium* species using a pathway that involves Co-enzyme A (CoA) activated intermediates. Several investigations describe the successful expression of butanol pathway genes in *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis* and *Saccharomyces cerevisiae* as a host using metabolic engineering (Atsumi *et al.*, 2008; Chen *et al.*, 2011). An

alternative approach for butanol production is to find and construct butanol biosynthesis pathway genes in a heterologous host, of butanol resistance (Kotaoka *et al.*, 2011). Suitable microbial hosts are to be used for metabolic engineering strategies with higher butanol tolerance capacity to improve butanol yields (Knoshaung *et al.*, 2009; Liu *et al.*, 2012). Organic solvent tolerant bacteria are newly discovered group of microorganism with butanol novel tolerance mechanisms, which is less than 0.3 % (Sardssai and Bhosle, 2002). Hence, the current research mainly focus to identify and characterize bacterial isolates with superior butanol tolerance capacity. Out of 976 isolates from 110 soil samples, only 30 isolates showed positive results, and among them potential six isolates were identified by 16S rDNA sequencing. In this study, 5 % butanol tolerance has not been previously reported in bacteria anywhere the highest butanol tolerance strains reported till now is (2.5-3 %) which produced 0.4 g⁻¹ butanol (Ting *et al.*, 2012). Hence, the mechanism for butanol production and thereby global demand can be easily worked out. The results of this initial screening and characterized for butanol tolerant organism was to engineer host for butanol production.

Materials and Methods

Isolation and identification of butanol tolerant bacteria: The waste soil samples were collected from sago industries in Salem, Kolli hills and Pachamalai the Eastern Ghats of Tamil Nadu, India. The samples were collected in sterile plastic containers and stored at 4 °C. Samples were plated on minimum salt agar medium (MSA) and incubated at 37 °C for 24 hr. Butanol tolerance was checked in MSA plates containing 1 to 5 % (v/v) butanol with the above said conditions. Several rounds of screening were performed to purify and isolate strains with high butanol tolerance (Sardesai and Bhosle, 2002; Knoshaug *et al.*, 2009). The isolated strain was stored at -20 °C for further use. Morphological features of colonies obtained on MSA media were studied. The biochemical characterization studies were carried out by Gram staining, catalase test, motility test, endospore stain and high salt tolerance test for the selected strains (Inui *et al.*, 2008).

Butanol tolerance assay: The butanol tolerance assay was performed to test the effect of butanol on the growth of microorganisms. Single colony was inoculated into fresh MSA medium and grown overnight at 37 °C and further inoculated into MSA media containing a series of butanol 1 to 5 %. Cell growth was measured at 600 nm using double beam spectrophotometer (Systronics, 2203, India). The relative cell growth rate was calculated in the presence or absence of butanol (Knoshaug *et al.*, 2009; Li *et al.*, 2010).

Biochemical analysis of butanol tolerant bacteria: The dry matter content of different samples, the buoyant densities of individual butanol tolerant bacterial cells and pellets were determined following the procedure of Bakken *et al.* (1983); Bratbak and Dundas (1984). The enzymatic hydrolysis experiments were conducted with starch as a substrate. Amylase activity was determined by starch hydrolysis method. One unit of enzyme activity (Unit number) is defined as the amount of enzyme releasing 1 mol maltose min⁻¹ that causes 1 % reduction of blue color intensity of starch iodine solution at 37 °C in 1 min (Roy *et al.*, 2012). The reducing sugars were estimated by dinitrosalicylic acid method. The absorbance was then converted to mg of glucose (Sago waste) from the standard curve of glucose (Chen *et al.*, 2011). Isolated bacterial cells were lysed using ultra sonicator (EN60; Ener Tech., India) as described by Deutscher, (1990). The cell pellet was resuspended in 3 M NaCl (50 mg of cell pellet ml⁻¹) and sonicated. Ultrasound was applied using a sonicator with an operating frequency of 50 kHz. The samples to be sonicated were subjected for 20 sec on/off pulses for 2 min at 20 % amplitude. The cell debris was removed by centrifugation at 10,000 x g for 10 min at 4 °C. The supernatant containing protein extracts was stored at -20 °C until further analysis. Protein content was measured by Bradford method (1976).

Phylogenetic analysis: Genomic DNA was extracted using phenol chloroform extractions method. The PCR parameters for

the amplification of 16S ribosomal DNA were optimized. 50 µl of PCR master mix contained universal primer set 27 F- (5'-AGAGT TGGATCMTGGCTCAG-3')/1492 R- (5'-GGYTACCTTGTACGA CTT-3'), 10 mM dNTPS, 10 X PCR Buffer, 1U Taq DNA polymerase, 2 mM Mg⁺ and (100-200 ng) template DNA. PCR steps include initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 2 min, elongation at 72°C for 1 min and final extension at 72°C for 10 min. Approximately, 1.5 kb amplicons were generated. The PCR product was purified using Gene JET PCR purification kit (Fermentas) according to manufacturer' instructions and sequenced at Eurofins Genomics India Pvt Ltd, Bangalore using AB DNA sequencer. Sequence search similarities were conducted using BLAST (Kotaka *et al.*, 2011; Edward *et al.*, 2011). Phylogenetic analysis of sequence data of bacteria under study were aligned with reference sequence homology from the NCBI database using multiple sequence alignment of MEGA 5.0 Program (Tamura *et al.*, 2007).

Results and Discussion

One hundred ten colonies grown in 1 % butanol concentration and the number of colonies decreased with increase in butanol concentration. Hence, 30 isolates were found growing in 5 % concentration after 4 days and out of these, six strains were found to be highly tolerant to butanol and hence these colonies were taken for further studies. Data representing the growth rate of those 6 strains (AS21-92.89, SD16-81.32, SD8-71.24, SS321-81.9, SS3 18-91.9 and AS35-84.89 %) were represented in Fig. 1. These results were more efficient than previously reported by Knoshaug and Zhang (2009) in which *Lactobacillus*, *Enterococcus*, *Pediococcus* and some other species also exhibited butanol tolerance; even though some of them were not grow in their optimal medium. But they are only 3 % butanol tolerable. Only high butanol tolerant *Bacillus* sp. showed growth within 4 days, suggesting that these strains possess

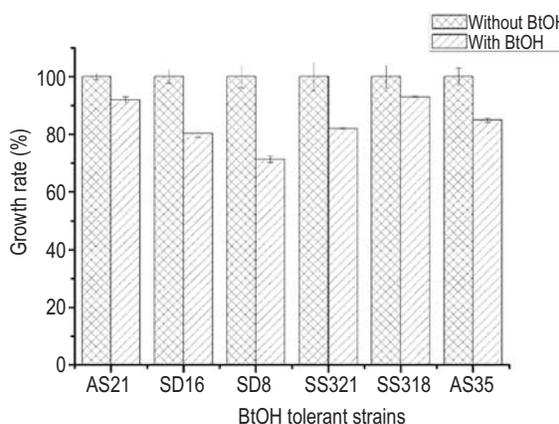


Fig. 1 : The growth of isolated highly butanol tolerant bacterial strains in 5 % butanol concentration in MSA medium at 37 °C at different hours. Values are indicated as mean ± SE from three independent analyses

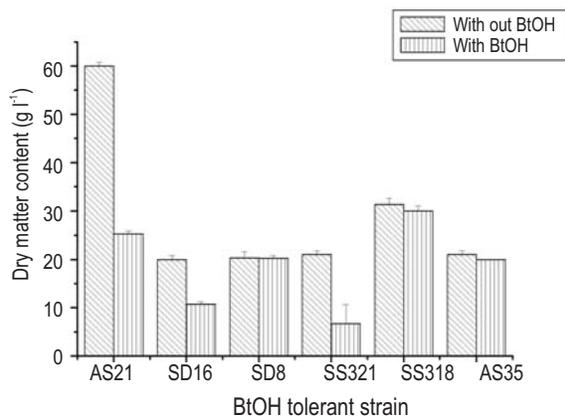


Fig. 2 (a) : Dry matter content of butanol tolerant bacterial strains

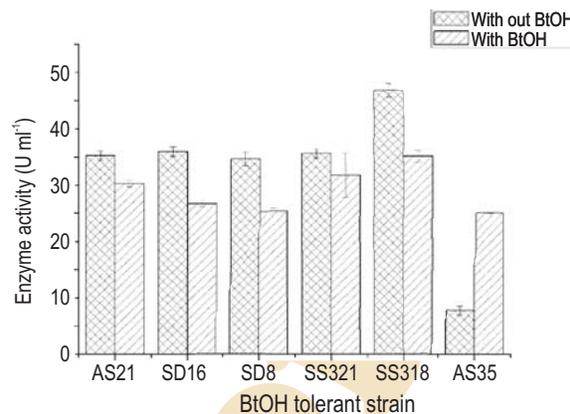


Fig. 2 (b) : Estimation of α-amylase by DNS method using butanol tolerant bacterial strains waste soil microorganisms in different sago starch concentration

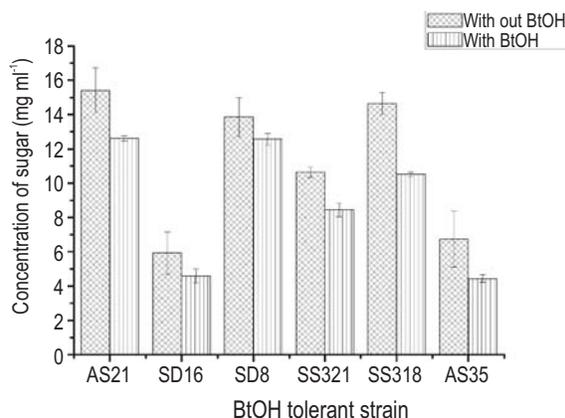


Fig. 2 (c) : Butanol tolerant bacterial strains waste soil microorganisms in different sago starch concentrations

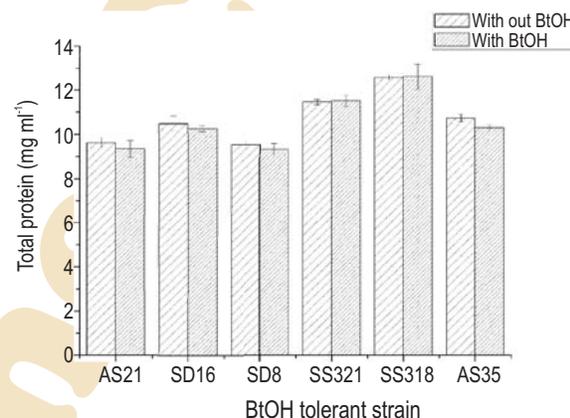


Fig. 2 (d) : Analysis of total protein by butanol tolerant bacterial strains waste soil

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 reported by Kotaoka *et al.* (2011) in which *Bacillus* sp. tolerated 1.2 % butanol and reported that it can be used as a host for the bio production of butanol. The major cellular impact of high butanol concentrations on microbes is the destruction of the cytoplasmic membrane and various macromolecules, accompanied by the loss of vital functions at the molecular level; butanol causes a stress response which is not yet fully understood.

Biochemical analysis of butanol tolerant bacteria: The dry weight of bacterial pellets ranged from 11 to 65 %, with an average of 20 % calculated from the percent dry weight of pellets and percent intercellular water. Buoyant density obtained by centrifugation denotes the density of cells on the medium. The

range of our estimate was mainly due to difference in dry mass percentage of the cells of the six bacterial strains used. The dry weight percent of pellets after suction was substantially higher than dry weight (Fig. 2a). Similar growth solvent tolerant strain, *B. thermophilus* was reported by Sarkar and Ghosh (2012). The dry matter content of butanol tolerant cells of a given strain differed with growth conditions. During early stages of fermentation, sago starch was hydrolyzed into maltose and glucose by the action of α-amylase secreted out into the medium by strains. Extracellular enzyme increased gradually and reached a maximum level after 24-48 hr. The maximum α-amylase obtained was 20 U ml⁻¹ in different sago starch concentration (Fig. 2b). Previously, it had been reported that *C. butylicum* TISTR 1032 produces low amount of acids, acetone, butanol and ethanol from cassava starch, exhibit very low amylase activity (1.85 U ml⁻¹) and could not utilise cassava starch effectively. Amylase activity produced by the co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 increased 16-fold or up to 30.5 U ml⁻¹ after 24 hr of incubation.

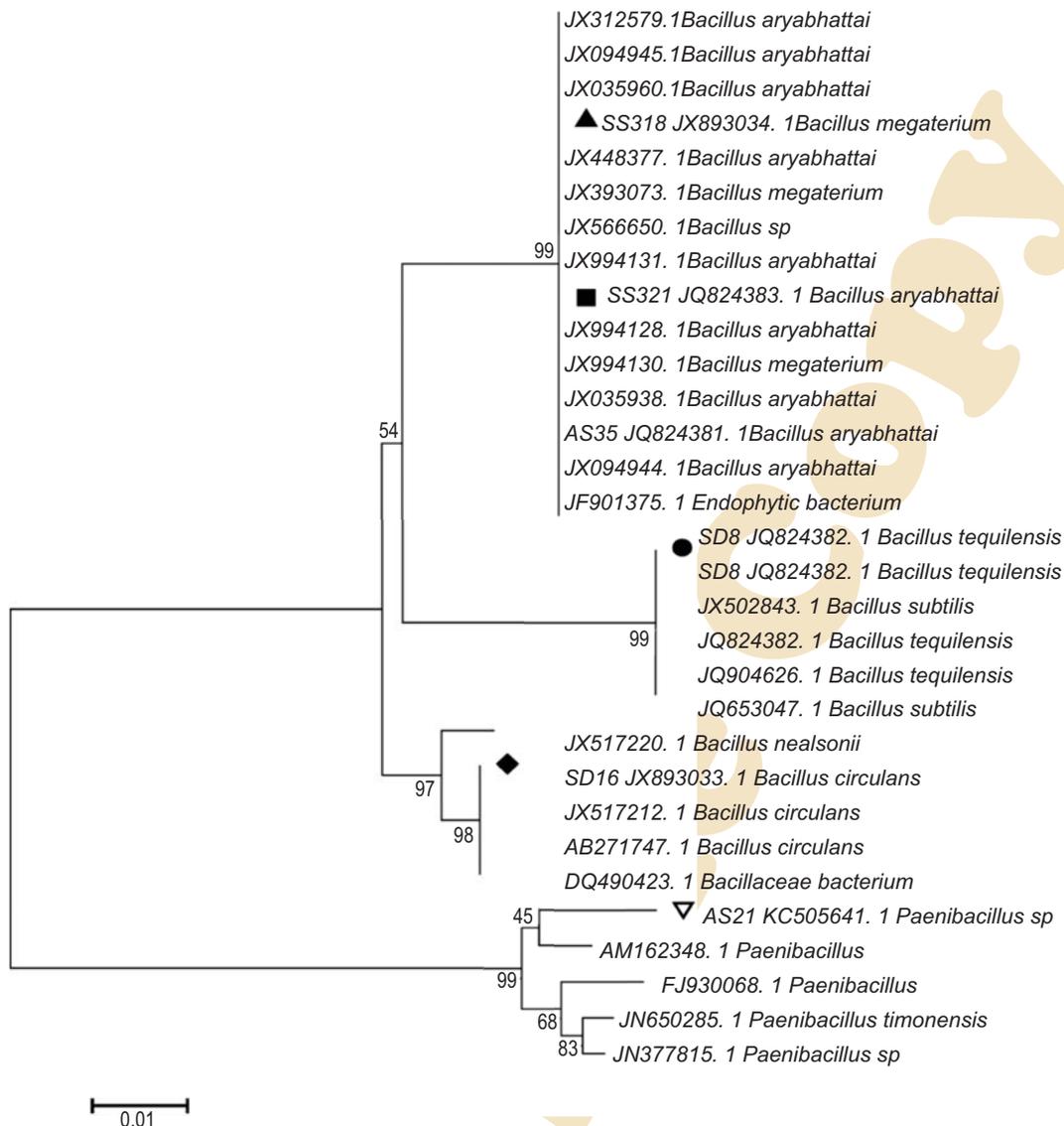


Fig. 3 : Phylogenetic tree based on homology sequence of 16S rDNA analysis. Each number on a branch indicates the bootstrap confidence values (100 replicates). The scale bar indicates 0.01 substitution per nucleotide position

High amylase activity in *Bacillus* apparently convert available starch to sugar rapidly, which stimulates the metabolism of *Clostridium* to grow and thus enhances both ABE production and its rate (Tran *et al.*, 2011). Bozi *et al.* (2011) reported maximum α -amylase production when starch was used as carbon source. The experiments carried out at different concentration of starch (1 %) and tryptone (0.5 %) showed that they were essential for maximum α -amylase production, inducing 5.2 U ml^{-1} of amylase secreted after 72 hr of cultivation. When the strain was cultivated in laboratory fermenter, α -amylase production of 5.2 U ml^{-1} at 24 hr

was found to remain constant during next 16 hr. The reducing sugar generation rate was positively proportional to starch concentration for the selected six strains. The strains showed high amount of reducing sugar at most concentrations, especially high sago starch concentration glucose was used as standard. The enzyme activities obtained for AS21, SD16, SD8, SS3 21, SS3 18 and AS3 5 were 2.1, 4.8, 12.2, 8.15, 11.5 and 5.04 mg ml^{-1} respectively. The reducing sugars produced by microbes were measured by adding DNS reagent, and is presented in Fig 2c. Accumulation of organic acids during fermentation of starch was

found to inhibit cell growth of *C. acetobutylicum*, and therefore sugar utilization remitted in decreased ABE production. It indicates the enzymatic hydrolysis of sago starch enhanced solvent production, yield and productivity (Al-Shorgani *et al.*, 2012). High concentration of reducing sugar $>12.2 \text{ mg ml}^{-1}$ were produced by the bacterial strains. Similar results were indicated by Chen *et al.*, 2011.

Protein quantification analysis demonstrated higher protein yield as compared to control (Fig. 2d). Protein expression in bacterial cell in particular plays an active role in determining the amount of butanol tolerant growth facts and other protein to be produced necessary for its growth so as to confer inhibitors or non-inhibitory effects (Vijayalakshmi *et al.*, 2011). These results are in agreement with an earlier study conducted by Mann *et al.* (2012).

The selected potential isolates were identified by partial sequencing of 16S rRNA gene. The obtained sequences were submitted to the BLAST in order to find out homology with the sequence in Gen Bank revealed that the six strains showed 99 % similarity to *B. megaterium* (JX893034.1), *B. aryabhatai* (JQ824381.1, JQ824383.1), *B. tequilensis* (JQ824382.1), *B. circulans* (JX893033.1) and *Paenibacillus* sp. (KC505641) (Fig. 3). All ambiguous positions were removed for each sequence pair. The six were rooted using *Bacillus* sp. strains as out group the stability of trees obtained from the above cluster analysis was assessed by using BOOTSTRAP program. The phylogenetic tree was constructed from the sequence data by neighbor joining methods. The bootstrap values (%) presented at the branches was calculated from 500 replications. *B. nealsonii* (JX517220.1) was used as an out-group. Scale bar indicated 0.01 substitutions per nucleotide position.

Out of 110 butanol tolerant strains five showed promising results as they could grow in 5 % butanol for more than 4 days adaptation. These bacterial strains were identified as *B. megaterium*, *B. aryabhatai*, *B. tequilensis*, *B. circulans* and *Paenibacillus* sp. 16S rDNA by sequencing. Further, investigation using comparative genomics based on genome sequencing and comparative proteomics based on two-dimension gel electrophoresis (2 DE) and mass spectrometry could be applied to identify butanol tolerant genes and get a novel target for strain improvement. Ultimately an ideal host allowing versatile manipulation such as responsive choice of enzyme, verification of gene expression and metabolic balance are to be broken.

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