Butanol tolerant bacteria: isolation and characterization of butanol tolerant *Staphylococcus sciuri sp*.

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Despite having tremendous potential as a fuel, butanol's toxicity to the microbes is a major bottleneck. An alternative microbial host for butanol production possessing higher level of tolerance has been isolated, which was able to tolerate up to 2.25% (v/v) butanol with 70% growth as compared to control. The isolate was identified as *Staphylococcus sciuri*. Improvement in tolerance to butanol through acclimatization by regular exposure to butanol did not result in any further significant improvement in tolerance. To the best of our knowledge in this study, we have reported highest growth of a microbe in the presence of 2.25% (v/v) butanol. Four possible gene products as universal stress protein (*UspA*), 2 general stress proteins (GSP), and one other stress protein (*GlsB*/*YeaQ*/*YmgE* family) have been predicted to play a possible role in butanol tolerance.

Keywords: bioenergy; biofuels; genetic engineering.

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Introduction

"Biofuels" produced from sustainable feed stocks seem to be the most promising alternative to increasing energy crisis and global warming. Currently ethanol is the most extensively used biofuel. Meanwhile, butanol is also gaining attention as an alternative biofuel because of its advantages over ethanol such as higher energy content, lower vapor pressure, less corrosive nature, and higher miscibility with gasoline [1, 2]. Chaim Weizmann industrialized the bio production of butanol using *Clostridium acetobutylicum* in 1961. Production of butanol by *Clostridium acetobutylicum* through Acetone Butanol Ethanol (ABE) fermentation yields Acetone:butanol:ethanol in the ratio of 3:6:1. But the major hurdles in ABE fermentation are anaerobic nature of *Clostridial sp*. which makes their handling difficult and low yield of butanol due to its severe toxicity to cells [3, 4]. Butanol accumulates in the plasma membrane of the cells which causes an increase in membrane fluidity resulting in loss of membrane potential and cellular growth [5]. Several heterologous hosts have been engineered for butanol production including *Escherichia coli* [6-14], *Sachharomyces cerevisiae* [15-18], *Pseudomonas putida* [9], *Bacillus subtilis* [9], *Lactobacillus brevis* [19] leading to production of butanol. But none of them could tolerate butanol beyond 2.0% (v/v) to show considerable growth. Later on, natural microbes *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas sp*. were also isolated, which were reported to tolerate up to 2.25 %, 3.0%, and 6.0 % (v/v) butanol respectively [20-22]. But the growth drops drastically down beyond 2.0% (v/v) butanol, so the severity of the problem existed. Therefore, the search of butanol tolerant

microbe is still required. In this study a novel *Staphylococcus sciuri* strain KM16 was isolated from soil which could tolerate butanol up to 2.25% (v/v) with relatively higher growth as compared to other reported microbes. Possible genes responsible for increased butanol tolerance were also predicted by performing genomic analysis of *Staphylococcus sciuri*. Four possible gene products as universal stress protein (*UspA*), 2 general stress proteins (GSP), and *GlsB*/*YeaQ*/*YmgE* family stress response protein have been identified which may contribute to high butanol tolerance.

Materials and Methods

Chemicals

Culture medium, solvents, enzymes, cloning vectors were all purchased from Himedia, Sigma Aldrich, and Thermo Fisher Scientific. Cultivation medium used were Luria broth (LB) and Luria agar (LA) (Himedia, Mumbai, India)). Soil samples were collected from a distillery in village Dharuhera, Haryana, India and a distillery in Meerut, Uttar Pradesh, India. *Clostridium acetobutylicum* ATCC 824 culture was purchased from National Collection of Industrial Microorganisms (NCIM) (Pune, India) and primers for butanol synthesis were designed in lab and purchased from Thermo Fisher Scientific (Bangalore, India).

Screening of soil sample for development of aerobic consortium and isolation of butanol tolerant bacteria

Two different soil samples were collected from alcohol distilleries of Dharuhera and Meerut India as described previously [23] and were named as KM and MT respectively. These soil samples were used to develop aerobic consortia by repeated enrichment technique at 37°C and 150 rpm with 0.5% (v/v) butanol. The process was repeated for six weeks at an interval of 15 days each to acclimatize the consortia. The developed consortia were then plated on LA with butanol concentration increasing from 1.0 - 3.0% (v/v) gradually at 37°C. After 48 hrs isolated and purified colonies on LA having 3.0% (v/v) butanol were inoculated into LB containing 1.0% (v/v) butanol and incubated for 48 hrs on a rotary shaker (150 rpm) at 37°C. These isolates were further tested for their growth in increasing concentration of butanol from 1.0% to 3.0% (v/v) in LB for 48hrs. Isolates showing maximum growth beyond 2.0% (v/v) butanol were again inoculated into their respective butanol concentration. This process was repeated for next 10 generations to acclimatize the isolates.

Molecular characterization of butanol tolerant bacteria

Genomic DNA of the selected bacteria was extracted as described by Kumar and Khanna [24]. 16S rRNA gene was amplified using genomic DNA as template with universal primers E8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer E1492R (5′-GGTTACCTTGTTACGACTT-3′) as described in literature [25]. The PCR reactions were prepared with 10X buffer, 0.2 mM deoxynucleoside triphosphate (dNTP), 1U Taq DNA polymerase, and 0.5 M each primer to yield final volumes of 25 µl, using 35 cycles of 94°C (60 s), 55°C (60 s), and 72°C (60 s) with an initial denaturation at 94°C (3 min) and a final extension at 72°C (8 min). Resulting PCR amplicons were subjected to restriction digestion with the help of enzymes MspI and HhaII in a mixed digestion reaction of 15 µl using 10X buffer, 10 U/µl enzyme at 37°C for 1hr.

Selected isolate was sent for 16S rRNA gene sequencing to Amnion Biosciences (Bangalore, India). Resulting sequence was BLAST searched through the National Centre for Biotechnology Information (NCBI) GenBank database for species identification of isolate. The 16S rRNA gene sequence of the bacterial strain reported in this study was deposited in the GenBank database with Accession number of MF151166. Phylogenetic tree was computed by using molecular evolutionary genetics analysis (MEGA) software with 1,000 bootstrap replicates for identification of the isolate KM16 [26]. Phylogenetic relatedness of isolate KM16 was

also studied with other bacterial species (including *Staphylococcus* species and heterologous hosts for butanol production) having tolerance to butanol and other organic solvents.

Growth and tolerance studies of the isolate

Growth of isolate KM16 was studied in LB for 48 hrs with different concentration of butanol in comparison to control (without butanol). The isolate was incubated in LB containing 0.0%, 2.0%, 2.25%, and 2.5% (v/v) butanol at 37°C and 150 rpm. Growth was observed by taking O.D. at 600 nm after regular interval of 1 hour. The growth of isolate in the presence of butanol from initial stage was chosen as the measure of butanol tolerance of isolate. For further improving the tolerance to butanol two approaches were used. In first approach, addition of minerals and vitamins solutions (For 1 L minerals solution: 1g $MnSO_4.2H_2O$, 0.2 g $CaCl_2.6H_2O$, 0.2 g $ZnSO_4$, 0.02 g $CuSO_4.5H_2O$, 0.2 g $Al_4(SO_4)_2$, 0.2 g H_3BO_3, 0.2 g $Na_2MoO_4.2H_2O$. For 100 ml (10x) vitamin solution: 2 mg Biotin, 2 mg Folic Acid, 1 ml Pyridoxine HCl, 5 ml Thiamine HCl, 5 ml Lipoic Acid, 1 mg Vitamin B12, 50 ml PABA) to the growth medium for increasing the nutrient content in the medium was done. Isolate was incubated overnight at 37°C and 150 rpm in the medium supplemented with vitamin and minerals solution. Other approach was to provide the exposure of UV rays (wavelength 253.4 nm) to the microbial cells to check for any further improvement in tolerance to butanol. Isolate was exposed to UV rays at a variable distance ranging from 5 cm up to 30 cm (5, 10, 15, 20, 25, 30 cm) for 0 min to 30 min at an interval of 5 min each and then incubated overnight at 37°C and 150 rpm with butanol [2.25%, 2.5%, 2.75%, and 3.0% (v/v)].

Genomic analysis of *Staphylococcs sciuri*

Genome analysis of *Staphylococcus sciuiri* was done to study the possible mechanisms or genes responsible for tolerance to butanol. Out of the whole genome sequence, genes chosen for comparative analysis were related to stress response, efflux pumps, and membrane modification. Analysis of the selected gene products and their homologues was done using NCBI protein BLAST against UniProtKB database.

Results and Discussion

Isolation and molecular characterization of butanol tolerant bacteria

Soil samples from alcohol distilleries were screened for the presence of butanol tolerant bacteria and enriched to develop a consortium. The consortium conKM and conMT developed at 0.5% (v/v) butanol yielded a total of 49 and 19 bacterial isolates respectively. Among the 19 isolates obtained from conMT only 2 isolates could tolerate 1% (v/v) butanol and none of them could tolerate beyond that (Data not shown). Among the 49 isolates obtained from conKM, 25 isolates were tolerant to 1% (v/v) butanol while 23 isolates to 1.5% (v/v), 3 isolates to 2.0% (v/v), and 3 isolates to 2.25% (v/v) butanol. None of the isolate could tolerate 3.0% (v/v) butanol. Among these three isolates (KM8, KM12 and KM16) which could tolerate butanol at 2.25% (v/v) over repeated cycles of growth, isolate KM16 was the most efficient (Figure 1).

Restriction digestion of 16S rRNA gene amplified using genomic DNA of isolates KM8, KM12, and KM16 resulted in three bands of 450 bp, 200 bp, and 100 bp each. All the three isolates showed similar band pattern. Microscopic study indicated that all the three isolates were gram positive bacteria. Thus, morphological and RFLP studies indicated that the three isolates seemed to be identical. Therefore, isolate KM16 was chosen for further studies because it showed maximum growth at 2.25% butanol. 16S rRNA gene sequence analysis and phylogenetic relatedness of isolate KM16 showed highest similarity (99%) and closeness with *Staphylococcus sciuri*. Isolate KM16 also showed 99% similarity to type strains *Staphylococcus sciuri subsp. scuiri* and *Staphylococcus sciuri subsp. rodentium* (Figure 2). Thus, the present culture was named as *Staphylococcus sciuri* KM16.

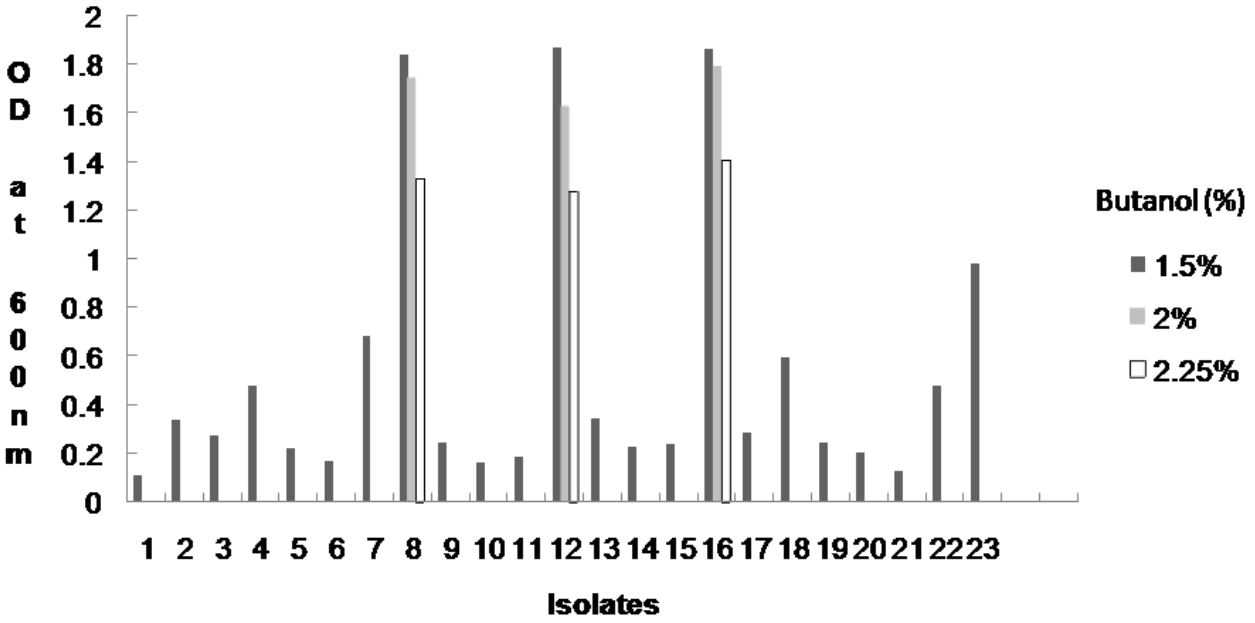

Figure 1. Growth profile of isolates obtained from conKM in presence of different concentration of butanol. Isolates (1-23) obtained after screening of conKM on Luria agar with 3.0% (v/v) butanol were inoculated in L.B with varying butanol concentrations at 1.5%, 2.0%, and 2.25% (v/v). Growth was monitored at 600 nm.

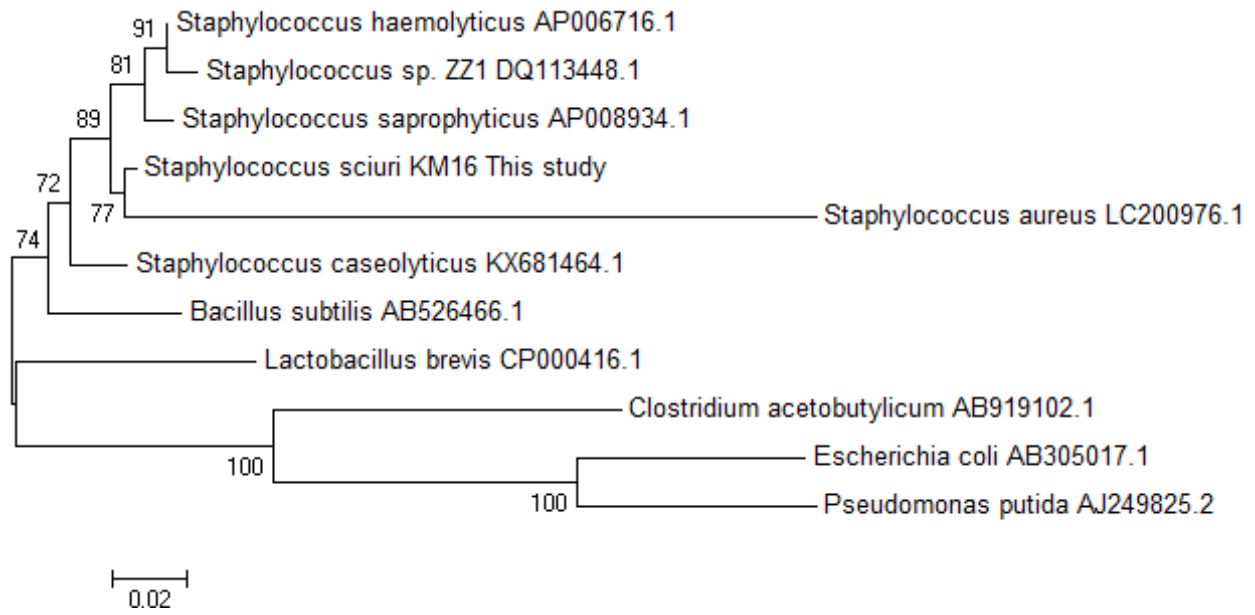

Figure 2. Phylogenetic relatedness of 16SrRNA gene amplified from isolate KM16. Dendrogram was constructed from a CLUSTALW alignment of related 16S rRNA gene sequences by neighbor-joining analysis using MEGA 5.2. Reference sequences from GenBank include the accession number. The scale bar represents substitutions per nucleotide.

Several studies reported the tolerance in *Staphylococoous sp*. with organic solvents other than butanol such as benzene, toluene, and hexane [27-32]. Phylogenetic relatedness of *Staphylococcus sciuri* KM16 was studied with other *Staphylococcus sp*. which are reported to

be tolerant to several organic solvents including butanol and also to various heterologous butanol tolerant bacterial species which have been studied as host for butanol production. Computation of phylogenetic tree with different organic solvent tolerant *Staphylococcus sp*. i.e. *Staphylococcus haemolyticus* (toluene), *Staphylococcus saprophyticus* (toluene) *Staphylococcus caseolyticus* (methanol, ethanol, and n-hexane), *Staphylococcus aureus* (butanol), *Staphylococcus ZZ1sp*., and other butanol tolerant bacterial hosts used for butanol production such as *Bacillus subtilis* (2.25%), *Lactobacillus brevis* (3.0%), *Pseudomonas putida* (3.0%), *E.coli* (1.5%), and native host *Clostridium acetobutylicum* (1.5%) revealed that *Staphylococcus sciuri* KM16 showed very close similarity with *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Staphylococcus caseolyticus*, and *Bacillus subtilis* (Figure 3).

According to past studies, tolerance to organic solvent in various *Staphylococcus sp*. has been attributed to changes in membrane composition or mechanism of lipase action leading to solvent degradation in response to stress similar to other gram-positive bacteria [27]. Tolerance to organic solvents in *Bacillus subtilis* has been attributed to membrane composition changes or stress response mechanisms [30]. Hence the close relatedness of *Staphylococcus sciuri* KM16 with these microbes indicated the possibility of a similar mechanism of tolerance in it.

Tolerance studies of the isolate

Growth studies with *Staphylococcus sciuri* KM16 revealed that optimum temperature and culture medium for its growth was 37°C and LB. Cell growth in the presence of butanol was used as a parameter for butanol tolerance. The growth profile studies showed that *Staphylococcus sciuri* KM16 showed 87%, 70%, and 18% growth at 2.0%, 2.25%, and 2.5% (v/v) butanol respectively as compared to the control. Negligible growth was observed at 3.0% (v/v) butanol (Figure 4).

It has been reported that acclimatization of cells and addition of mineral and vitamins solution to the growth medium resulted in improved tolerance to butanol in *Bacillus subtilis* [20], and exposure to UV rays led to improvement in tolerance of *Clostridium acetobutylicum* [33]. However, contrastingly in our study, none of these led to any improvement in tolerance. (Data not shown)

Butanol toxicity has been a bottleneck in its microbial production. Several studies have been reported aimed at genetic engineering to improve the tolerance of native *Clostridial sp*. It has been reported that random mutation using NTG and UV rays and targeted mutations led to improvement in tolerance of *Clostridial sp*. to butanol leading to increase in yield from 7.6 g/L to 17.6 g/L and 10.46 g/L to 14.15 g/L [33, 34]. Over expression of *groESL* (heat shock protein) in *Clostridium acetobutylicum* increased the tolerance to butanol hence leading to 30% increase in yield of approximal 17 g/L [35]. Deletion of novel protein SMB_G1518 having conserved region to modulate butanol tolerance resulted in increase in 70% tolerance at 1% (v/v) butanol [36]. Expression of glutathione biosynthetic genes (*gshAB* gene) assumed to protect central metabolic pathway of cell under butanol stress resulted in increase in butanol yield from 11 g/L to 15 g/L by improvement in tolerance [37]. Apart from these strategies attempts were made to simultaneously extract butanol from *Clostridial* fermentation broth to overcome butanol toxicity through *in situ* product removal by gas stripping, gas stripping coupled to liquid-liquid extraction, and bioreactor bleeding resulting in maximum production of up to 113 g/L, 610 g/L, and 461.3 g/L butanol respectively have been reported [38, 39].

Strict anaerobic growth, low yield of cells, and end production inhibition are certain limitations associated with *Clostridium sp*. Therefore, different microbial species were explored for butanol production including *E. coli*, *Pseudomonas putida*, *Lactobacillus brevis*, and *Bacillus subtilis*. Initial studies were done in *E. coli* because of its easy genetic manipulation and

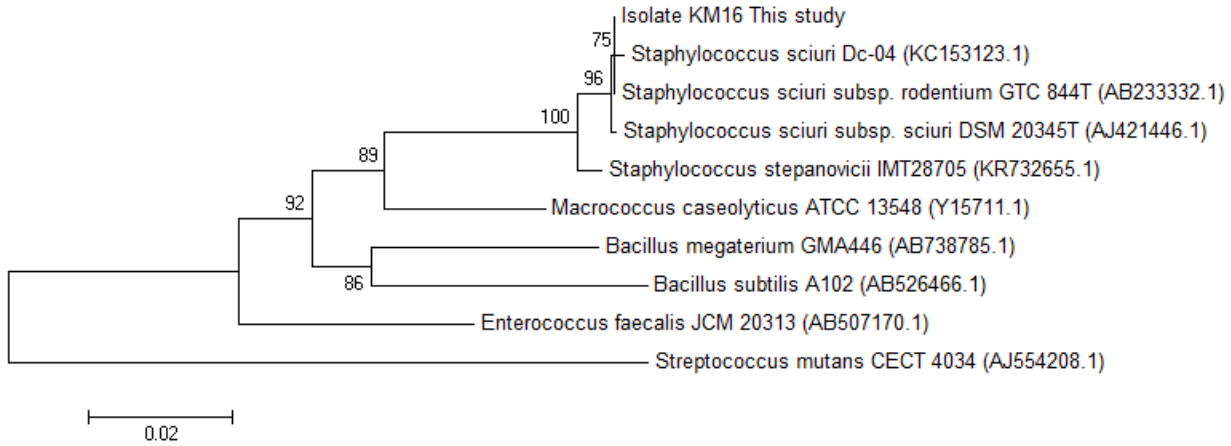

Figure 3. Phylogenetic relatedness of 16S rRNA gene amplified from *Staphylococcus sciuri* KM16 and organic solvent tolerant bacterial sp. Dendrogram was constructed from a CLUSTALW alignment of related 16S rRNA gene sequences by neighbor-joining analysis using MEGA 5.2. Reference sequences from GenBank include the accession number. The scale bar represents substitutions per nucleotide.

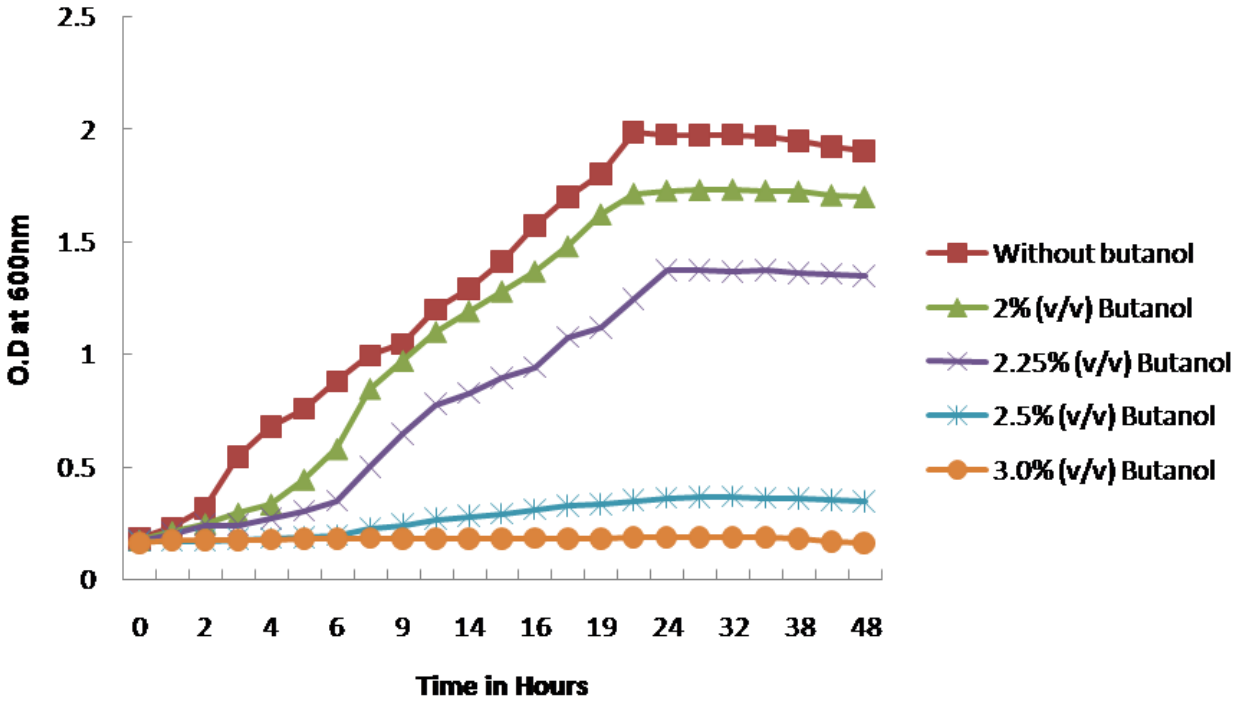

Figure 4. Growth profile of Staphylococcus sciuri KM16 in different concentration of butanol. Growth of the *Staphylococcus sciuri* KM16 was monitored at 600 nm in absence of butanol as well as in presence of different concentrations of butanol at 0%, 2%, 2.25%, and 2.5% (v/v) butanol.

high growth. However, it also lacked the ability to tolerate butanol beyond 1.0% (v/v) [40]. To overcome butanol toxicity in *E. coli*, successful attempts to improve the tolerance through *in situ* product removal were reported resulting in maximum production of 50 g/L butanol [7]. Expression of *groESL* (heat shock proteins) in *E. coli* resulted in 56% increased cellular growth at 1% (v/v) butanol [41]. Expression of *TMT* (Tilapia metallothionein) fused with *OmpC* (outer membrane protein C precursor) led to reduced oxidative stress caused by ROS (reactive oxygen

species) produced as a result of butanol stress in *E.coli* and resulted in 3 times improved cellular growth at 1.5% (v/v) butanol [42]. Expression and manipulation of efflux pumps *AcrB* in *E. coli* for improved butanol tolerance resulted in 25% increased cellular growth at 1.5% (v/v) butanol [43].

These genetic manipulations and *in situ* product removal techniques created additional cost burden on the fermentation process. Alternative microbes such as *Lactobacillus brevis*, *Bacillus subtilis*, *Pseudomonas putida*, and *Sachharomyces cerevisiae* were explored to be butanol production hosts because of their reported tolerance to organic solvents [18, 40]. But none of them could tolerate butanol beyond 2.0% (v/v) showing considerable growth [19, 20, 40]. Apart from this deficiency of redox balance, in case of *E. coli* and *Lactobacillus brevis* was also a limitation in using these microbes as hosts as they both lack reducing equivalent NADH which is required for butanol production [8, 19]. Therefore, a new host which can overcome all these limitations and tolerate butanol naturally is very important for its high production.

Natural butanol tolerance has been reported in *Lactobacillus sp*. (3.0%) [19, 44, 40], *Bacillus subtilis* (2.25%) [20], *Staphylococcus aureus* (3.0%) [21], and *Pseudomonas sp*. (6.0%) [22]. Tolerance to butanol in these reports has been assessed by exposing the mid or late exponential stage cells to butanol and then estimating their viable cell numbers or by measuring the maximum inhibitory concentration of butanol for their growth. On the other hand, dynamic growth measurement of *Lactobacillus sp*., *Bacillus subtilis*, *Staphylococcus aureus*, and *Pseudomonas putida* showed that despite their tolerance the cell number on exposure to butanol beyond 2.0% (v/v) resulted in drastic 50-80% reduction in growth. As during fermentation butanol is formed gradually so the cells should have the ability of tolerance during entire growth and not only during exponential phase. In our study growth of the *Staphylococcus sciuri* KM16 was measured dynamically from initial stage in the presence of butanol and the isolate showed growth equivalent to 70% in the presence of 2.25% (v/v) butanol of that observed without butanol. This level of tolerance by *Staphylococcus sciuri* KM16 is higher than any other reported strain in the presence of 2.25% (v/v) butanol.

Genome analysis and prediction of genes for butanol tolerance

Genome analysis and protein BLAST predicted the presence of four genes in the genome of *Staphylococcus sciuri* which may contribute towards butanol tolerance. The gene products of these four genes are universal stress protein *UspA* (WP_048540223.1), General stress protein (WP_048541491.1), (WP_048540132.1), and *GlsB*/*YeaQ*/*YmgE* family stress response protein (WP_048539880.1)

UspA showed 53% similarity with Universal stress protein YxiE (P42297) encoded by *yxiE* gene present in *Bacillus subtilis*. Homologue of this gene i.e. *YheK* has been reported by Petersohn et al., (2001) to contribute towards improved ethanol tolerance in *Bacillus subtilis* [45]. General stress response protein (WP_048541491.1) showed 66% similarity to General stress protein 17M (GSP17M) (P80241) encoded by *YflT* gene present in *Bacillus subtilis*. This gene has been reported to be induced under σ^{B} regulon on exposure to heat salt and ethanol [45]. General stress response protein (WP_048540132.1) showed 62% similarity to General stress protein 13 (GSP13M) (P80870) encoded by *YugI* gene in *Bacillus subtilis*. *YugI* gene showed increased mRNA level under cold shock by providing temperature downshift [46]. *GlsB*/*YeaQ*/*YmgE* family stress response protein (WP_048539880.1) showed 73% similarity with UPF0410 protein YwzA (O32282) encoded by *ywzA* gene in *Bacillus subtilis*. This gene is also reported to be induced under oxidative stress [47].

Generally, there are three possible mechanisms reported to overcome the butanol toxicity including expression of stress/heat shock

proteins or anti oxidative enzymes in response to stress caused by butanol, expression of efflux pumps to actively flush the toxic compound out of the cell, and change in membrane composition in response to butanol toxicity to maintain the stability of cell membrane. According to previous studies, there can be combination of various cellular mechanisms inside a microbe which can contribute to organic solvent tolerance [41]. In the present study, genome comparison of *Staphylococcus sciuri* with other related solvent tolerance genes present in various microorganism revealed that the tolerance in our isolate might be attributed to various stress protein expression as it might help to reduce the oxidative and general stress caused by butanol in the cell. This fact is supported by previous studies done in *Clostridium acetobutylicum* and *E. coli* [41, 42].

Conclusion

Butanol can prove to be a potential next generation biofuel, but it is considered highly toxic to microbes. Therefore, its toxicity is a major hurdle in its biological production through microbial fermentation. The aim of this study was to find a microbe which can naturally tolerate butanol at a higher level than *Clostridia*. Although there are vast number of approaches which have been used in the past to improve the tolerance of the microbes to butanol, these approaches were either aimed at genetic manipulations in microbial hosts or *in situ* product removal techniques to overcome the butanol toxicity problem. A natural butanol tolerant microbe will have the potential to be a superior host for butanol production. This study reports a novel strain *Staphylococcus sciuri* KM16 with remarkably high level of tolerance to butanol and thus it may be an ideal host for butanol production. To further improve the tolerance of the microbe, it is very crucial to understand the possible mechanism behind butanol tolerance in that organism. The genomic study of *Staphylococcus sciuri* revealing its similarity with stress response genes present in *Bacillus subtilis* also supports the previous studies and offers more scope to further improve the tolerance of microbe to butanol.

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