Enhanced ethanol tolerance in *Lysinibacillus* sp.

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ABSTRACT

Alcohol-tolerant microbes are the prime requirement for industrial-scale production of biofuels and beverages. Tolerance is a complex phenomenon that is achieved by mutational changes at several points in the genome. Since a network of genes and pathways are involved in adapting to ethanol tolerance, it is, therefore, more preferable to obtain ethanol tolerance phenotype by adaptive evolution. Adaptive evolution ensures genotypic changes which result in the evolution of a phenotypically tolerant strain. In the present work, *Lysinibacillus* sp. isolated from the estuarine area was subjected to adaptive evolution under ethanol stress that resulted in an increase in ethanol tolerance from 1.6% to 6.4%.

1. INTRODUCTION

In the era of biofuels, ethanol is considered as an alternative source of energy. Several ethanol-producing microbes have been screened [1–3] as well as genetic engineering strategies [4–6] have been designed to achieve high productivity of ethanol at industrial scale. However, the main limitation at the industrial-scale production is the lower tolerance of microbes toward the accumulated ethanol [7–9]. The major effect that ethanol has on microbes is the increase in permeability of the plasma membrane and its complete disruption at very higher concentrations [10,11]. Also, it has been reported that ethanol affects transcriptional as well as translational process to various levels, thereby disturbing the metabolic activity of the microbes [12]. To counter this, several approaches like screening of ethanol-tolerant strain by the random method, adaptive evolution to ethanol tolerance as well as engineering of tolerance in the microbes have been tested [13–15].

Ethanol affects at multiple cellular levels and, therefore, only fragmented information on the mechanism of ethanol toxicity is known to date [16]. So, designing ethanol tolerance at genetic level becomes critical [17–19]. It is difficult to achieve ethanol tolerance by modifying at a single gene or enzyme level [20].

To achieve complete fitness, a more robust method like adaptive evolution needs to be designed. During evolution, microbes adapt to the immediate environment by modifying its metabolism. This is achieved by acquiring mutations in the genome and subsequent selection during the evolutionary process [21–24].

Using the same phenomenon, in the present study, a *Lysinibacillus* sp. was tested for ethanol tolerance and, subsequently, the strain was evolved under ethanol as selection pressure.

2. MATERIALS AND METHODS

2.1. Strains used in the study

The bacterial isolates used in this study have been isolated from the soil sediment of Khandia estuary located at 21°19′1.65″N and 86°53′32.99″E in Balasore district near the mouth of Khandia river which is about 15 km away from Chandipur sea beach. The samples were inoculated on Nutrient Agar media and isolated colonies were obtained.

2.2. Screening of ethanol tolerant strain

The six isolates obtained from the estuary were studied for their ethanol tolerance capacity. For this, Terrific Broth (TB) media was supplemented with different concentrations of ethanol (0.2%, 0.4%, 0.8%, 1.6%, and 3.2%) and the isolates were inoculated. They were allowed to incubate at 37°C for 24 hours post-inoculation and their final biomass was measured.

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2.3. Phylogenetic analysis of the ethanol tolerant strain

The molecular characterization of the isolate KEI10 was done by 16S rRNA sequencing. The 16S rRNA gene was amplified from the genomic DNA of all the isolates. The primers used in the study [25,26] were as follows:

BAC27F   AGAGTTTGATCCTGGCTCAG

BAC1492R   GGTTACCTTGTTACGACTT

The PCR was carried out at initial denaturation of 95°C/5min, denaturation 95°C/30 sec, annealing 42°C/1min, extension 72°C/1 minute 30 seconds, and Final Extension 72°C/5 minutes. The sequencing of the purified PCR product was done using the BAC27F forward primer. The work was outsourced from SciGenom Labs Pvt. Ltd. Kerala, India. The sequence was submitted to EZ BioCloud [27] for identification purpose. Nearest matched and validly published sequences were downloaded from the EZ BioCloud system and used for the formation of a phylogenetic tree. MEGA 7 software [28] was used to prepare the phylogenetic tree. *Escherichia coli* 16S rRNA sequence [29] was taken as an outgroup. The sequences were aligned using Muscle software [30]. The tree was prepared using Neighbor-joining method with a bootstrap value of 1,000.

2.4. Adaptive laboratory evolution of ethanol tolerant strain

Isolate KEI10 showed the highest tolerance for ethanol and, therefore, was selected for the Adaptive laboratory evolution experiment. The strain was inoculated in TB medium supplemented with 1.6% ethanol and allowed to grow for 24 hours. Post incubation, the growth rate was measured and the culture was serially diluted into media containing 3.2% ethanol. The process was repeated until the cells regained their specific growth rate. At 6.4% ethanol concentration, the cells could tolerate ethanol stress and post that the cells could not maintain the specific growth rate.

2.5. Antibiotic susceptibility test

Antibiotic susceptibility test was performed by measuring the zone of inhibition produced by the antibiotics. Ampicillin, kanamycin, tetracyclin, streptomycin, and penicillin were tested for their susceptibility at 50 μg/μl concentration.

3. RESULTS AND DISCUSSION

3.1. Screening of ethanol-tolerant strain

Presently, microbes are extensively utilized for bio-ethanol production. However, the major drawback in the production process is the ability of the organism to tolerate ethanol. The tolerance level of microbes for ethanol is very low because of its harmful effect on the cell. As the ethanol keeps on accumulating in the media during the production process, it leads to severe decline in biomass formation and ultimately leads to cell lysis. Therefore, to increase the titer of ethanol production, ethanol-tolerant strains should be utilized in the production process. In the present study, after incubation of isolates in TB media with different concentrations of ethanol for 24 hours, the final biomass was measured. It was observed that in all isolates, there

was a continues decline in biomass as the ethanol concentration increased, but in isolate KEI10, there was no significant change in biomass even at a higher concentration of ethanol. Also, the overall biomass of isolate KEI10 was more in comparison to other isolates (Fig. 1). This indicates that isolate KEI10 is more tolerant to ethanol in comparison to other isolates.

3.2. Isolate identification and phylogenetic analysis

Amplified 16S rRNA gene sequence of KEI10 submitted in EZ BioCloud (https://www.ezbiocloud.net/identify) for identification showed the nearest matched bacteria was *Lysinibacillus fusiformis* with 98.10% similarity, indicating that KEI10 may be a new species of *Lysinibacillus* genera. However, we will designate isolate KEI10 as *Lysinibacillus* sp. KEI10. Twenty nearest matched and validly published sequences were downloaded from the EZ BioCloud system and used for the formation of a phylogenetic tree. Out of 20, only the last 2 sequences were of *Bacillus* and the rest of the sequences were of *Lysinibacillus*. In the phylogenetic tree, *Lysinibacillus* sp. KEI10 clusters with *L. fusiformis*, also isolated from the soil [31] (Fig. 2), confirming the identification result of the EZ BioCloud system. *Escherichia coli* being different from *Lysinibacillus* and *Bacillus* made an outgroup, indicating the correctness of tree.

3.3. Adaptive laboratory evolution of ethanol-tolerant strain

Adaptive laboratory evolution experiments, as shown in this work, were performed for a sufficient time period to generate an apparently stable phenotype. During adaptive evolution, several phenotypic as well as genotypic changes take place, which are generally associated with increased fitness. However, for industrial production, parameters such as specific growth rate (μ_{max}), survival rates in toxic concentrations of certain chemical compounds, and absolute biomass yield are appropriate fitness criteria. In this case, the specific growth rate (μ_{max}) has been considered as a test to evaluate the fitness of the evolved strain. Generally, the number of generations is taken as the timescale for adaptive laboratory evolution experiments. Usually, mutations are accumulated during successive rounds of cell division in growing cultures, and, therefore, the cumulative number of cell divisions
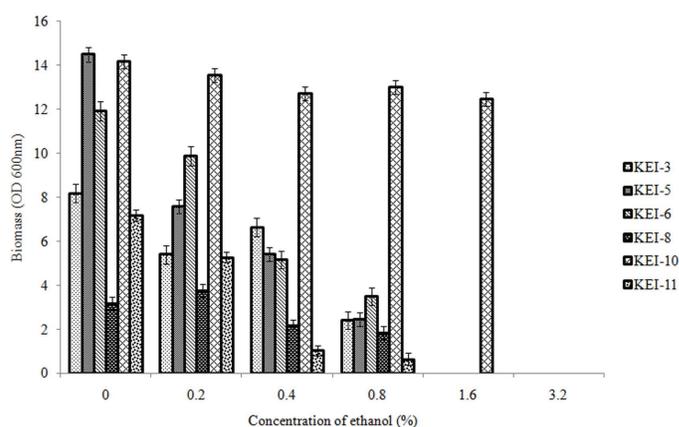

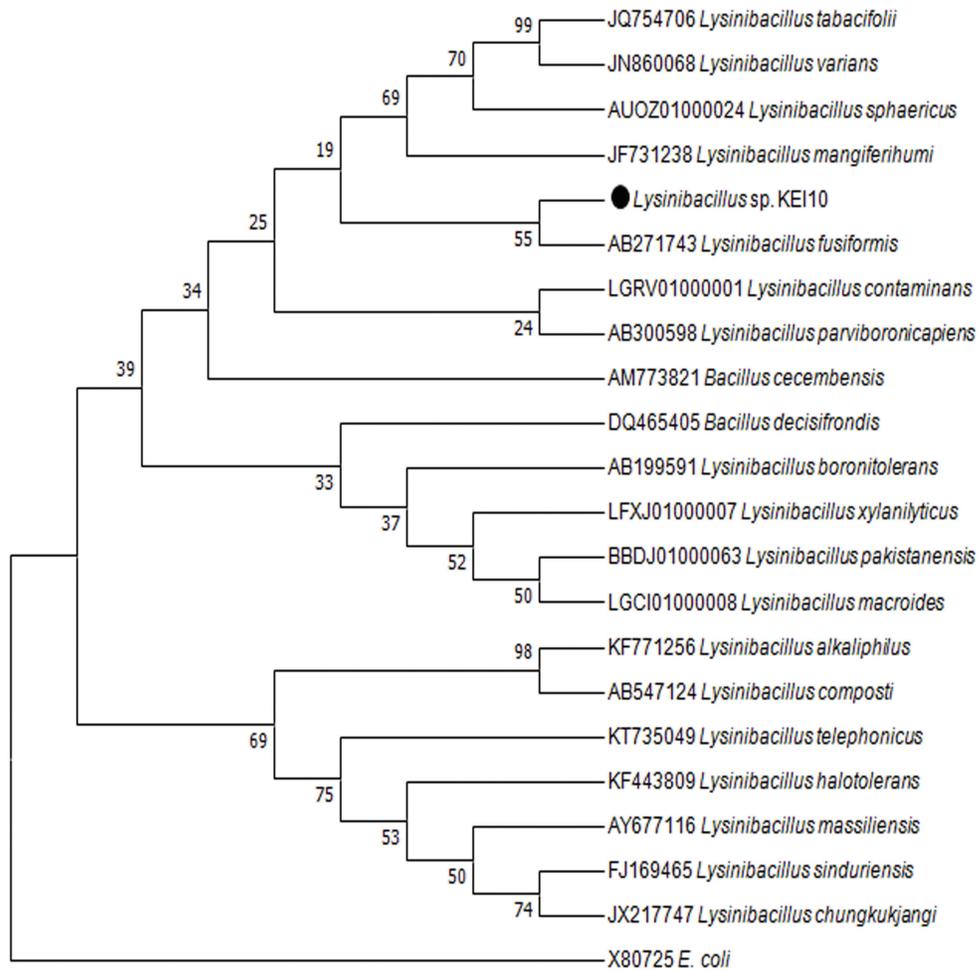**Figure 1:** Biomass profile of selected isolates in different concentrations of Ethanol.

Figure 2: Tree showing the phylogenetic relationship of *Lysinibacillus* sp. KEI10 with 20 other nearest matched species of *Lysinibacillus* and *Bacillus*. The tree was prepared using Neighbor-Joining method with a bootstrap value of 1,000.

is considered as an alternative way to analyze evolution over a period of time [32]. To further increase the ethanol tolerance of the KEI10 strain, an adaptive laboratory evolution experiment was set up; where *Lysinibacillus* sp. KEI10 was sequentially subcultured in increasing concentration of ethanol till it attains an increase in specific growth rate. The isolate was grown in 1.6% ethanol supplemented TB medium and then inoculated into serially higher concentrations. Initially, there was a fall in specific growth rate but after growing in the same concentration for few more generations; the isolates recovered its specific growth rate. This was continued till 6.4% ethanol concentration (Fig. 3). Beyond that, such as 7.2%, the cells could not recover their specific growth rate. So, it can be inferred that there was an increase in tolerance from 1.6% to 6.4%. The isolate with increased tolerance to 6.4% was named as KEI10_ET6.4. A background review on ethanol tolerance has revealed that the most common ethanol production hosts like *E. coli*, *Clostridium acetobutylicum*, *Pseudomonas putida*, etc., have shown as high as 2% tolerance toward several alcohols, and several genetic engineering strategies have been implemented to achieve high tolerance, high MIC, or increased colony count [7]. With inverse engineering strategies [33], *Saccharomyces*
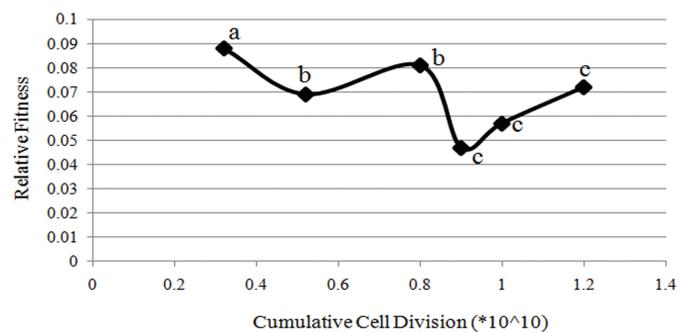

Figure 3: Fitness profile of the KEI10 isolate as obtained after Adaptive laboratory Evolution in increasing concentration of ethanol. a–c represents the percentage of alcohol at which the respective fitness level is achieved. (a) 1.6%, (b) 3.2%, and (c) 6.4%.

cerevisiae has shown increased tolerance till 5%. Similarly, an alcohol dehydrogenase mutant of *Thermoanaerobacter ethanolicus* showed 8% ethanol tolerance [34]. There are also reports of *Clostridium thermocellum* adapted to 8% ethanol tolerance [35] and evolutionary engineering strategies yielding

12% ethanol-tolerant *Saccharomyces cerevisiae* strains [36]. So, it is noteworthy that the ethanol-tolerant strain KEI10_ET6.4 developed in this study is on par with the reported tolerant and production strains. Also, adaption till 6.4% is achieved by simple laboratory-scale adaptive evolutionary experiments unlike the strenuous genetic engineering experiment.

3.4. Analysis of glucose uptake

Glucose uptake is an indirect measurement of the cellular physiology. Glucose uptake and subsequent increase in biomass explain healthy cellular physiology. Also, glucose is the primary precursor for ethanol fermentation. Therefore, in this experiment, different concentration of glucose was utilized to check the glucose uptake ability of the wild type and ethanol-tolerant strains. For this, TB media was supplemented with various concentrations (0.2%, 0.4%, 0.8%, 1.6%, and 3.2%) of glucose and both wild type (Control strain KEI10) and ethanol-tolerant strains (KEI10_ET6.4) were inoculated. Control was inoculated with glucose-supplemented media and KEI10_ET6.4 was inoculated in glucose and ethanol-supplemented media. After incubation for 24 hours, the final biomass was measured at 600 nm. It was observed that the ethanol-tolerant strain showed a slight decline in final biomass as compared to control. But interestingly, the ethanol-tolerant strain could grow well till 0.8% glucose (Fig. 4), signifying that the tolerant strains have a normal glucose uptake and metabolism.
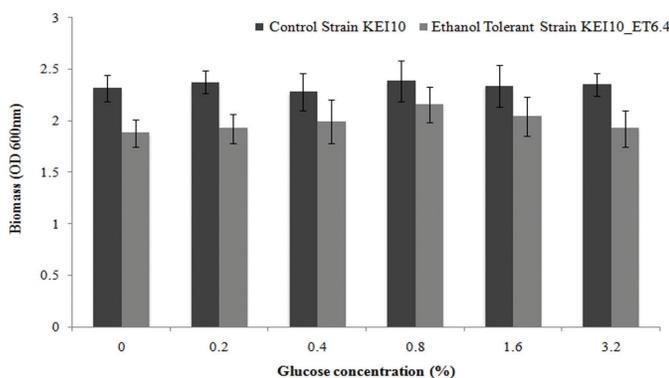

Figure 4: Biomass profile of the control strain KEI10 and ethanol-tolerant strain KEI10_ET6.4 under the increasing concentration of glucose.
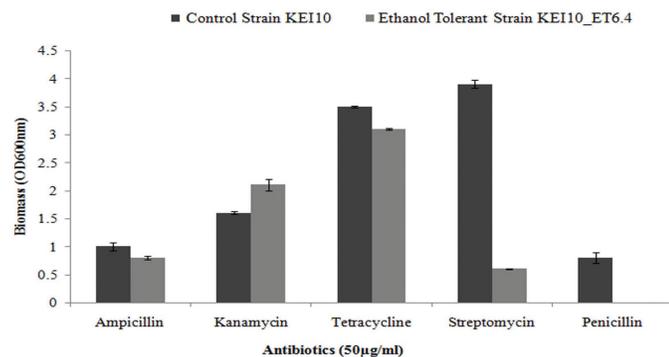

Figure 5: Zone of Inhibition generated by control strain KEI10 and ethanol-tolerant strain KEI10_ET6.4 when treated with various antibiotics.

3.5. Antibiotic susceptibility test

Previously, it has been reported that ethanol tolerance leads to change in the cell membrane permeability. Change in cell membrane permeability is associated with differential expression of the multidrug resistance gene. Therefore, it could lead to change in the degree of resistance and susceptibility of microbes toward antibiotics [37,38]. Previously, it has also been reported that overexpression of the multidrug resistance gene increases ethanol tolerance and fermentation performance in yeast [39]. In this context, antibiotic susceptibility of the ethanol-tolerant strain KEI10_ET6.4 was tested for ampicillin, kanamycin, tetracyclin, streptomycin, and penicillin. It was observed that for ampicillin and tetracycline, there was a very slight decline in the zone of inhibition, whereas, in case of kanamycin, there was a slight increase in zone of inhibition, which suggests that there is no differential change in susceptibility of ethanol-tolerant strain KEI10_ET6.4 toward ampicillin, tetracycline, and kanamycin. Interestingly in the case of streptomycin, there was a sharp decline in the zone of inhibition in ethanol-tolerant strain. Similarly, in the case of penicillin, no zone of inhibition could be measured (Fig. 5). This indicates that the ethanol tolerant strain has gained resistance for both streptomycin as well as penicillin at a concentration of 50 μg/μl.

4. CONCLUSION

Microbial physiology is highly affected by ethanol. Most of the bacteria are tolerant in the range of 1%–10% [7]. Ethanol toxicity becomes the primary problem for its production via microbial fermentation [40]. Numerous studies have been conducted to find, modify, and construct an optimal host with high tolerance to ethanol [9,41]. While the construction of an ethanol biosynthesis pathway in several heterologous hosts has been reported, the major obstacle limiting their achievement is due to the low tolerance of the host to ethanol toxicity. In this context, the aim of this work was to search for and develop an ethanol-tolerant bacterium as a host for further application in the bioproduction of alcohol. We have used adaptive evolution to generate spontaneous ethanol-tolerant strains of *Lysinibacillus* sp. The KEI-10 isolate has been identified to be *Lysinibacillus* sp. KEI-10 and it has maximum similarity to *L. fusiformis* (98.10%). This strain isolated from Khandia estuary of Balasore district, had a maximum ethanol tolerance of 3.2%. By using the adaptive laboratory evolution experiment, its ethanol tolerance could be increased up to 6.4% which is almost double. Since ethanol leads to membrane permeability and change in antibiotic sensitivity due to differential expression of multi drug exporters present on the membrane, therefore the tolerant strain was tested for its antibiotic sensitivity. While there was no significant change in ampicillin, kanamycin, and tetracycline resistance, the resistance for streptomycin and penicillin increased as observed from the decreased zone of inhibition as compared to control strain. Also, the tolerant strain had no altered glucose metabolism, which makes it a potential ethanol production strain. Further biochemical and genetic characterization could be performed for the ethanol-tolerant strain KEI-10_ET6.4 and its ability to ferment ethanol under various carbon sources could be analyzed to use it as an industrial strain for ethanol fermentation.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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