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°19ʹ1.65″N and 86°53ʹ32.99″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.
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 (μmax), survival rates in toxic concentrations of certain chemical compounds, and absolute biomass yield are appropriate fitness criteria. In this case, the specific growth rate (μmax) 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
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 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.

### 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.

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

### 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.
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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

REFERENCES
1. Tangtua J, Techapun C, Pratanaphon R, Kuntiya A, Chaiyasu T, Hammangai P, et al. Screening of 50 microbial strains for production of ethanol and (R)-phenylacetylcarnobil. Chiang Mai J Sci 2013;40(2):299–304.
2. Metsoviti M, Paramithiotis S, Drosinos EH, Galiotou-Panayotou M, Nychas GJ, Zeng AP, et al. Screening of bacterial strains capable of converting biodiesel-derived raw glycerol into 1, 3-propanediol, 2, 3-butanediol and ethanol. Engr Life Sci 2012;12(1):57–68.
3. Elshaghabee FM, Bockelmann W, Meske D, de Vreeze M, Walte HG, Schrezenmeir J, et al. Ethanol production by selected intestinal microorganisms and lactic acid bacteria growing under different nutritional conditions. Front Microbiol 2016;7:47.
4. Dienes BS, Cotta MA, Jeffries TW. Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 2003;63(3):258–66.
5. Yoshikawa K, Toya Y, Shimizu H. Metabolic engineering of Synechocystis sp. PCC 6803 for enhanced ethanol production based on flux balance analysis. Bioproc Biosyst Eng 2017;40(5):791–6.
6. Jojima T, Naburu Y, Sasaki M, Tajima T, Suda M, Yukawa H, et al. Metabolic engineering for improved production of ethanol by Corynebacterium glutamicum. Appl Microbiol Biotechnol 2015;99(3):1165–72.
7. Mukhopadhyay A. Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. Trends Microbiol 2015;23(8):498–508.
8. Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA. The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. J Appl Microbiol 2010;109(1):13–24.
9. Dunlop MJ. Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels 2011;4(1):32.
10. Ingram LO. Ethanol tolerance in bacteria. Crit Rev Biotechnol 1989;9(4):305–19.
11. Huffer S, Clark ME, Ning JC, Blanch HW, Clark DS. Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria, and archaea. Appl Environ Microbiol 2011;77(18):6400–8.
12. Haff RJ, Keating DH, Schaegawa T, Schwalbach MS, Vinokur J, Tremaine M, et al. Correcting direct effects of ethanol on translation and transcription machinery confers ethanol tolerance in bacteria. Proc Natl Acad Sci 2014;111(25):E2576–85.
13. Biswas R, Prabhu S, Lynd LR, Guss AM. Increase in ethanol yield via elimination of lactate production in an ethanol-tolerant mutant of Clostridium thermocellum. PLoS One 2014;9(2):e86389.
14. Suzuki T, Seta K, Nishikawa C, Hara E, Shigeno T, Nakajima-Kambe T. Improved ethanol tolerance and ethanol production from glycerol in a streptomycin-resistant Klebsiella varicola mutant obtained by ribosome engineering. Bioresearch Technol 2015;176:156–62.
15. Tian L, Cervenka ND, Low AM, Olson DG, Lynd LR. A mutation in the Adhe alcohol dehydrogenase of Clostridium thermocellum increases tolerance to several primary alcohols, including isobutanol, n-butanol and ethanol. Sci Rep 2019;9(1):1736.
16. Cao H, Wei D, Yang Y, Shang Y, Li G, Zhou Y, et al. Systems-level understanding of ethanol-induced stresses and adaptation in E. coli. Sci Rep 2017;7:44150.
17. Chong H, Huang L, Yeow J, Wang I, Zhang H, Song H, et al. Improving ethanol tolerance of Escherichia coli by rewiring its global regulator cAMP receptor protein (CRP). PLoS One 2013;8(2):e57628.
18. Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 2006;314(5805):1565–8.
19. Brown SD, Guss AM, Karpintis TV, Parks JM, Smolin N, Yang S, et al. Mutant alcohol dehydrogenase leads to improved ethanol tolerance in Clostridium thermocellum. Proc Natl Acad Sci 2011;108(33):13752–7.
20. Kasavi C, Eraslan S, Arga KY, Oner ET, Kirdar B. A system based network approach to ethanol tolerance in Saccharomyces cerevisiae. BMC Syst Biol 2014;8(1):90.
21. Chen S, Xu Y. Adaptive evolution of Saccharomyces cerevisiae with enhanced ethanol tolerance for Chinese rice wine fermentation. Appl Biochem Biotechnol 2014;173(7):1940–54.
22. Horinouchi T, Suzuki S, Hirasawa T, Ono N, Yono T, Shimizu H, et al. Phenotypic convergence in bacterial adaptive evolution to ethanol stress. BMC Evol Biol 2015;15(1):180.
23. Voordeekers D, Kominek J, Das A, Espinoza-Cantu A, De Maeyer D, Arslan A, et al. Adaptation to high ethanol reveals complex evolutionary pathways. PLoS Genet 2015;11(11):e1005635.
24. Winkler JD, Kao KC. Recent advances in the evolutionary engineering of industrial biocatalysts. Genomics 2014;104(6):406–11.
25. Karakasidou K, Nikolouli K, Amoutzias GD, Pournou A, Manassis C, Tsiamis G, et al. Microbial diversity in biodeteriorated Greek historical documents dating back to the 19th and 20th century: a case study. Microbiol Open 2018;7(5):e00596.
26. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol 2008;74(8):2461–70.
27. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, et al. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 2017;67(5):1613.
28. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016;33(7):1870–4.
29. Cilia V, Lafay B, Christen R. Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol Biol Evol 1996;13(3):451–61.
30. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32(5):1792–7.
31. Ahmed I, Yokota A, Yamazoe A, Fujitawa T. Proposal of Lysinibacillus boronitolerans gen. nov. sp. nov., and transfer of Bacillus fusiformis to Lysinibacillus fusiformis comb. nov. and Bacillus sphaericus to Lysinibacillus sphaericus comb. nov. Int J Syst Evol Microbiol 2007;57(5):1117–25.
32. Lee DH, Feist AM, Barrett CL, Palsson BØ. Cumulative number of cell divisions as a meaningful timescale for adaptive laboratory evolution of Escherichia coli. PLoS One 2011;6(10):e26172.
33. Hong ME, Lee KS, Yu BJ, Sung YJ, Park SM, Koo HM, et al. Identification of gene targets eliciting improved alcohol tolerance in Saccharomyces cerevisiae through inverse metabolic engineering. J Biotechnol 2010;149(1–2):52–9.
34. Burdette DS, Jung SH, Shen GJ, Hollingsworth RI, Zeikus JG. Physiological function of alcohol dehydrogenases and long-chain (C30) fatty acids in alcohol tolerance of Thermoanaerobacter ethanolicus. Appl Environ Microbiol 2002;68(4):1914–20.
35. Zhu X, Cui J, Feng Y, Fa Y, Zhang J, Cui Q. Metabolic adaption of ethanol-tolerant Clostridium thermocellum. PLoS One 2013;8(7):e70631.
36. Koppram R, Albers E, Olsson L. Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. Biotechnol Biofuels 2012;5(1):32.
37. Dötsch A, Becker T, Pommerenke C, Magnowska Z, Jänsch L, Häussler S. Genomewide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2009;53(6):2522–31.

38. Delcour AH. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 2009;1794(5):808–16.

39. Teixeira MC, Godinho CP, Cabrito TR, Mira NP, Sá-Correia I. Increased expression of the yeast multidrug resistance ABC transporter Pdr18 leads to increased ethanol tolerance and ethanol production in high gravity alcoholic fermentation. Microbial Cell Factories 2012;11(1):98.

40. Azhar SH, Abdulla R, Jambo SA, Marbawi H, Gansau JA, Faik AA, et al. Yeasts in sustainable bioethanol production: a review. Biochem Biophys Rep 2017;10:52–61.

41. Carreón-Rodriguez OE, Gutiérrez-Ríos RM, Acosta JL, Martinez A, Cevallos MA. Phenotypic and genomic analysis of *Zymomonas mobilis* ZM4 mutants with enhanced ethanol tolerance. Biotechnol Rep 2019;23:e00328.

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