https://doi.org/10.48047/AFJBS.6.14.2024.1031-1052

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Exploring the Ethanol Tolerance Potential of Bacteria Isolated from Refinery Oily Sludge and Contaminated Soil

Janayita Biswa Sarma[1], Saurov Mahanta[2*], Hemen Deka[3], Bhaben Tanti[3]

[1]Department of Energy Engineering, Assam Science & Technology University, Jalukbari, Tetelia, Guwahati-781013, Assam, India

ORCiD: 0000-0002-8134-2146

[2]National Institute of Electronics and Information Technology, Guwahati, 781022, Assam, India

[3]Department of Botany, Gauhati University, Jalukbari, Guwahati-781014, Assam, India

Corresponding Author*: saurov.mahanta@gmail.com (+919435047023)

Volume 6, Issue 14,2024

Received: 10 JULY 2024

Accepted: 31 JULY 2024

Published: 02 AUG 2024

doi:10.48047/AFJBS.6.14.2024.1031-1052

Abstract

In this study, ethanol tolerant bacteria have been isolated from refinery oily sludge contaminated soil by employing enrichment culture technique. All total 29 of the bacterial isolates have been obtained and screened for ethanol tolerating abilities at different concentrations of ethanol. The results showed that all the bacterial isolates could tolerate upto 5% v/v. A total of 13 bacterial isolates showed resistance to 10% v/v ethanol stress. On the other hand, 4 isolates can tolerate ethanol stress upto 15% v/v. The result also showed that only 1 bacterial isolate was able to withstand ethanol stress up to 30% v/v. The characterization of the bacterial isolate has been done which showed ethanol tolerance upto 30% v/v. Therefore, there are possibilities that by using the ethanol tolerant isolate, biofuel production can be achieved.

Key words: Alternative source, Bio-ethanol, Bioenergy, Ethanol-tolerant bacteria, Refinery oily sludge.

Introduction

Fossil fuel concentrations are rapidly diminishing, and the world's energy demand is growing. This has encouraged the development of alternative fuel to counteract the increased atmospheric deposition of greenhouse gases, which has resulted in significant climate changes. Sea levels and temperatures may rise as an effect of these changes, which may result

in catastrophic consequences eventually as reported by Kumar and Kumar (2017). Ethanol and other biofuel have become popular sources of alternative energy. Ethanol is a desirable alternative fuel since it is capable of being combined with petrol to generate clean alcohol for engines with higher octane numbers and vaporisation temperatures (Srivastava et al,. 2015; Demirbas 2004 and Chovau et al., 2013). Biofuels are considered as ecologically friendly and sustainable since they are produced in huge quantities using renewable resources. Its significance is highlighted by the possibility that bacterial biofuel production could provide a sustainable and renewable alternative to conventional fossil fuel (Ramos et al., 2022). Utilising biofuel is more environmentally benign than using fossil fuel because of the low sulphate concentration of the atmosphere (Palit et al., 2011). Alcohols and other biofuel are energy compounds produced with the help of microorganisms. The effect of biofuel on climate change is far less than that of fossil fuel. Studies show that bioethanol can cut hydrocarbon emissions by half as reported by Bentley (2002) and Cavallo (2002). When compared to conventional fossil fuel, biofuel generated by bacteria frequently have smaller carbon footprints. Because of the approximate equalization of the carbon dioxide taken by the plants during their growth and emitted after combustion, they can aid in the reduction of greenhouse gas emissions (Ramos et al., 2022). Bacteria are the primary focus in producing biofuel. Sustainable biofuel are essential to offer a consistent, reliable supply of energy for individuals and businesses. With the help of advanced biofuel, we can reduce our environmental impact and dependency on fossil fuel. Due to the growth of industry and global population, there is a steady demand for high energy. Biofuel are widely seen as viable alternative transportation fuel which may contribute to reduce the effects of climate change and decrease dependency on fuel derived from petroleum (Sims 2014 and Jeswani et al., 2020). The transportation industry's energy usage has played a role in a global issue. The issue of the depletion of fossil fuel is growing deeper as the world's energy needs rise daily (Costagliola et al., 2016). Bioethanol contributes to a reduction in greenhouse gas emissions in the transportation sector. It has been suggested that ethanol might be used in place of liquid transportation fuel according to Liu and Qureshi (2009). Yusuf and Inambao (2019) suggested that in order to improve efficiency and lower greenhouse gas emissions, the development of new strategies that concentrate on energy consumption, optimisation and alternative sources of fuel must be prompted. Ethanol is crucial for addressing the energy crisis and preserving the environment. Nowadays, alcohol-tolerant bacteria are a major requirement for the large-scale industrial production of bioethanol since ethanol causes the plasma membrane to become more permeable and, in extreme cases, it completely breaks,

which is the main effect on microorganisms (Ingram 1990 and Huffer et al., 2011). The fact is widely known that ethanol, in particularly, disintegrates the membrane. This leads to the unregulated movement of solutes, which can lower the proton influx throughout the membrane and result in the leaking of critical cofactors like magnesium (Ingram 1976 and Cartwright et al., 1986). Additionally, ethanol can deactivate cytosolic and membrane enzymes, such as glycolytic and ATPase, which slows down cell growth (Ingram 1976; Banat et al., 1998 and Ding et al., 2009). The ability of certain bacterial strains to resist the effects of ethanol stress suggests that ethanol modifies the physiology of cells and aids in the production of a diffusible component, ethanol, which permits bacterial growth to higher densities. This indicates that ethanol has the ability to function as a signal as well as a carbon source, activating one or more pathways and altering the physiology and survival of bacteria. Studies show that ethanol is both necessary and sufficient to promote the growth of bacteria (Smith et al., 2004). The cell membranes of bacteria that are resistant to ethanol may change to allow less ethanol to enter the system. This may involve modifications to the lipid content or the addition of certain proteins which provide stability to the membrane. Several studies reported that bacteria exposed to ethanol might have modifications in their metabolism or metabolic pathways to reduce the generation of harmful intermediates or to increase energy output. Moreover, the ethanol tolerance of different bacteria is influenced by a number of physiological parameters, including temperature, osmotic pressure, intracellular ethanol deposits, and the way of substrate feeding. Considering the complexity of ethanol toxicity, it is likely that the ethanol tolerance mechanism involves multiple genes. Such strains' isolation and characterisation may improve the understanding of how well they are able to withstand ethanol as reported by D'amore and Stewart (1987). During the process of ethanol fermentation, bacteria face a variety of dynamic environmental problems, such as high substrate concentrations, nutritional shortages, acetic acid sensitivity, swift pH and temperature changes, and high substrate concentrations. Even though they are desirable for efficient product recovery, alcohol accumulations frequently cause the fermenting bacteria to grow less and eventually die. The methods that bacteria utilize to tolerate ethanol in their habitats must be investigated and understood at the cellular and molecular levels in order to create novel tolerant strains as suggested by Liu and Qureshi (2009). As a potential response to the problems presented by climate change and depleting fossil fuel supplies, the production of biofuel by bacteria is significant since it contributes to ecologically friendly, sustainable, and renewable energy sources. In the current era of biofuel, bioethanol has also been taken into consideration as a different energy source (Metsoviti et al., 2012; Tangtua et al., 2013

and Elshaghabee et al., 2016). Producing biofuel from refinery waste can be an inexpensive process. Reducing the total cost of production, bacteria that are acclimated to this environment can effectively transform the sludge's organic content into biofuel. To attain the high efficiency of ethanol at the industrial level, a number of studies have noted the screening of ethanol-tolerant bacteria. The decreased tolerance capability of the bacteria, however, is the main issue in the industrial production of ethanol (Stanley et al., 2010; Dunlop 2011; Mukhopadhyay 2015 and Mahalik et al., 2020). Refinery sludge bacteria are frequently adapted to extreme environments, such as high salinity, high temperatures and the presence of harmful substances. They serve as excellent possibilities for the manufacture of biofuel from industrial waste because of their tolerance, which enables them to survive in harsh environments. These bacteria break down and use petrochemicals and hydrocarbons as carbon sources. When compared to conventional refinery waste disposal techniques, using bacteria from refinery sludge for biofuel generation can be a more environmentally responsible solution. The concepts of resource recovery and waste-to-value are in line with this strategy. To enhance the commercial utility in the bioethanol sectors, an effort has been made in the present work to isolate and screen out an enhanced ethanol stress tolerant bacterial isolate from petroleum refinery oily sludge and contaminated soil samples.

Material and methods

1. Sample collection and isolation of bacteria

Environmental samples were collected from the refinery nearby discharge sites of Guwahati Refinery, Bongaingaon Refinery and Numaligarh Refinery, Assam, India. Samples were collected in air tight plastic bottle and kept at 4°C for isolation of bacterial candidates. The enrichment culture method was employed for isolation of ethanol tolerant bacteria according to the protocol described by Yomano and Ingram (1998) and Mukred et al., 2008. For isolation of ethanol producing bacteria, Mineral Salt Media (MSM) broth was used. This enrichment method using MSM broth supplemented with 1g of 5% (v/v) ethanol was performed to enrich bacteria capable of ethanol tolerance. 1g of dry contaminated soil samples and sludge samples was added into the 100 ml of sterile broth in a 250 ml conical flask supplemented with 5% ethanol respectively and was incubated in continuous shaking condition at 180 rpm at 30°C±1 for 6-7 days (Ambust et al., 2021). Following incubation, serial dilution was performed by transferring 1 mL of the above aliquot into 9 mL of sterile

distilled water. Spread plate technique was performed according to Yomano and Ingram (1998) and Prathyusha et al., 2016 by supplementing the agar plates containing Mineral Salt Medium agar media and modified Luria Bertani agar media with glucose and chloramphenicol. After the incubation period, morphologically distinct bacterial colonies that appeared on all the culture plates inoculated with soil samples and sludge samples were recorded. Plates showing ~30 to 300 colonies were considered ideal for determining colony-forming unit per mL of soil and sludge. The total colony forming units (CFUs) for each dilution of the soil sample and sludge sample in the respective media were calculated using the following formula.

$$\text{Colony Forming Unit}\frac{\text{CFU}}{\text{mL}} in\ original\ stock = \frac{Number\ of\ colonies}{Volume\ of\ culture\ plated\ (mL)} \times dilution\ factor$$

The pure culture of the isolates was maintained by subsequent subculturing in nutrient agar slants. All isolated bacteria were stored at 4ºC for further use and for long term preservation 20% glycerol stocks were prepared and kept at - 80°C.

2. Efficacy for ethanol tolerance of the bacterial isolates

Ethanol tolerance of the isolates was examined in terms of growth in nutrient broth containing different concentrations (0%, 10 %, 15 %, 18%, 20%, 25% and 30 % (v/v)) of absolute ethanol. During the screening process, 200µl of inoculum from 24 hours bacterial broth culture were amended with different ethanol concentration ranging from 0 (control) to 30% (v/v) were used in the screening procedure. The inoculated test tubes were incubated at 35℃ at 180 rpm at shaking incubator for 48 hours. After incubation, the absorbance at 600nm was measured in a UV-Vis Spectrophotometer (Ire et al., 2016). The bacterial isolates with highest OD readings at higher ethanol concentration were taken for further analysis. The blank was created using nutrient broth medium without any bacterial inoculation, and the treatments were repeated three times. The cell mass or growth is directly correlated with optical density (Iticha 2016).

3. Qualitative assay for ethanol production of the ethanol tolerant isolates

The bacterial isolates tolerating ethanol stress were subjected to qualitative assay for confirming ethanol production by using the 2, 3, 5-triphenyltetrazolium chloride (TTC)

double media method (Lu et al., 2013). With slight modifications, the TTC medium was introduced with the bacterial isolates and incubated for two to three hours at 28°C.

4. Molecular identification by sequencing of 16S rRNA

The bacterial isolates were cultured in Luria Bertani medium for 24 hours at 35±2℃ under shaking condition at 180 rpm. The bacterial cell pellets were recovered by centrifugation to perform the DNA extraction. DNA extraction was performed by a standard phenol chloroform method as described by Sambrook et al., 1989. The absorbance ratios A_{260}/A_{280} were measured spectrophotometrically to determine the yield and purity of the extracted DNA. PCR amplification of the 16S rRNA of the selected bacterial isolates was performed by Thermal Cycler and by using Master Mix. Universal primers 27F (Forward: 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (Reverse: 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a 1500bp fragment and which corresponds to the genes of the bacterial 16S rRNA (Chen et al., 2016). The PCR reaction was performed in 25µl reaction volume containing 22µl of PCR Master Mix, 0.4µl of each primer (27F and 1492R) and 2µl DNA. During the PCR reaction, the samples were subjected to initial denaturation by 95℃ for 3min, followed by 30 cycles of denaturation at 95℃ for 30sec, annealing at 55℃ for 40sec, extension at 72℃ for 14sec and finishing with 7min final extension at 72℃. The PCR products were detected by electrophoresis in 1% (w/v) agarose gel and the visualization was done by ethidium bromide staining (Lee et al., 2012). The reaction products were purified with the Purification kit, following the manufacturer's recommendations. The purified products were sent for sequencing at Unipath Speciality Laboratory Ltd., Ahmedabad, India using primers 27F and 1492R.

5. Phylogenetic Analysis

Phylogenetic analysis of the identified strains based on the 16S rRNA gene sequences was performed. The sequence was BLAST-ed against the NCBI database to identify the organism and determine its taxonomic classification. After obtaining the fasta files for the closest-matched sequences, a complete alignment was performed using the Clustal X sequence alignment tool. Multiple sequence alignments and phylogenetic trees were performed using the MEGA7 software and the Neighbour Joining method (Saitou and Nei 1987; Kumar et al., 2016 and Es-sbata et al 2021). The transcripts were submitted to NCBI GenBank in order to get their accession number.

6. Statistical analysis

Every experiment was carried out in triplicates. The mean values in addition to the standard deviation were used to express the results. Using the least significant difference (LSD) method, the differences between the categories were analysed and a confidence interval of 95% or 99% was determined.

Results

1. Enumeration of bacterial isolates using enrichment media

The abundance of ethanol tolerant bacteria was enumerated followed by isolation of pure culture strains using specific enrichment isolation procedure and results are presented graphically in figure 1. A total of 29 bacterial isolates were isolated from the contaminated sample collected from three different petroleum refineries. Out of these a total of 17 bacterial isolates were obtained from refinery oily sludge samples whereas a total of 12 bacterial isolates were obtained from the contaminated soil samples. By utilising MSM agar media, on an average, a total of 4.6 $\times 10^5$ cfu/ml bacterial population was found in contaminated soil samples and 5.4 $\times 10^4$ cfu/ml in refinery oily sludge samples. In addition, bacterial population in contaminated soil samples was calculated to be 1.0 $\times 10^5$ cfu/ml and the population in refinery oily sludge samples was 6.4×10^5 cfu/ml when modified LB agar media was used for the enumeration.

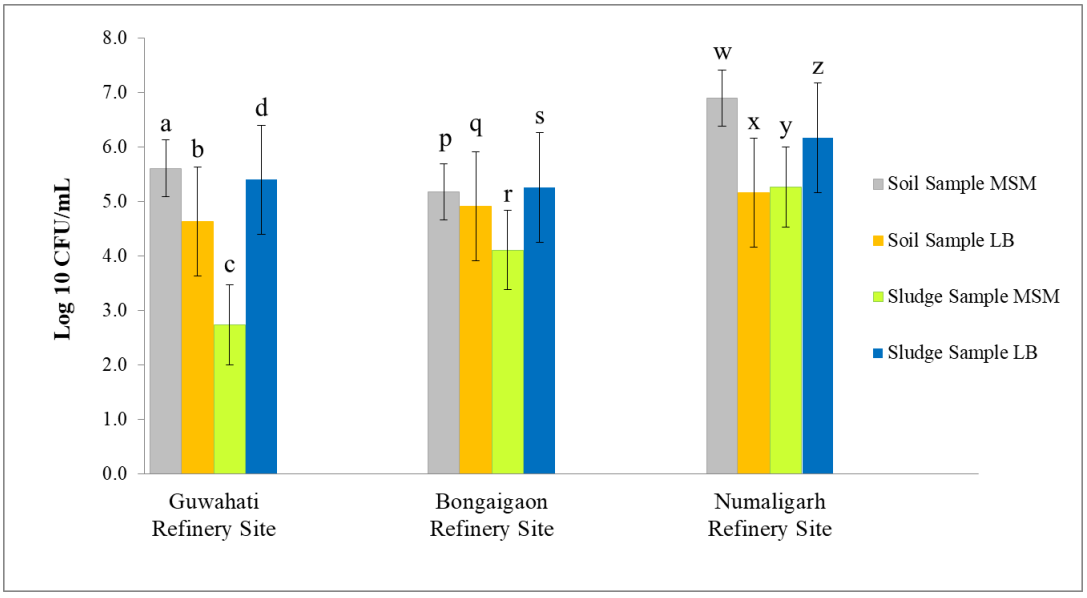

Figure 1. Culturable bacterial population number (log_{10} CFU/mL) isolated from the refinery oil sludge and refinery oily sludge contaminated soil samples collected from nearby refinery sites in two culture media. The bars represent the SD of the mean of three replicates of the values. Different lower case letters above the bars indicate statistically significant differences (ANOVA, LSD test, $p \leq 0.05$).

2. Concentration dependent ethanol tolerance abilities of the bacterial isolates

The ethanol tolerance abilities of the bacterial isolates were tested at various concentrations of ethanol from 0% to 30% v/v. During the experiment, initially the isolates were tested at lower concentration of ethanol. Further, the isolates which can tolerate lower concentration were subjected to higher concentration of ethanol. The result revealed that out of 29 bacterial isolates, only 13 bacterial isolates- BRSO1, BRSO5, BRSO10, BRSL2, BRSL14, GRSO8, GRSL2, GRSL5, GRSL7, GRSL8, GRSL9, GRSL11 and NRSO1 were able to grow in 10% (v/v) ethanol concentration when compared with control which has been presented in table 1 and 2. Further, these 13 bacterial isolates were tested for ethanol stress of 15% v/v, but only 4 of them- BRSL2, BRSL14, GRSL7 and GRSL11 showed increase in growth to the given ethanol stress (table 1).

Table 1: Ethanol tolerance level of bacterial isolates obtained from refinery oily sludge samples at $O.D_{600nm}$

Isolate	**Different EC %**						
	0%	**10%**	**15%**	**18%**	**20%**	**25%**	**30%**
GRSL1	1.203 ± 0.08	0.984 ± 0.12	0.782 ± 0.07	0.405 ± 0.09	0.199 ± 0.11	-	-
GRSL2	0.746 ± 0.11	0.912 ± 0.07	0.647 ± 0.10	0.381 ± 0.13	0.123 ± 0.07	-	-
GRSL3	1.197 ± 0.10	0.673 ± 0.07	0.313 ± 0.15	0.221 ± 0.08	-	-	-
GRSL4	1.165 ± 0.07	0.698 ± 0.07	0.295 ± 0.06	-	-	-	-
GRSL5	0.374 ± 0.07	0.553 ± 0.53	0.329 ± 0.06	-	-	-	-
GRSL7	0.611 ± 0.07	0.660 ± 0.10	0.782 ± 0.08	0.712 ± 0.09	0.823 ± 0.07	0.562 ± 0.14	0.157 ± 0.07

GRSL8	0.655 ± 0.10	1.249 ± 0.10	0.572 ± 0.07	0.205 ± 0.10	-	-	-
GRSL9	0.539 ± 0.08	0.547 ± 0.08	0.454 ± 0.08	0.295 ± 0.07	-	-	-
GRSL10	1.436 ± 0.11	1.071 ± 0.08	0.865 ± 0.05	0.568 ± 0.14	0.253 ± 0.15	-	-
GRSL11	1.035 ± 0.15	1.170 ± 0.18	1.255 ± 0.17	1.228 ± 0.11	1.405 ± 0.13	1.286 ± 0.15	1.739 ± 0.15
BRSL1	1.144 ± 0.14	0.722 ± 0.06	0.487 ± 0.10	0.203 ± 0.15	-	-	-
BRSL2	0.377 ± 0.07	0. 424 ± 0.05	0.539 ± 0.14	0.262 ± 0.07	0.146 ± 0.09	-	-
BRSL9	1.462 ± 0.10	0.815 ± 0.11	0.265 ± 0.05	-	-	-	-
BRSL13	0.972 ± 0.09	0.604 ± 0.08	0.357 ± 0.07	-	-	-	-
BRSL14	0.403 ± 0.08	0. 562 ± 0.06	0.432 ± 0.07	0.201 ± 0.11	0.119 ± 0.07	-	-
BRSL16	1.437 ± 0.13	0.821 ± 0.15	0.421 ± 0.18	0.272 ± 0.08	0.213 ± 0.10	-	-
NRSL1	1.606 ± 0.09	0.667 ± 0.07	0.327 ± 0.07	-	-	-	-

Key: EC% (Ethanol Concentration (v/v)). (-) denotes no growth.

Table 2: Ethanol tolerance level of bacterial isolates obtained from oily sludge contaminated soil samples at $O.D_{600nm}$

Isolate	**Different EC %**						
	0%	**10%**	**15%**	**18%**	**20%**	**25%**	**30%**
GRSO1	0.925 ± 0.09	0.588 ± 0.08	0.188 ± 0.10	-	-	-	-
GRSO7	1.378 ± 0.12	1.227 ± 0.11	0.621 ± 0.14	-	-	-	-
GRSO8	1.047 ± 0.13	1.110 ± 0.10	0.816 ± 0.10	0.324 ± 0.08	-	-	-
BRSO1	1.302 ± 0.09	1.408 ± 0.08	0.797 ± 0.08	0.235 ± 0.09	-	-	-
BRSO4	1.517 ± 0.10	1.029 ± 0.14	0.528 ± 0.09	-	-	-	-
BRSO5	0.955 ± 0.10	1.153 ± 0.17	0.883 ± 0.10	0.289 ± 0.14	-	-	-
BRSO8	1.206 ± 0.09	0.677 ± 0.10	0.388 ± 0.08	-	-	-	-

BRSO10	1.429 ± 0.12	1.565 ± 0.10	0.853 ± 0.09	0.312 ± 0.10	-	-	-
BRSO11	1.357 ± 0.14	0.962 ± 0.05	0.412 ± 0.10	-	-	-	-
BRSO13	1.324 ± 0.11	0.995 ± 0.12	0.329 ± 0.12	-	-	-	-
NRSO1	0.690 ± 0.08	0.738 ± 0.14	0.480 ± 0.08	0.202 ± 0.11	-	-	-
NRSO2	0.659 ± 0.14	0.377 ± 0.11	-	-	-	-	-

Key: EC% (Ethanol Concentration (v/v)). (-) denotes no growth.

In comparison of thirteen bacterial isolates with ethanol stress upto 15% v/v, the 4 bacterial isolates- BRSL2, BRSL14, GRSL7 and GRSL11 have been considered as potent ethanol tolerant isolates for further ethanol tolerant test. These 4 bacterial isolates were the most resolved isolates when compared to control. After an additional increase of ethanol stress upto 18% v/v and 20% v/v, only 2 bacterial isolates- GRSL7 and GRSL11 out of the 4 isolates, were able to withstand the ethanol stress. Further, with an additional increase of 5% v/v more ethanol concentration, the isolates- GRSL7 and GRSL11 were subjected to 25% v/v and 30% v/v ethanol test. The observation revealed that the isolate GRSL11could survive in the ethanol stress condition showing an increased growth pattern while GRSL7 was not able to withstand the increased ethanol stress showing a decrease in growth pattern. Therefore, the isolate GRSL11 showed a significant increase in growth pattern as well as increase in ethanol tolerance level at 0%, 10%, 15%, 18%, 20%, 25% and 30% v/v ethanol concentrations (table 1) as ethanol is metabolised by certain bacteria as a source of carbon and energy.

3. Morphological, biochemical and molecular characterization of the potent bacterial isolate

The result of morphological characters revealed that the isolate GRSL11 is a gram positive rod. The pigmentation of this isolate was creamy white and was wet in texture. Furthermore, GRSL11 was found to be opaque with regular edges and have smooth entire margin. Additionally, the GRSL11 was observed as motile isolate. Furthermore, the results of biochemical characteristics for triple sugar iron test revealed that the isolate GRSL11 showed fermentation of glucose and sucrose and no fermentation of lactose. For indole production and phosphate solubilizing test the isolate GRSL11 showed negative results whereas for catalase test it showed positive result. Again, the isolate GRSL11 showed positive results for both starch hydrolysis and citrate utilization test. In case of methyl red test and urease test,

the isolate showed negative response. Again, the isolate GRSL11 showed positive response for Voges Proskauer test. In addition, the isolate GRSL11 showed negative result for both O_2/CO_2 production as well as H_2S production. Out of the 29 bacterial isolates obtained for this experiment, one strong ethanol tolerant isolate was identified by the use of 16SrRNA sequencing analysis. The purified GRSL11 bacteria's genomic DNA was found to have a purity of 1.7. Thus, the strain was identified as *Bacillus cereus* strain GRJBSBT-1, whose 16S rRNA sequence was submitted at NCBI GenBank with an accession number of PP702364. The phylogenetic tree of the identified bacterial isolate is presented in figure 2.

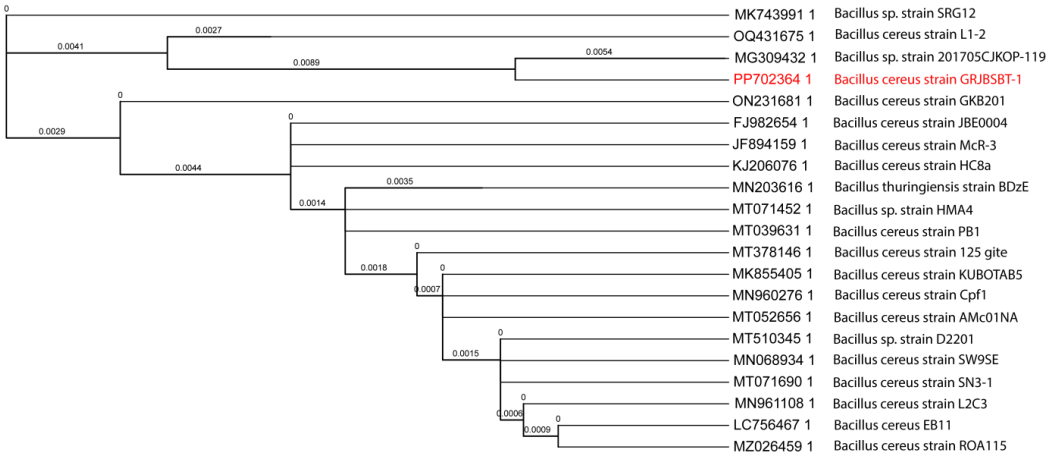

Figure 2. Molecular phylogenetic analysis showing the relation of Bacillus cereus strain GRJBSBT-1 with other related taxa based on available 16S rRNA gene sequences.

4. Conformation for ethanol production

The conformation of production of ethanol by the ethanol tolerant isolates was done by growing all the bacterial isolates on 2, 3, 5-triphenyltetrazolium chloride (TTC) medium agar plates for qualitative assay (figure 3). The results revealed that, out of 29 isolates, the bacterial isolate GRSL11, displayed rapid growth and positive response indicating dark red strains.

Figure 3. Ethanol tolerant bacterial isolate showing positive result for ethanol production by qualitative assay.

Discussion

1. Enumeration of bacterial isolates using enrichment media

Among the different media used for enumeration of ethanol tolerant bacteria, the MSM agar medium was found to be most efficient when compared with modified LB agar media in case of contaminated soil samples whereas for refinery oily sludge samples, the modified LB agar media was found to be more effective than MSM agar media. Henceforth, using modified LB agar media in contaminated sludge sample and MSM agar media in contaminated soil sample, the most diverse bacterial population number was discovered. Several reports are there where it confirms the remarkable increase in growth of bacterial counts during enrichment for ethanol tolerance level (Leahy and Colwell 1990; Kostka et al., 2011; Sarkar et al., 2016 and Sarkar et al., 2017). It has been established that during enrichment, there is an increase in the bacterial abundance as it utilises the major sources of carbon as well as other essential nutrients (K, P, Mg, Zn, Cu, Mn etc.) present (Kostka et al., 2011 and Sarkar et al., 2017).

2. Concentration dependent ethanol tolerance abilities of the bacterial isolates

A significant factor to consider while evaluating the potential for biofuel production of bacterial isolates is their concentration-dependent ethanol tolerance capabilities. Increase in ethanol concentration considerably reduce the growth and optical density, as a result at higher ethanol concentrations it is apparent that the cell growth will be further supressed. In contrary to this, the growth activity of the 4 potent bacterial isolates in different ethanol concentrations has been discussed in details. The bacterial isolate BRSL2 showed significant tolerant growth at 15% (v/v) ethanol stress as compared to control; nevertheless, a deteriorating growth pattern was seen at 18% and 20% v/v ethanol stress, respectively. The BRSL14 bacterial isolate, however, was resistant to 15% (v/v) ethanol stress. Though, this resistance was significantly reduced in comparison to 10% ethanol stress, and further growth patterns were recorded to be altered in 18% and 20% ethanol stress. In addition to this, the bacterial isolate GRSL7 could tolerate up to 20% v/v of ethanol stress. On the other hand, a pattern of

decreased tolerance level was observed when ethanol stress was introduced at 25% and 30% v/v. In contrast, the isolate GRSL11 was able to withstand 30% v/v ethanol stress. Therefore, in terms of its ability to tolerate ethanol, GRSL11 has been regarded as the most potent bacterial isolate. The isolate GRSL11 has been identified to be *Bacillus cereus* strain GRJBSBT-1 which is a gram-positive bacterium from the phylum Firmicutes constitute up the *Bacillus cereus* group of microorganisms. There are at least eight closely related species in the category of rod-shaped, facultatively anaerobic, aerobic, spore-forming bacteria: *B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides, B. cytotoxicus, B. weihenstephanensis, B. anthracis,* and *B. toyonensis* (Liu et al., 2015). With sizes ranging from 5.2-5.5 Mb and extremely comparable 16S rRNA gene sequences, the genomes of the *B. cereus* group members are highly conserved (Ehling-Schulz et al., 2019). In a study, *B. cereus* has been reported as an ethanol tolerant capable of tolerating 5% v/v ethanol concentration at incubation phase (Abood et al., 2021). In addition, *Bacillus cereus* strain GBPS9 has been reported to be ethanol tolerant was investigated using feedstock sugarcane bagasse and cassava peels. This study has demonstrated the efficient ethanol stress tolerance by *B. cereus* at 6% (v/v) ethanol concentration (Ezebuiro et al., 2015). Another study regarding membrane tolerance strategy to ethanol in *Bacillus subtilis* has been reported. Even short exposure to 3% (v ⁄ v) ethanol significantly impacted *B. subtilis*'s cytoplasmic membranes: growth rate and membrane protein synthesis were significantly lowered, and no adaptive changes in phospholipids were found. After a prolonged 3% (v ⁄ v) ethanol stress, there was a small acceleration of cell growth along with a significant reconstruction of the polar head group and fatty acid content of membrane phospholipids. These results highlight the function of membrane lipids in developing ethanol tolerance (Seydlová et al., 2012). It is also reported that cell membrane integrity is compromised by ethanol, which alters permeability and fluidity. In order to preserve membrane stability and function under ethanol-induced stress, *Bacillus* strains evolved to withstand high ethanol concentrations by changing the lipid composition of their membranes and increasing the fraction of saturated fatty acids. Furthermore investigation revealed about the comparable study of the viability of *Bacillus subtilis* 168 (*trp*$^{-}$) wild type strain and the recombinant mutant strain. The finding showed the viability of both the strains decreased when exposed to ethanol with lower concentrations ranging from 0 to 40%. So, this finding could potentially be considered as a typical sensitivity and reaction to the reported ethanol stress factor rising, resulting in a consistent reduction in the rate of bacterial proliferation for survival and self-defense (Yuan et al., 2014). Additionally, there are reports on ethanol tolerance in *Bacillus thuringiensis* strains

when treated upto 10% ethanol. When the ethanol concentration was subjected to ≥ 6%, there was no visible growth, hence indicating its survival and tolerance level at 2% as well as at 4% ethanol concentrations when compared with the growth rate with no ethanol (Zhou et al., 2006). Also, another observation revealed that *Bacillus* strains having the capacity to tolerate up to 12% ethanol (Dung and Huynh 2013). Furthermore, a particular ethanol tolerant gene study has been done by expressing the β-glucosidase gene of *Bacillus cellulosilyticus* into *Escherichia coli* BL21. This particular strain displayed a high tolerance to 15% v/v ethanol (Wu et al., 2018). In a study, *Pseudomonas putida* was able to survive in the presence of ethanol and the survival rate was found to be 50% at 5% v/v ethanol concentration when compared to ethanologenic *E.coli* strain (Nikel and Lorenzo 2014). Another study revealed that in low concentration of ethanol stress, certain *Pseudomonas* sp showed long-term survivability (Ferraro and Finkel 2019). This implies that ethanol can be a valuable resource as a source of carbon that can be used to increase the survival rate of bacteria (Crocker et al., 2019). There are also additional reports on bacteria with 8% ethanol tolerance levels, including *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum*. More studies revealed that isolates of *Lactobacillus fermentum* and *L. vini* could grow in liquid media containing up to 10% ethanol in a laboratory experiment (Lucena et al., 2010). According to a recent study, *Lactobacillus buchneri* NRRL B-30929, which was isolated from a fuel ethanol production facility, showed a strong tolerance to natural ethanol content and there was no discernible decline in growth rate when the media's ethanol concentrations increased from 10% to 14% v/v (Liu et al., 2008). Strains of *Acetobacter cerevisiae* and *A. malorum* have also been shown to have 10% (v/v) and 12% (v/v) ethanol tolerance as reported by Es-sbata et al., 2021. Further studies revealed that, certain strains *Oenococcus oeni* G39 can grow well up to 15% v/v ethanol whereas G46, G63 and G71 were up to 17% v/v ethanol. At 19% v/v ethanol, only G63 was able to survive (Jin et al., 2022). The survival and adaptive mechanisms that *O. oeni* developed under harsh conditions may be influenced by the fact that the membrane composition of this bacterium has been found to be dependent on the concentration of ethanol (Teixeira et al., 2002). According to a different study, strains of *Lactobacillus heterohiochii* and *Lactobacillus homohiochii* could thrive in media containing 18% v/v ethanol (Ingram 1990; Liu and Qureshi 2009). According to similar studies, the *Lactobacillus hilgardii* strain grew well on media with 19% v/v ethanol (Jin et al., 2022). Since, lower concentrations of ethanol helped in development to a higher cell density while acting as a signalling molecule. Therefore, these 4 bacterial strains which could withstand the ethanol stress indicate that ethanol alters cell physiology and helps these isolates to produce a

diffusible component that allows them to proliferate to higher densities (Smith et al., 2004). Also, it indicates that there is an increase in membrane unsaturated fatty acids, mixtures of proteins and vitamins in the growth media which supplements the increase in the ethanol tolerance level in the isolates (D'amore and Stewart. 1987). Ethanol-tolerant bacteria may develop regulatory mechanisms to control gene expression as well as adjust ethanol concentrations. They can create organized cell communities like biofilms, promoting nutrient exchange and signaling chemicals. Ethanol-metabolizing enzymes like acetaldehyde and alcohol dehydrogenases indicate tolerance activity. Ethanol volume in the medium affects bacteria growth, and extrinsic addition promotes growth. Different bacterial species' environmental conditions may impact these mechanisms, as ethanol tolerance involves multiple processes to protect cells from harmful effects. Exposure to ethanol has been found to modify the composition, shape, and function of the inner and plasma membranes as well as the morphology of cells. It has also been found to restrict cell division, reduce cell viability, and decrease metabolic activity (Jones et al., 1981; Salgueiro et al., 1988; Alexandre et al., 1994 and Kubota et al., 2004). Since there are several ways for bacteria to become resistant to high ethanol concentrations and different bacterial species may have distinct resistance mechanisms. However, genes linked to ethanol metabolism and stress response are often upregulated as a typical adaptation. It has also been reported that under ethanol stress, the cellular physiological state and ethanol concentration determine the membrane composition (Teixeira et al., 2002; Thackray and Moir 2003).

3. Conformation for ethanol production

The rapid growth and dark red colouration of the isolate suggested the involvement of a dehydrogenase enzymatic system particularly the ubiquinol-cytochrome c oxidoreductase (complex III) within the bacterial cell during incubation (Tanaka et al., 2021). It indicates that the bacterial cell membrane is broken down by the 2, 3, 5-triphenyltetrazolium chloride (TTC) molecule. The process of penetration can be either passive diffusion or assisted movement, depending upon the characteristics of the bacterial species and the level of TTC present in the surrounding medium. The respiratory chain or other intracellular reducing agents enzymatically reduce TTC once it has entered the bacterial cell. When NADH or NADPH are oxidised, which happens during metabolic activities like glycolysis and the citric acid cycle, the electrons required for this reduction are usually obtained. The reddish substance formazan, which is the reduced form of TTC, builds up inside the bacterial cells.

Within the bacterial colonies or suspensions, formazan tends to precipitate and is insoluble in water, forming crimson deposits. The appearance of red tint in bacterial suspensions or colonies is an indication of cell viability and metabolic activity. This conversion demonstrates that the bacterial cells are able to produce energy through the citric acid cycle and glycolysis, indicating that they are actively engaged in metabolism (Tanaka et al., 2021). In comparison to dormant or non-viable cells, metabolically active bacterial cells that can reduce TTC will create more formazan and have a deeper coloration of red.

Conclusion

The information above emphasises the importance of ethanol-tolerant bacteria that are naturally found in polluted soils around refineries and oily sludge from refineries. The bacterial strain *Bacillus cereus* strain GRJBSBT-1 in this work demonstrates its ethanol tolerance capability, which would improve the development of bioethanol technology to address the accumulation of oily sludge in petroleum refineries. The shift to sustainable energy options depends heavily on ethanol-tolerant microorganisms. Both environmental restoration and the production of renewable energy are benefited by their capacity to transform waste materials into clean energy. The application of these microbial systems will be crucial in helping to resolve issues with conventional fuel in the future as science and technology develop, paving up the possibility to a more ecologically friendly and sustainable energy landscape.

REFERENCES

1. Kumar, R. and Kumar, P. (2017). Future microbial applications for bioenergy production: a perspective. *Frontiers in Microbiology* 8, 450.
2. Srivastava, N., Rawat, R., Singh Oberoi, H. and Ramteke, P. W. (2015). A review on fuel ethanol production from lignocellulosic biomass. *International Journal of Green Energy* 12(9), 949-960.
3. Demirbas, A. (2004). Combustion characteristics of different biomass fuels. *Progress in Energy and Combustion Science* 30(2), 219-230.
4. Chovau, S., Degrauwe, D. and Van der Bruggen, B. (2013). Critical analysis of techno-economic estimates for the production cost of lignocellulosic bio-ethanol. *Renewable and Sustainable Energy Reviews* 26, 307-321.

5. Ramos, J. L., Pakuts, B., Godoy, P., García-Franco, A. and Duque, E. (2022). Addressing the energy crisis: using microbes to make biofuels. *Microbial Biotechnology* 15(4), 1026-1030.
6. Palit, S., Chowdhuri, A. K. and Mandal, B. K. (2011). Environmental impact of using biodiesel as fuel in transportation: a review. *International Journal of Global Warming* 3(3), 232-256.
7. Bentley, R. W. (2002). Global oil & gas depletion: an overview. *Energy Policy* 30(3), 189-205.
8. Cavallo, A. J. (2002). Predicting the peak in world oil production. *Natural Resources Researc*h 11(3), 187-195.
9. Sims, R. E. (2014). Renewable energy and climate change mitigation: an overview of the IPCC Special Report. *Weather Matters for Energy* 91-110.
10. Jeswani, H. K., Chilvers, A. and Azapagic, A. (2020). Environmental sustainability of biofuels: a review. *Proceedings of the Royal Society A*, 476(2243), 20200351.
11. Costagliola, M.A., Prati, M.V., Florio, S., Scorletti, P., Terna, D., Iodice, P., Buono, D. and Senatore, A. (2016). Performances and emissions of a 4-stroke motorcycle fuelled with ethanol/gasoline blends. *Fuel* 183, 470-477.
12. Liu, S. and Qureshi, N. (2009). How microbes tolerate ethanol and butanol. *New Biotechnology* 26(3-4), 117-121.
13. Abdu Yusuf, A. and L Inambao, F. (2019). Bioethanol production techniques from lignocellulosic biomass as alternative fuel: a review. *International Journal of Mechanical Engineering and Technology* 10(6).
14. Ingram, L. O. (1989). Ethanol tolerance in bacteria. *Critical Reviews in Biotechnology* 9(4), 305-319.
15. Huffer, S., Clark, M. E., Ning, J. C., Blanch, H. W. and Clark, D. S. (2011). Role of alcohols in growth, lipid composition, and membrane fluidity of yeasts, bacteria, and archaea. *Applied and Environmental Microbiology* 77(18), 6400-6408.
16. Cartwright, C. P., Juroszek, J. R., Beavan, M. J., Ruby, F. M., De Morais, S. M. and Rose, A. H. (1986). Ethanol dissipates the proton-motive force across the plasma membrane of Saccharomyces cerevisiae. *Microbiology* 132(2), 369-377.
17. Ingram, L. (1976). Adaptation of membrane lipids to alcohols. *Journal of Bacteriology* 125(2), 670-678.

18. Banat, I. M., Nigam, P., Singh, D., Marchant, R. and McHale, A. P. (1998). Ethanol production at elevated temperatures and alcohol concentrations: Part I–Yeasts in general. *World Journal of Microbiology and Biotechnology* 14, 809-821.
19. Ding, J., Huang, X., Zhang, L., Zhao, N., Yang, D. and Zhang, K. (2009). Tolerance and stress response to ethanol in the yeast Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology* 85, 253-263.
20. Smith, M. G., Des Etages, S. G. and Snyder, M. (2004). Microbial synergy via an ethanol-triggered pathway. *Molecular and Cellular Biology* 24(9), 3874-3884.
21. D'amore, T. and Stewart, G. G. (1987). Ethanol tolerance of yeast. *Enzyme and Microbial Technology* 9(6), 322-330.
22. Tangtua, J., Techapun, C., Pratanaphon, R., Kuntiya, A., Chaiyaso, T., Hanmuangjai, P., Seesuriyachan, P. and Leksawasdi, N. (2013). Screening of 50 microbial strains for production of ethanol and (R)-phenylacetylcarbinol. *Chiang Mai J. Sci.* 40(2), 299-304.
23. Metsoviti, M., Paramithiotis, S., Drosinos, E. H., Galiotou-Panayotou, M., Nychas, G. J. E., Zeng, A. P. and Papanikolaou, S. (2012). Screening of bacterial strains capable of converting biodiesel-derived raw glycerol into 1, 3-propanediol, 2, 3-butanediol and ethanol. *Engineering in Life Sciences* 12(1), 57-68.
24. Elshaghabee, F. M., Bockelmann, W., Meske, D., De Vrese, M., Walte, H. G., Schrezenmeir, J. and Heller, K. J. (2016). Ethanol production by selected intestinal microorganisms and lactic acid bacteria growing under different nutritional conditions. *Frontiers in Microbiology* 7, 47.
25. Mahalik, S., Mohanty, A. and Kumar, D. (2020). Enhanced ethanol tolerance in Lysinibacillus sp. *Journal of Applied Biology & Biotechnology* 8(02), 78-83.
26. Mukhopadhyay, A. (2015). Tolerance engineering in bacteria for the production of advanced biofuels and chemicals. *Trends in Microbiology* 23(8), 498-508.
27. Stanley, D., Bandara, A., Fraser, S., Chambers, P. J. and Stanley, G. A. (2010). The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. *Journal of Applied Microbiology* 109(1), 13-24.
28. Dunlop, M. J. (2011). Engineering microbes for tolerance to next-generation biofuels. *Biotechnology for Biofuels* 4, 1-9.
29. Mukred, A. M., Abd Hamid, A., Hamzah, A. and Yusoff, W. M. W. (2008). Growth Enhancement of Effective Microorganisms for Bioremediation of Crude Oil Contaminated Waters. *Pakistan Journal of Biological Sciences* 11(13), 1708-1712.

30. Yomano, L. P., York, S. W. and Ingram, L. O. (1998). Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production. *Journal of Industrial Microbiology and Biotechnology* 20(2), 132-138.
31. Ambust, S., Das, A. J. and Kumar, R. (2021). Bioremediation of petroleum contaminated soil through biosurfactant and Pseudomonas sp. SA3 amended design treatments. *Current Research in Microbial Sciences* 2, 100031.
32. Prathyusha, K., Jagan Mohan, Y. S. Y. V., Sridevi, S. and Sandeep, B. V. (2016). Isolation and characterization of petroleum hydrocarbon degrading indigenous bacteria from contaminated sites of Visakhapatnam. *International Journal of Advanced Research* 4(3), 357-362.
33. Ire, F. S., Ezebuiro, V. and Ogugbue, C. J. (2016). Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse. *Bioresources and Bioprocessing* 3, 1-12.
34. Iticha, T. N. (2016). Isolation and screening of ethanol tolerant yeast for bioethanol production in Ethiopia. *Global Journal of Life Sciences and Biological Research* 2(2), 1-7.
35. Lu, Z. T., Lin, S. X., Zhang, D. W., & Dong, H. (2013). Screening of microorganisms capable of producing ethanol by direct fermentation of D-xylose. *Applied Mechanics and Materials* 291, 230-233.
36. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.
37. Chen, Y., Bai, Y., Li, D., Wang, C., Xu, N. and Hu, Y. (2016). Screening and characterization of ethanol-tolerant and thermotolerant acetic acid bacteria from Chinese vinegar Pei. *World Journal of Microbiology and Biotechnology* 32, 1-9.
38. Lee, P. Y., Costumbrado, J., Hsu, C. Y. and Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments* (62), e3923.
39. Es-sbata, I., Lakhlifi, T., Yatim, M., El-Abid, H., Belhaj, A., Hafidi, M. and Zouhair, R. (2021). Screening and molecular characterization of new thermo-and ethanol-tolerant Acetobacter malorum strains isolated from two biomes Moroccan cactus fruits. *Biotechnology and Applied Biochemistry* 68(3), 476-485.

40. Kumar, S., Stecher, G. and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33(7), 1870-1874.
41. Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4), 406-425.
42. Sarkar, P., Roy, A., Pal, S., Mohapatra, B., Kazy, S. K., Maiti, M. K. and Sar, P. (2017). Enrichment and characterization of hydrocarbon-degrading bacteria from petroleum refinery waste as potent bioaugmentation agent for in situ bioremediation. *Bioresource Technology* 242, 15-27.
43. Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C. and Huettel, M. (2011). Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. *Applied and Environmental Microbiology* 77(22), 7962-7974.
44. Leahy, J. G. and Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. *Microbiological Reviews* 54(3), 305-315.
45. Sarkar, J., Kazy, S.K., Gupta, A., Dutta, A., Mohapatra, B., Roy, A., Bera, P., Mitra, A. and Sar, P., 2016. Biostimulation of indigenous microbial community for bioremediation of petroleum refinery sludge. *Frontiers in Microbiology* 7, 1407.
46. Liu, Y., Lai, Q., Göker, M., Meier-Kolthoff, J.P., Wang, M., Sun, Y., Wang, L. and Shao, Z., 2015. Genomic insights into the taxonomic status of the Bacillus cereus group. *Scientific Reports* 5(1), 14082.
47. Ehling-Schulz, M., Lereclus, D. and Koehler, T. M. (2019). The Bacillus cereus group: Bacillus species with pathogenic potential. *Microbiology Spectrum* 7(3), 10-1128.
48. Abood, M. F., Hamzah, H. M. and Al-Rawii, D. F. (2021,). Production of Cellulase and bioethanol by ethanol-tolerant coculture of Bacillus cereus and Fusarium solani. In Journal of Physics: Conference Series. IOP Publishing.
49. Ezebuiro, V., Ogugbue, C. J., Oruwari, B. and Ire, F. S. (2015). Bioethanol production by an ethanol-tolerant Bacillus cereus strain GBPS9 using sugarcane bagasse and cassava peels as feedstocks. *Journal of Biotechnology & Biomaterials* 5(4), 1.
50. Seydlová, G., Halada, P., Fišer, R., Toman, O., Ulrych, A. and Svobodová, J. (2012). DnaK and GroEL chaperones are recruited to the Bacillus subtilis membrane after short-term ethanol stress. *Journal of Applied Microbiology* 112(4), 765-774.

51. Yuan, Y., Feng, F., Chen, L., Yao, Q. and Chen, K. (2014). Surface display of Acetobacter pasteurianus AdhA on Bacillus subtilis spores to enhance ethanol tolerance for liquor industrial potential. *European Food Research and Technology* 238, 285-293.
52. Zhou, Y., Ye, W. X., Zhou, Y., Zhu, C. G., Sun, M. A and Yu, Z. N. (2006). Ethanol tolerance, yield of melanin, swarming motility and growth are correlated with the expression levels of aiiA gene in Bacillus thuringiensis. *Enzyme and Microbial Technology* 38(7), 967-974.
53. Dung, N. T. P. and Huynh, P. X. (2013). Screening Thermo-and Ethanol Tolerant Bacteria for Ethanol Fermentation. *American Journal of Microbiological Research* 1(2), 25-31.
54. Wu, J., Geng, A., Xie, R., Wang, H. and Sun, J. (2018). Characterization of cold adapted and ethanol tolerant β-glucosidase from Bacillus cellulosilyticus and its application for directed hydrolysis of cellobiose to ethanol. *International Journal of Biological Macromolecules* 109, 872-879.
55. Nikel, P. I. and de Lorenzo, V. (2014). Robustness of Pseudomonas putida KT2440 as a host for ethanol biosynthesis. *New Biotechnology* 31(6), 562-571.
56. Ferraro, C. M. and Finkel, S. E. (2019). Physiological, genetic, and transcriptomic analysis of alcohol-induced delay of Escherichia coli death. *Applied and Environmental Microbiology* 85(2), 2113-2118.
57. Crocker, A.W., Harty, C.E., Hammond, J.H., Willger, S.D., Salazar, P., Botelho, N.J., Jacobs, N.J. and Hogan, D.A. (2019). Pseudomonas aeruginosa ethanol oxidation by AdhA in low-oxygen environments. *Journal of Bacteriology* 201(23), 10-1128.
58. Lucena, B.T., dos Santos, B.M., Moreira, J.L., Moreira, A.P.B., Nunes, A.C., Azevedo, V., Miyoshi, A., Thompson, F.L. and de Morais, M.A. (2010). Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiology* 10, 1-8.
59. Liu, S., Skinner-Nemec, K. A. and Leathers, T. D. (2008). Lactobacillus buchneri strain NRRL B-30929 converts a concentrated mixture of xylose and glucose into ethanol and other products. *Journal of Industrial Microbiology and Biotechnology*, 35(2) 75-81.
60. Jin, G., Jiranek, V., Hayes, A. M. and Grbin, P. R. (2022). Isolation and characterization of high-ethanol-tolerance lactic acid bacteria from Australian Wine. *Foods* 11(9), 1231.

61. Teixeira, H., Goncalves, M. G., Rozès, N., Ramos, A. and San Romao, M. V. (2002). Lactobacillic acid accumulation in the plasma membrane of Oenococcus oeni: a response to ethanol stress?. *Microbial Ecology* 146-153.
62. Alexandre, H., Rousseaux, I. and Charpentier, C. (1994). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. *FEMS Microbiology Letters* 124(1), 17-22.
63. Salgueiro, S. P., Sá-Correia, I. and Novais, J. M. (1988). Ethanol-induced leakage in Saccharomyces cerevisiae: kinetics and relationship to yeast ethanol tolerance and alcohol fermentation productivity. *Applied and Environmental Microbiology* 54(4), 903-909.
64. Kubota, S., Takeo, I., Kume, K., Kanai, M., Shitamukai, A., Mizunuma, M., Miyakawa, T., Shimoi, H., Iefuji, H. and Hirata, D. (2004). Effect of ethanol on cell growth of budding yeast: genes that are important for cell growth in the presence of ethanol. *Bioscience, Biotechnology, and Biochemistry* 68(4), 968-972.
65. Jones, R. P., Pamment, N. and Greenfield, P. F. (1981). Alcohol fermentation by yeasts-the effect of environmental and other variables. *Process Biochem*. 16(3).
66. Thackray, P. D. and Moir, A. (2003). SigM, an extracytoplasmic function sigma factor of Bacillus subtilis, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. *Journal of Bacteriology* 185(12), 3491-3498.
67. Tanaka, J., Kiyoshi, K., Kadokura, T., Suzuki, K. I. and Nakayama, S. (2021). Elucidation of the enzyme involved in 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining activity and the relationship between TTC staining activity and fermentation profiles in Saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering*, 131(4), 396-404.