CHAPTER-5

Isolation, characterization, and applications of efficient LCW degrading bacterial strains from vermibeds and earthworm intestines as consortium candidates and ethanol production.

5.1. Introduction

One of the biggest challenges of the modern world is to meet the ever-increasing energy demands. As non-renewable fossil fuels are mainly used for energy generation, the quest for a sustainable energy supply has gained considerable attention in the present [1]. Consequently, renewable and sustainable energy generation has become integral to the UN's SDGs [2]. In this context, the pathways for bioethanol production from wasted lignocellulosic biomass through the utilization of a microorganism-mediated fermentative process, is a sustainable option for fossil fuel substitution [3]. Conventionally, bioethanol production from lignocellulosic biomass involves steps like pretreatment for feedstock fortification and enzymatic hydrolysis with the help of externally added enzymes for converting cellulosic materials to sugars [4]. However, the process's success depends on the microbial activity and their compatibility with the feedstock and exposure conditions [3]. Thus, experimentation for optimizing the bioethanol production process by step reduction and using novel and efficient microbial species should be continued considering the environmental superiority of the technology. In particular, applying food waste as feedstock reduces the ecological risk of dumping such waste on land [5].

Research has demonstrated that composting and vermicomposting processes foster a diverse microbial community capable of performing crucial biochemical functions. These systems offer a sustainable approach to managing agricultural and organic waste while enhancing soil fertility and plant growth. For instance, N-fixing bacteria such as *Azotobacter* and *Rhizobium* have been isolated from composts enriched with LCW, showcasing their ability to convert atmospheric nitrogen into forms accessible to plants [6]. Similarly, P&K-solubilizing microorganisms, including strains of *Pseudomonas* and *Bacillus*, have been identified, which enhance the bioavailability of these essential nutrients from organic matter [7].

Furthermore, the production of siderophores organic compounds that bind and

solubilize iron by microorganisms such as *Pseudomonas* spp. and *Streptomyces* spp. isolated from compost and vermicompost is of particular interest. These siderophores facilitate the uptake of iron by plants, which is crucial for their growth and development [8]. IAA-producing bacteria, including various species of *Enterobacter* and *Azospirillum*, have also been reported in these composting systems, playing a pivotal role in promoting root elongation and enhancing plant hormone levels [9]. The degradation of cellulose, a primary component of LCW, is another critical function performed by cellulolytic microorganisms such as *Trichoderma* and *Cellulomonas*, which break down complex carbohydrates into simpler sugars, thereby accelerating the composting process and improving the overall quality of the compost [10].

Lignocellulosic wastes (e.g., crop residues and fruit peels) have been widely utilized as raw materials for bioethanol production [11]. Fruit peels and discarded pulps are mainly disposed of in landfills owing to their low commercial value [11]. Although these materials are compostable, the economic viability of the process could be better due to time, space, and labor intensiveness. Generally, yeasts (*Saccharomyces sp.*) are the most dominant microorganism utilized for sugar fermentation for decades [12]. The metabolic efficiency of S*accharomyces* is greatly affected in peptone (xylose, arabinose, etc.) dominated feedstocks [13]. Moreover, the most popular yeast, *Saccharomyces cerevisiae*, cannot utilize cellulose and hemicelluloses [14]. Hence, the quest for alternative microorganisms for efficient ethanol production from bio-wastes has gained considerable attention in the recent past. Several bacterial communities (thermophilic, lactic acid producing, volatile fatty acid producing, etc.) bacteria have shown high promise for economically viable bioethanol production [15, 16, 17]. As such, earthworm guts and earthworm-mediated lignocellulosic feedstock-based vermicomposting systems may also serve as potential sources of fermentative microorganisms because the gut environment stimulates their growth [18]. However, vermicomposting beds and earthworm gut have never been utilized to identify ethanol-producing microorganisms. In particular, the vermicomposting bed can be an easy source of beneficial fermentative vis-à-vis ethanologenic bacteria.

One of the vital qualities of ethanologenic microorganisms is their osmo-adaptation. The growth rate of several yeasts is significantly inhibited in high-concentration sugar solutions due to the saturation of the sugar uptake capacity of microbial cell [19, 20]. However, no study is available about osmo-tolerant characteristics in ethanol-producing

bacteria. As such, the sedimentation rate, ethanol, and sugar tolerance properties are crucial attributes for the evaluation of the industrial applicability of the ethanologenic microorganisms [20]. In this context, assessing waste conversion and economic efficiencies is essential to appreciate the sustainability and eco-compatibility of technology. A few enzymes (Acetyl-CoA synthase, alcohol dehydrogenase, pyruvate decarboxylase, pyruvate kinase, and pyruvate decarboxylase complex) are also essential regulators of industrially scalable bioethanol production. However, these enzymatic pathways have mainly been studied in the yeast-mediated bio-ethanol production system. Hence, analyzing the activation dynamics of these enzymes in bacteria-mediated schemes will be intriguingly novel.

Adopting a novel approach, the present study explores earthworm-mediated vermibeds to isolate and utilize potent bacterial strains with multi-dimensional plant growth promoting traits from the vermicompost systems. As detailed in Chapter 4 of this thesis, vermicomposting experiment was conducted in two stages, accommodating various types of lignocellulosic biomass. At the first stage, the knowledge acquired from NGS-derived taxonomic and functional information was utilized for the first time to isolate and characterize a few bacterial strains with promising plant-growth-promotional traits. The vermicomposting experiment conducted at the second stage was utilized to isolate ethanologenic bacteria using biosolids with high lignocellulosic substances. Previously, our published work identified and reported the prolific plant growth-promoting roles of a few earthworm gut-derived bacterial species [21]. Hence, the ethanologenic property of those bacteria has been explored in the present investigation. In addition, a lignocellulosic feedstock-based vermicompost reactor was searched to isolate new ethanol-producing bacterial strains. Initially, several bacterial strains were qualitatively screened based on their cellulose and carbohydrate degrading capacity, and a few selected isolates were for assessing their bio-ethanol-producing potentials. Eventually, the ethanol-producing ability of the characterized bacteria was evaluated in sugar solution and lignocellulosic fruit waste (banana peel) compared with yeast. Moreover, the activities of different key enzymes were assessed to appreciate the underlying mechanism of microbe-mediated bioethanol production. Finally, the membrane permeability kinetics of the selected bacteria was compared with yeast through flow cytometry and fluorescence microscopy to understand the osmo-adaptation mechanism.

5.2. Materials and Methods

5.2.1. Isolation of plant growth promoting bacterial strains from vermicompost and earthworm intestines

The results of the NGS-based functional metagenomic analyses revealed high predominance of functions related to plant growth promoting soil-based microbial communities. Therefore, microbial species with plant growth promotional traits were targeted to isolate from vermicompost and earthworm guts. Four mature earthworm specimens were collected from the vermibeds after the incubation period. The collected earthworm specimens were washed with sterile water to clean the intestinal debris and kept on hydrated filter paper overnight. Eventually, one gram of intestinal materials was dissected after disinfecting the worms with 70% ethanol. Subsequently, the gut materials were suspended in sterile NaCl solution (0.85%) and vortexed for fifteen minutes, and then the supernatant of the mixture was collected [22]. Microbial colonies were developed on nutrient agar Petri dishes following serial dilution techniques.

Similarly, air-dried vermicompost samples (1g) were dissolved in sterile water (10ml) and vortexed for 15 min. The bacterial colonies were then developed on nutrient agar Petri dishes using serial dilution techniques. Subsequently, pure colonies of the most dominant bacterial strains found in the highest order (10^{-6}) dilution plates were developed. The dominance of the pure colonies was calculated by deriving the relative dominance (RD %), as reported earlier [21]. The formula for the RD is given below.

$$\text{RD (\%)} = \frac{\text{Colony count of a particular strain}}{\text{Total colony count}} \times 100$$

5.2.2. Screening of cellulose-degrading and carbohydrate-utilizing bacterial strains

Based on the results of the microbial assessment, the vermicompost samples were searched for isolating potential cellulose-degrading and carbohydrate-utilizing bacterial strains. Initially, 14 profusely growing bacterial strains in NA media were isolated from vermicompost and earthworm gut. Eight well-characterized and previously reported bacterial species isolated from earthworm gut were also assessed for their carbohydrate and cellulose utilization properties [21]. These 22 dominant bacterial strains were evaluated for their carbohydrate utilization efficiency using the Hi-Media Carbo Kit. Based on the outcome of the qualitative carbohydrate solubilizing assessment, a few

strains were selected for assessing their cellulose degradation potential. These isolates were allowed to grow for 24 hours at 37°C in Carboxymethyl cellulose and 1% agar media plates, followed by Congo-red staining according to the technique standardized by Smibert and Krieg (1994) [23].

5.2.3. Gas chromatographic estimation of ethanol production from sugar solution and banana peel

Sugar solutions (5% w/v) were initially fermented at 37°C for 48 hours in an anaerobic chamber. The sugar solution was then sterilized at 121°C and 210 kPa for 20 minutes in an autoclave. Later, 1% inoculum of the selected bacterial strains was inoculated into the sugar solutions at room temperature. Glucose was the sole energy source for the bacterial inoculums used in this study. Subsequently, after 48 hours, the turbid microbiological media was transferred to sterilized falcon tubes. A strain of yeast (*Saccharomyces cerevisiae* MTCC 170), procured from MTCC, was used as a positive control to compare the ethanol production potency of the screened bacterial isolates.

Moreover, the ethanol production potential of the selected bacterial isolates was evaluated using non-edible banana (*Musa* spp.) epicarps as substrates. The banana epicarps weighing 20 g were homogenized with water at 1:2 ratio, and the homogenate (15 ml) was subjected to centrifugation at 8000 rpm for 15 minutes. The supernatant of the centrifuged contents and the filtrate of the sonicated samples were collected in fresh falcon tubes (5 ml in each tube). After that, 1% of the bacterial cultures were inoculated in each tube and allowed to ferment at 28°C. After 48 hours of incubation, the tubes were sealed and used to measure ethanol content.

5.2.3.1. Gas-chromatography

The extracted ethanol was analyzed using a Gas chromatograph (GC) (Model: Agilent 7890A) following the procedure described by Ebersole (2016) [24]. The method of the GC was set as follows:

- Inlet: 160 ^{0}C, mode: split, Split flow: 80 mL min^{-1}
- Oven: Initial-50 ^{0}C, hold time of 1 min and the Ramp (10 ^{0}C min^{-1}) till temperature goes to 200 ^{0}C and hold time of 1 min
- Detector: 200 ^{0}C, Flame Ionisation Detector (FID)

- Carrier gas: N_2, 7 mL min^{-1}
- Injection volume: 100 to 200 µL

5.2.3.2. QA-QC

HPLC-grade ethanol was used as the standard for the calibration of the instrument. The lowest concentration of the working standard taken daily was considered as the limit of detection (LOD) (defined as the lowest concentration of the working standard with a signal-to-noise ratio equal to or exceeding 10:1) and the limit of quantification (LOQ). For this method, the LOD was 1.0% (v/v), and the LOQ was determined to be 0.789 mg L^{-1}.

5.2.4. Sedimentation rate, ethanol tolerance, and sugar tolerance of the microbial cultures

The sedimentation rate of the yeast and the selected bacterial isolates was estimated with minor modifications of the method standardized by previous works [20, 25]. The yeast-extract potato dextrose (YPD) medium was used to grow the yeast cells. Bacterial cells were cultured in Luria Bertani (LB) agar medium for 24 hours, centrifuged (14000 × g) for 10 minutes, and pellets were obtained. This method modification was necessary because bacterial cells cannot grow in the YPD medium. Subsequently, the pellets were suspended in NaCl (0.89%) solution for two hours, the change in absorbance at 600 nm was recorded in a UV-Vis spectrophotometer (Cary 60), and the sedimentation rate, expressed in percentage (SRP), was derived using formula according to Moneke et al. (2008) [25].

$$Sedimentation\ rate\ percentage\ (SRP) = \left(1 - \frac{Drop\ in\ absorbance\ after\ 2\ hours}{Absorbance\ at\ 0\ hour}\right) \times 100$$

The ethanol (10% and 15%) and sugar tolerance (5%, 8%, and 12%) of the selected bacterial strains were assessed in comparison with yeast (*Saccharomyces cerevisiae* MTCC 170). The ethanol tolerance was measured based on the extent of cell viability after exposure to different concentrations of ethanol following the method detailed by Moneke et al. (2008) [25]. The survival percentage (SP) in different ethanol solutions was calculated as formula:

$$Survival\ percentage\ (SP) = \frac{Number\ of\ alive\ cells\ after\ 2\ hour\ exposure}{Number\ of\ cells\ in\ control\ (sterilized\ water)} \times 100$$

To assess the sugar tolerance limit, sugar solutions of 5, 8, and 15 % concentration (w/v) were sterilized using an autoclave. Once the solutions reached room temperature, 1% yeast culture and selected bacterial strains were inoculated. The inoculums were allowed to grow at 37°C for 72 hours in an anaerobic chamber. After every 24 hours, the optical density of the cultures was noted at 280 nm using the UV-Vis spectrophotometer (Cary 60).

5.2.5. Identification of microorganisms through Gram staining and 16S rRNA sequencing

Initially, Gram staining of single colonies of the dominant microbial strains was performed by the standard method [26], and the morphological characteristics of the isolated strains were studied under a light microscope. Then, the isolated strains were inoculated into 25 ml of broth medium and incubated on a rotary shaker (200 rpm) for 2–4 days. The biomass was harvested by centrifugation at 3000 rpm for 10 min, washed twice with sterilized MilliQ water, and centrifuged at 3000 rpm for 10 min to collect the microbial cells. The microbial genomic DNA from 65 mg of each bacterial pellet was extracted using a Genomic DNA Mini prep kit (Axygen Biosciences, USA). The quantity of DNA was measured at 260 nm using a Nano-Drop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA). Eventually, agarose gel electrophoresis was performed (0.8% w/v) to confirm the success of the DNA extraction process. Then, the DNA extracted from the respective microbes was used as PCR templates. PCR was conducted to amplify the full-length 16S rRNA gene fragment using universal bacterial primers 27FA (5'-AGAGTTTGATCATGGCTAG-3') or 27FC(5'-AGAGTTTGATCCTGGCTAG-3') and U1492R (5'-GTTACCTTGTTACGACTT-3'). The stepwise PCR protocol was initiated with (1) denaturation at 94 °C for 2 min, (2) 30 cycles of denaturation at 94 °C for 60 sec, (3) annealing at 45 °C for 60 sec, (4) extension at 72 °C for 90 sec, and (4) a final extension at 72 °C for 15 min. The PCR product was purified with the gel elution technique using a QIA quick gel extraction kit (QIAGEN, Germany). The quantity and quality of the DNA extracts were checked by spectrophotometry and electrophoresis on 1 % agarose gels. Later, the DNA was stored at –20 °C until further use. Partial nucleotide sequencing was

performed at 1st BASE (Malaysia) using Sanger's method with the 27FA or 27FC and U1492R primers [27]. The obtained sequences were identified with the help of the NCBI database's Basic Local Alignment Search Tool. Sequence alignment was performed with ClustalW software from the EMBL server (http://www.ebi.ac.uk/). The aligned sequences were analyzed using MEGA software [28]. The phylogenetic tree was constructed by following a precise algorithm [29], and the strength of the tree topologies was confirmed with 1000 bootstrap replications [30]. The never-joining method was used to derive the evolutionary history of the sequences from the bacterial isolates. Here, it is essential to mention that the selected isolates' three (*K. ascorbata* S8, *Rhizobium sp*. S10, and *Bacillus sp*. S12) were previously reported as N-fixing strains [21]. However, one among the three newly isolated strains (i.e., C5) was identified by fatty acid methyl ester (FAME) analysis because the broth culture of the organism was unsuccessful after several efforts.

The FAME analysis was conducted by outsourcing from Royal Life Sciences Pvt. Ltd. (affiliated with MIDI Sherlock, USA). In short, the colonies of the C5 strains were grown on Trypticase soy broth Agar at 28 ^{o}C for 24 hours. Then about 40 mg of bacterial cells were harvested in sterilized Petri plates by streaking. Then FAMEs were extracted in a step-wise manner following the standard protocol (saponification, methylation, extraction, and aqueous wash) [31]. The extracted FAMEs were analyzed by gas chromatography. The gas chromatography-derived FAME profiles were used as standard profiles. Subsequently, the organism's genus and species were identified with the assistance of the Sherlock software. The strain with a single match of at least 0.600 similarity indexes or 0.600 with < 0.100 distance from the nearest choice was considered a dependable species match.

5.2.6. Assessing activity of ethanol-specific enzymes

Activities of five enzymes [Acetyl-CoA synthetase (AS), pyruvate dehydrogenase (Pdh), pyruvate kinase (Pyk), pyruvate decarboxylase (PyD), and alcohol dehydrogenase (AD)] that are associated with the central metabolism of ethanologenic microorganisms were studied in the early exponential phase and at the onset of the stationary phase of growth. AS, Pdh, Pyk, and AD activities were measured regarding the NADH generation at 340 nm (εNADH = 6.220 $M^{-1}cm^{-1}$) following standard methods [32,33,34]. The activity of PyD was assessed following the method of Hoppner and Doelle (1983) [34], and the

enzyme unit of pyruvate decarboxylase activity was expressed as 1.0 μM of acetaldehyde min^{-1} at 340 nm [34]. The details of all these methods have been provided below.

5.2.6.1. Enzyme assays

Five different enzyme activities associated to the central metabolism were studied in the early exponential phase and at the onset of the stationary phase of growth. The enzyme activities were assayed by the following method. All the values were taken out in a spectrophotometer (Agilent Technologies). Enzyme activity was defined as μmol of substrate consumed per minute and mg of protein (U/ mg) at 37 °C.

5.2.6.1.1. Acetyl-CoA synthetase

Acetyl-CoA synthetase assay was performed following Lin et al., 2006 [35] The reactant buffer used in the assay was100 mM of Tris-HCl (pH 7.8). The reaction mixture prepared to measure the Acs activity includes 5 mM D, L-Malate, 1 mM ATP, 2.5 mM MgCl2, 0.1 mM coenzyme A, 3 mM NAD+, 2.5 U mL^{-1} malate dehydrogenase, 1.25 U mL^{-1} citrate synthase and 100 mM sodium acetate. The increase in NADH absorbance was noted at 340 nm (εNADH = 6.220 $M^{-1}cm^{-1}$). One enzyme activity unit was defined as the enzyme generating 1 μmol of NADH per min.

5.2.6.1.2. Pyruvate dehydrogenase complex

Pyruvate dehydrogenase activity was measured following the standard protocol of Brown & Perham, 1976 [36]. The determination of Pdh enzyme activity utilizes 50 mM potassium phosphate (pH 8.0) as a reactant buffer and 1 mM $MgCl_2$, 0.5 mM thyamine pyrophosphate, 0.5 mM L-cysteine, 2.5 mM NAD+0.1 mM coenzyme A and 10 mM sodium pyruvate as the reaction mixture. The enzyme activity was measured by calculating the difference in increase in NADH absorbance at 340 nm (εNADH = 6.220 $M^{-1}cm^{-1}$). One enzyme activity unit was taken to be the enzyme required to generate 1 μmol of NADH per min.

5.2.6.1.3. Pyruvate kinase

Pyruvate kinase assay was conducted by following Peng & Shimizu, 2003 [37] with slight modification. The reaction buffer and reaction mixture prepared to determine the Pyk activity was 50 mM (pH 6.5) bis-tris buffer, and 25 mM $MgCl_2$, 10 mM KCl, 0.25 mM Dithiotreitol, 0.5 mM NADH, 2.5 mM ADP, 2.5 U/ml L-lactic dehydrogenase and 5 mM phosphoenol pyruvate respectively. The enzyme activity was measured by calculating the difference in increase in NADH absorbance at 340 nm (εNADH = 6.220 $M^{-1}cm^{-1}$). One enzyme activity unit was taken to be the enzyme required to generate 1 µmol of NADH per min.

5.2.6.1.4. Alcohol dehydrogenase

Alcohol dehydrogenase activity was measured by following the modified protocol by Tamaki et al. (1977) and Johansson and Sjostrom (1984) [33,38]. The assay was conducted at 25 °C in cuvettes using 20 µL of crude extract. A reaction mixture of 2.98 ml was prepared with the help of 100 mM sodium pyrophosphate buffer of pH 8.5, 1 mM NAD^+, 70 mM semicarbazide, and 2 mM a-mercaptoethanol. The enzyme units are expressed as µmol of NADH formed/min.

5.2.6.1.5. Pyruvate decarboxylase

The assay on Pyruvate decarboxylase was measured by following the protocol of Hoppner and Doelle (1983). The chemicals used to prepare the reaction mixture prepared are 400 mM tris-HCl buffer pH, 10 mM thiamine pyrophosphate, 10 mM $MgCl_2 \cdot 2H_2O$, 1.0 M sodium pyruvate, and 4.0 mM NADH. The assay was measured at spectrophotometer at 340 nm. The enzyme unit of pyruvate decarboxylase activity was expressed as 1.0 µmole of acetaldehyde/minute.

5.2.7. Flow cytometry and confocal microscopy: cellular function for sugar tolerance

The fluorescence-activated cell sorting (FACS) and confocal microscopy techniques were utilized to study cellular responses of the yeast (*Saccharomyces cerevisiae* MTCC 170) and selected bacterial isolates following methods described by Malakar et al. (2008) and Mukherjee et al. (2014) [39, 40]. The yeast and bacterial cells were incubated with 0 (i.e., control), 5, and 15% sugar solutions for 24 hours. Then, the treated and untreated (i.e., control) cells were stained using an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol and subjected to flow cytometry analysis (BD Accuri, BD Biosciences). Apoptosis phases were detected by distinct double staining patterns: viable (Annexin V- and PI-, lower left square), early apoptotic (Annexin V+ and propidium iodide (PI)-, lower right square), late apoptotic (Annexin V+ and PI+, upper right square) and necrotic cells (Annexin V- and PI+, upper left square). To evade the coincidence of cells, the flow rate was adjusted to the lowest setting mode (data rate, 200–300 events per second). At least 10,000 events were recorded for each sample with three replicates. Cells were collected on the forward scatter with logarithmic amplifiers for 5,000 events to determine the cell size. As the membrane potential of different organisms varies based on their cell size, all data were expressed as the ratio of membrane potential according to their size. The acquired data were accumulated as list mode files and examined offline using the System II V.3 software (Beckman-Coulter).

For determining the membrane permeability, the 0 (i.e., control), 5, and 15% sugar-exposed cells were stained by two nucleic acid staining reagents, propidium iodide (PI) and FITC, using a Live/Dead BacLight kit (Molecular Probes, Invitrogen, Cergy-Pontoise, France). The membrane integrity of the yeast and bacterial cells was assessed by staining the cells at first with green fluorescing FITC, which can enter all cells when used alone, and then with the red-fluorescing PI that specifically invades cells with injured cytoplasmic membranes. The appropriate mixture of the FITC and PI stains enables differentiation between live organisms with intact cytoplasmic membranes and dead organisms with permeable cellular membranes. The method has been adequately standardized for observing apoptotic response in bacteria and yeast in previous studies [41,42]. However, the staining exercise was repeated several times for the yeast cells because a few cells were not responding uniformly to the PI. The morphology of the

stained organisms was studied with the help of a Zeiss LSM 510 confocal fluorescence microscope with a 63X objective.

5.2.8. Statistical Analysis

The temporal data on the bio-composting experiment was analyzed for two-way ANOVA with three observations per cell followed by the Least Significant Difference (LSD) test to differentiate the efficiency of various treatments at the probability level of $p < 0.05$ using SPSS. One-way ANOVA followed by an LSD posthoc test was also performed for all other experiments.

5.3. Results and discussion

5.3.1. Stage-1 vermicomposting experiment - Identification and isolation of bacteria with plant-growth-promoting traits: A metagenome-linked exploration

According to the NGS-based taxonomic and functional study of microbial communities, earthworms in vermireactors considerably altered the diversity of the microbial community in lignocellulosic feedstocks. Earthworms release intestinal microbes through their excreta, which significantly modifies the structure of microbial communities in the vermibeds [43]. Using the information from our NGS analyses, we tried to isolate a few beneficial microbial species from the vermicomposting systems. The RD values (Fig.5.1a, b) indicate that some bacterial species were highly dominant in both earthworm guts and vermicompost samples. Interestingly, colonies of 21 and 15 strains exhibiting high RD (> 1) were detected in earthworm guts (Fig. 5.1a) and vermicompost samples (Fig. 5.1b), respectively. Eventually, pure cultures of strains with more than four RD values in the earthworm gut samples were studied qualitatively for their ability to promote plant growth in various ways (N-fixation, P-solubilization, K-solubilization, IAA production, siderophore production, cellulose degradation, and ethanol production). The RD values for the vermicompost-derived strains were in the order B8 > PB1 >B3> others (Fig. 5.1a). On the other hand, in the earthworm gut samples, the highest RD was for the T24 strain, followed by OS6 and

OS8 (Fig. 5.1b).

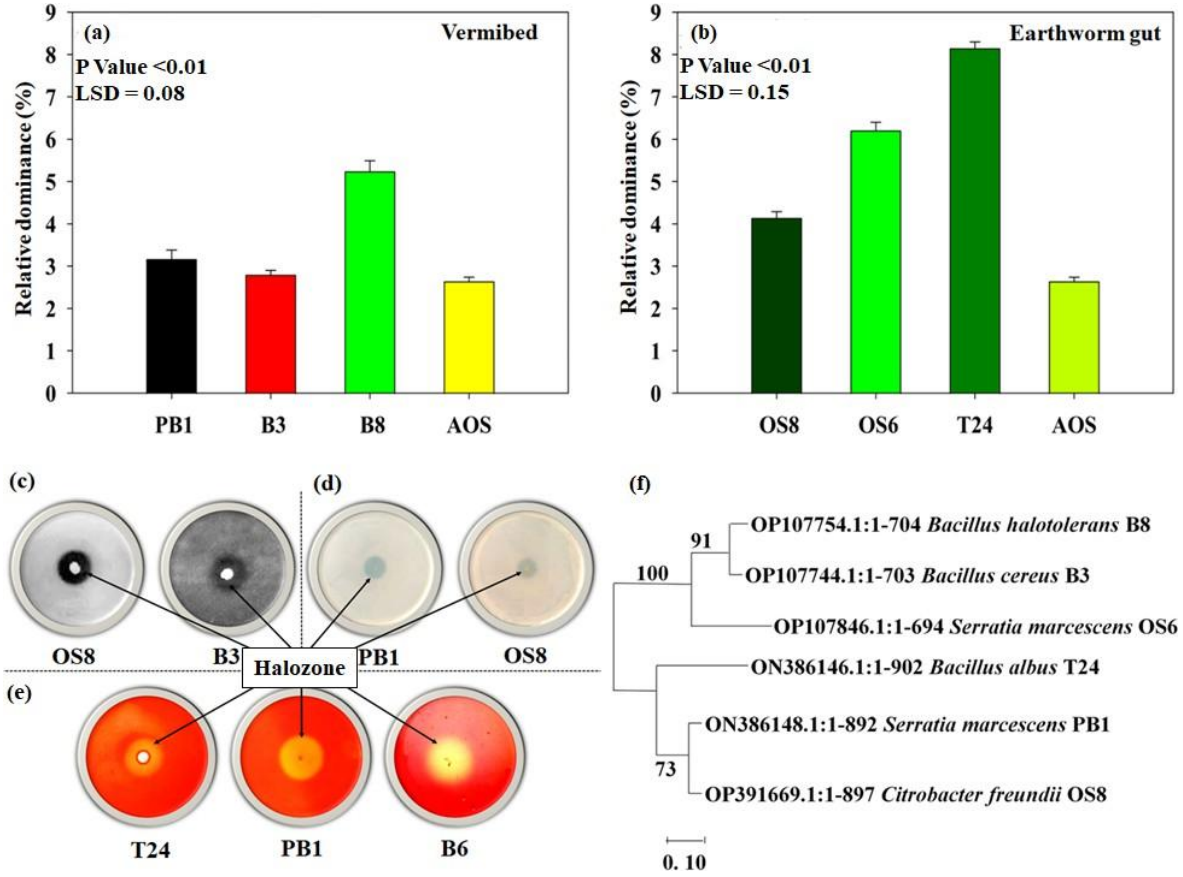

Figure 5.1: Isolation of bacterial strains with plant-growth-promoting traits from vermibeds and earthworm guts. a. Relative dominance of vermibed-isolated strains compared with all other strains; b. Relative dominance of earthworm gut–isolated strains compared with all other strains; c. Phosphate solubilizing potential; d. Potassium solubilizing potential; e. Identification of cellulolytic bacteria using Congo red test; and f. Phylogenetic tree of potential plant-growth-promoting isolates.

The characteristics and identification of these dominant strains were important for understanding how the whole metagenome analysis is linked to the real data. The rod-shaped Gram-positive strain T24, isolated from *E. fetida* guts, exhibited N-fixing, P- and K-solubilizing, IAA producing, and cellulose-degrading traits. This strain was identified as *Bacillus albus* via 16s rRNA sequencing (Table 5.1).

Table 5.1: Strain information of different potential isolates and their morphological and biochemical properties (mean±standard deviation)

Order	Organism	Accession Number	Differential Staining	IAA Conc. (µg/ml)	N-fixation	P-Solubilization (SI)	Siderophore Production	K-Solubilization (HI*)	Congo red (HI in mm)
1	*Citrobacter freundii* OS8	ON391669	Gram Negative	213.14±1.4	Positive	3.24±0.15	Positive	2.87±0.08	Negative
2	*Serratia marcescens* OS6	OP107846	Gram Negative	69.43±1.7	Positive	4.27±0.20	Negative	1.86±0.06	2.5±0.11
3	*Bacillus albus* T24	ON386146	Gram Positive	61.55±0.9	Positive	2.84±0.12	Negative	4.17±0.15	2.29±0.36
4	*Serratia marcescens* PB1	ON386148	Gram Negative	114.75±0.47	Positive	1.91±0.03	Positive	3.05±0.05	2.6±0.28
5	*Bacillus cereus* B3	OP107744	Gram Positive	54.58±0.3	Positive	3.45±0.15	Positive	2.26±0.2	Negative
6	*Bacillus halotolerans* B8	OP107754	Gram Positive	53.89±1.6	Positive	2.084±0.08	Positive	1.92±0.04	Negative
	P value	<0.01							
	LSD			2.13		0.09		0.11	0.7

*HI – Halo zone index

B. albus belongs taxonomically to *Firmicutes, Bacilli, Bacillales*, and *Bacillaceae* at the phylum, class, order, and family levels, respectively as reported in Chapter 4 of the thesis (Fig. 4.4a–d). Two rod-shaped Gram-positive strains (*B. cereus* B3 and *B. halotolerans* B8) under similar taxonomic groups (i.e., *Bacilli, Bacillales,* and *Bacillaceae*) were also isolated from the vermicompost (Table 5.1). Contrastingly, the genes of firmicutes and their lineages (i.e., *Bacilli, Bacillales,* and *Bacillaceae*) were identified in meager amounts in the whole metagenome analysis. In the present investigation, two rod-shaped Gram-negative *Serratia marcescens* isolates (PB1 and OS6) were identified. OS6 was isolated from earthworm guts, and PB1 was found in vermicompost (Table 5.1). This is strong evidence of earthworm-induced modification of microbial community profiles in LCW feedstocks during vermicomposting and agrees with the findings of a study conducted in warm and humid Indian conditions [44]. According to the literature, *S. marcescens* belongs to the *Pseudomonadota* (*Proteobacteria*) phylum, *Gammaproteobacteria* class, and *Enterobacterales* order [45]. OS8, isolated from earthworm guts, was identified as the *Citrobacter freundii* strain after gene sequencing (Table 5.1). This rod-shaped Gram-negative strain belongs to the same taxonomic lineage as *Serratia marcescens* [45]. Interestingly, the whole metagenome-based

bioinformatics analysis revealed that the candidates under the *Proteobacteria* (*Pseudomonadota*) phylum, *Gamma proteobacteria* class, and *proteobacteria* class, and *Enterobacterales* order were the most dominant communities in the vermireactor. However, the Illumina-based metagenomic analyses did not detect the dominant species that could be cultured from the vermicompost and earthworm gut samples, as detailed in the previous section. Therefore, this comparative bioinformatics-based analysis provided new information about the overall diversity of microbial communities with respect to the phylum, class, and order level taxonomic abundance in environmental samples. Nevertheless, it is difficult to assess the authenticity of the species-level metagenomic information because the metagenome database did not detail the genes of the truly dominant bacterial isolates. The phylogenetic tree developed from the evolutionary taxa of the six bacterial strains revealed that *Bacillus halotolerans* B8, *Bacillus cereus* B3, and *Serratia marcescens* OS6 originated from two distinct groups of evolutionary resemblance and had similar origins. At the same time, the other three (*B. albus* T24, *S. marcescens* PB1, and *C. freundii* OS8) were closely related to each other (Table 5.1). Interestingly, strains isolated from vermicompost and earthworm guts were present in the both groups, implying that earthworm gut–associated microflora significantly modified the indigenous microflora of the LCW feedstock in the vermireactors, as observed previously [45]. Eventually, the strains isolated and characterized via fundamental metagenomic analyses were verified for their plant-growth-promotional traits. The IAA production capabilities of the bacterial strains were found to be in the order *C. freundii* OS8 >*S. marcescens* PB1 >*S. marcescens* OS6 >*B. albus* T24 >*B. cereus* B3 = *B. halotolerans* B8 ($P<0.01$; LSD=2.13; Table 5.1). All the strains exhibited positive N-fixation potential. The two strains of *S. marcescens* (OS6 and PB1) followed by *B. albus* T24 were also found to be significant cellulose degraders (Table 5.1). Moreover, *S. marcescens* PB1, *C. freundii* OS8, *B. cereus* B3, and *B. halotolerans* B8 showed significant siderophore production potential (Table 5.1). Siderophores are secondary metabolites secreted by endophytic microorganisms and can play a vital role in improving the bioavailability of iron by reducing insoluble $Fe^{3+}$to the soluble ferrous form [46]. On the other hand, IAA (i.e., auxin) is a well-known phytohormone produced by some microorganisms, in addition to aerial plant parts [47]. The bacterial isolates also showed high P- and K-solubilization potential in the order of: P-solubilization—*C. freundii* OS8 >*S. marcescens* OS6 >*B. albus* T24 >*S. marcescens* PB1 >*B. cereus* B3 >*B. halotolerans* B8 ($p<0.01$; LSD =0.09; Table 5.1); K-solubilization —

C. freundii OS8 >*S. marcescens* OS6 >*B. albus* T24 >*S. marcescens* PB1 >*B. cereus* B3 >*B. halotolerans* B8 ($p < 0.01$; LSD =0.11; Table 5.1). As endophytes, these bacterial strains can thrive inside plants to facilitate plant growth in stressful conditions [48]. Therefore, all the isolated strains were easy to culture and could be used as plant growth promoting agents. We have thus demonstrated that metagenomic approaches can be used to specify appropriate habitats in which to meaningfully explore microorganisms with multi-dimensional plant-growth-promotional traits.

5.3.2. Stage-2 vermicomposting experiment - Screening of cellulose degrading (Congo red assay) and carbohydrate solubilizing efficiency

Based on the results of the composting and vermicomposting experiments, 14 isolates were initially screened out from the vermibeds considering their high relative dominance (RD) in Nutrient agar plates (Fig. 5.2 (ii)). The RD is an authentic and dependable parameter for assessing the aggressivity of microbial strains in congregations [21]. Eight previously reported strains were also considered for the present study because the data about their molecular identity and general characteristics were readily available. Cellulolytic capability in microorganisms signifies the effectiveness of the organisms for rapid transformation of obstinate cellulose-rich biomass [49]. On the other hand, the extent of carbohydrate solubilization efficiency in microorganisms indicates their ability to derive energy from recalcitrant substrates [50]. Hence, 22 strains were selected to study their cellulose degradation and carbohydrate solubilization potentials (Fig. 5.3 & Table 5.2 (a & b)).

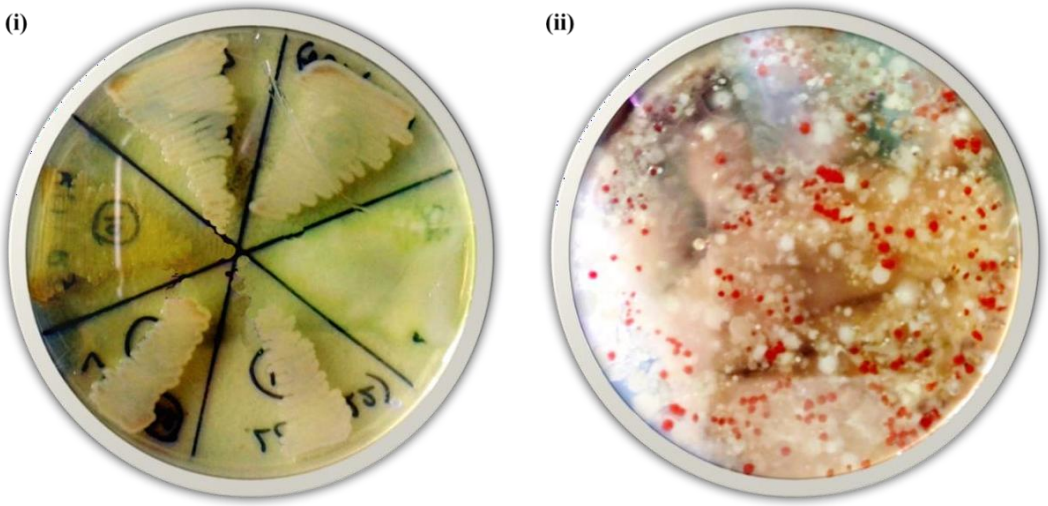

Fig. 5.2: (i) Isolated Strains grown in Burks Media for N-Fixing Potential; (ii) Relative dominance of Isolated Strains grown in Burks Media for N-Fixing Potential.

Organism Name	Strain name	Halozone index
Kluyvera ascorbata	S8 (IN2)	2.8
Rhizobium sp.	S10 (IN4)	3.9
Bacillus sp.	S12 (IN5)	3.3
Burkholderia sp.	IP2	3.2
Serratia marscescens	IP3	3.9
Bacillus cereus	IP4	3.5
Kluyvera ascorbata	IN3	2.9
Serratia sp.	IN6	3.6
Kosakonia sacchari	C1	3.4
Enterobacter cloacae	C3	2.8
Bacillus alcalophilus	C5	4.2
Unidentified Strain	T3	2.0
Unidentified Strain	T20	2.1
Unidentified Strain	B5	2.4
Unidentified Strain	T17	1.1
Unidentified Strain	T10	1.3
Unidentified Strain	T7	1.5
Unidentified Strain	OS6	1
Unidentified Strain	OS7	2.5
Unidentified Strain	Os3	1.2
Unidentified Strain	Os9	0.4
Unidentified Strain	Os10	0.8
P_{Value}	<0.01	
LSD	0.039	

*** The strain names in parentheses are previously reported by Hussain et al.**

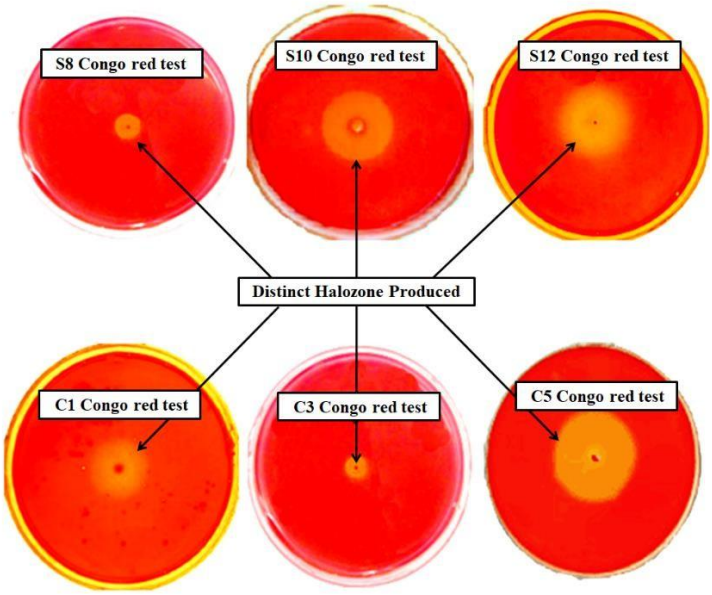

Fig. 5.3: Cellulose degradation efficacy of different microbial strains isolated from vermicompost and earthworm gut (Congo red assay)

Overall, six strains were able to solubilize ~28-30 different types of sugars (glycerol, mannitol, adonitol, etc.), and the C1, C5, and S10 could solubilize 30-31 out of 35 tested carbohydrates (Table 5.2 (a & b)). The C5 and S10 exhibited significantly higher cellulose degrading efficiency (i.e., halo zone Index) compared to other strains (Fig. 5.3; $p<0.01$; LSD = 0.039). On the other hand, a few different strains (T3, T20, OS7, and B5) exhibited high carbohydrate

Table 5.2(a): Carbohydrate solubilization potentials of eleven different microbial strains

		Strain name										
Sl No.	**Test**	**S 8**	**S 10**	**S 12**	**IP2**	**IP3**	**IP4**	**IN3**	**IN6**	**C1**	**C3**	**C5**
1	**Lactose**	-	-	-	-	-	-	-	-	-	-	-
2	**Xylose**	+	+	+	+	+						
3	**Maltose**	+	+	+	+	+	+	+	+	+	+	+
4	**Fructose**	+	+	+	+	+	+	+	+	+	-	+
5	**Dextrose**	+	+	+	+	+	+	+	+	+	-	+
6	**Galactose**	+	+	+	+	+	+	+	+	+	+	+
7	**Raffinose**	-	+	-	+	+	+	+	+	+	+	+
8	**Trehalose**	+	+	+	+	+	+	+	+	+	+	+
9	**Melibiose**	+	+	+	+	+	+	+	-	+	+	+
10	**Sucrose**	+	+	+	+	+	+	+	+	+	+	+
11	**L-Arabinose**	+	+	+	+	+	+	+	+	+	+	+
12	**Mannose**	+	+	+	+	+	+	+	+	+	+	+
13	**Inulin**	+	+	+	+	+	+	+	+	+	+	+
14	**Sodium gluconate**	-	+	-	+	+	+	+	-	+	+	+
15	**Glycerol**	+	+	+	+	+	+	+	+	+	+	+
16	**Salicin**	+	+	+	+	+	+	+	+	+	+	+
17	**Dulcitol**	+	+	+	+	+	+	+	+	-	+	+
18	**Inositol**	+	+	+	+	+	+	+	+	+	+	-
19	**Sorbitol**	+	+	+	+	+	+	+	+	+	+	+
20	**Mannitol**	+	+	+	+	+	+	+	+	+	+	+
21	**Adonitol**	+	+	+	+	+	+	+	+	+	+	+
22	**Arabitol**	+	-	+	+	+	+	+	+	+	+	+
23	**Erythritol**	-	-	-	+	+	+	+	+	-	+	+
24	**α-Methyl-D-Glucoside**	+	-	+	+	+	+	-	+	-	+	+
25	**Rhamnose**	-	+	-	-	-	-	-	-	-	+	+
26	**Cellobiose**	+	+	+	-	-	-	-	-	+	+	+
27	**Melezitose**	+	+	+	-	-	-	-	-	+	-	+
28	**α-Methyl-D-Mannoside**	-	+	-	-	-	-	-	-	+	-	+
29	**Xylitol**	+	+	+	+	+	+	+	+	+	+	+
30	**ONPG**	+	+	+	-	-	-	-	-	+	+	+
31	**Esculin Hydrolysis**	+	+	+	+	+	+	+	+	+	+	+
32	**D-arabinose**	+	+	+	-	-	-	-	-	+	+	+
33	**Citrate Utilization**	+	+	+	+	+	+	+	+	+	+	+
34	**Malonate Utilization**	+	+	+	-	+	-	-	-	+	+	+
35	**Sorbose**	-	+	+	-	-	-	-	-	+	+	-
36	**Control**	-	-	-	-	-	-	-	-	-	-	-

	Total	**28**	**31**	**29**	**26**	**27**	**26**	**25**	**24**	**30**	**30**	**31**

Table 5.2(b): Carbohydrate solubilization potentials of eleven different microbial strains

		Strain name										
Sl No.		**T3**	**T20**	**B5**	**T17**	**T10**	**T7**	**OS6**	**OS7**	**Os3**	**Os9**	**Os10**
	Test											
1	**Lactose**	-	-	-	-	-	+	-	+	+	-	-
2	**Xylose**				-	-	-	+	-	-	-	-
3	**Maltose**	+	+	-	-	-	+	+	+	+	-	-
4	**Fructose**	+	+	+	+	+	-	-	+	-	-	+
5	**Dextrose**	+	+	+	+	+	-	-	+	-	+	+
6	**Galactose**	+	+	+	-	-	-	-	+	-	-	-
7	**Raffinose**	-	-	+	-	-	+	+	-	+	+	-
8	**Trehalose**	+	+	+	+	+	-	+	+	-	-	+
9	**Melibiose**	-	-	+	-	-	-	+	+	-	+	-
10	**Sucrose**	+	+	+	+	+	-	+	-	-	-	+
11	**L-Arabinose**	+	+	+	+	+	-	+	+	-	-	+
12	**Mannose**	+	+	-	+	+	-	+	+	-	+	+
13	**Inulin**	+	+	-	-	-	-	+	+	-	+	-
14	**Sodium gluconate**	-	+	+	-	-	-	-	-	-	-	-
15	**Glycerol**	+	+	+	-	-	-	+	+	-	-	-
16	**Salicin**	+	+	+	+	+	-	+	+	+	+	+
17	**Dulcitol**	-	-	+	-	-	-	-	-	-	-	-
18	**Inositol**	+	-	+	-	-	-	-	+	-	-	-
19	**Sorbitol**	+	-	+	-	-	-	-	-	-	+	-
20	**Mannitol**	+	+	+	+	+	-	+	+	-	-	+
21	**Adonitol**	-	-	-	-	-	-	-	+	-	-	+
22	**Arabitol**	-	+	-	-	-	-	-	+	+	+	-
23	**Erythritol**	-	-	+	-	-	-	-	-	-	-	-
24	**α-Methyl-D-Glucoside**	+	-	+	+	+	+	-	-	+	+	+
25	**Rhamnose**	-	-	+	-	-	-	+	-	-	-	-
26	**Cellobiose**	+	+	+	-	-	-	-	+	-	+	-
27	**Melezitose**	+	+	-	-	-	-	-	-	-	-	-
28	**α-Methyl-D-Mannoside**	-	-	-	-	-	-	-	+	-	-	-
29	**Xylitol**	-	+	+	-	-	-	-	+	-	-	-
30	**ONPG**	+	-	-	+	+	-	-	-	-	+	+
31	**Esculin Hydrolysis**	+	+	+	+	+	+	-	+	+	-	+
32	**D-arabinose**	+	+	-	-	-	-	+	+	-	+	-
33	**Citrate Utilization**	+	+	+	+	+	+	-	+	+	-	+
34	**Malonate Utilization**	+	+	-	-	-	-	-	-	-	-	-
35	**Sorbose**	-	-	-	-	-	-	-	-	-	-	-
36	**Control**	-	-	-	-	-	-	-	-	-	-	-
	Total	**22**	**21**	**23**	**12**	**12**	**6**	**14**	**22**	**8**	**12**	**13**

solubilization efficiency (table. 5.3).

Table 5.3: Relative dominance for gut isolated and vermibeds isolated bacteria.

Organism Name	Strain name	Source	Relative Dominance(%)
Kosakonia sacchari	C1	Vermicompost	5
Enterobacter cloacae	C3	Vermicompost	4.6
Bacillus alcalophilus	C5	Vermicompost	6
Unidentified Sequence	T3	Earthworm gut	3.7
Unidentified Sequence	T20	Vermicompost	2.8
Unidentified Sequence	B5	Earthworm gut	2.4
Unidentified Sequence	T17	Vermicompost	1
Unidentified Sequence	T10	Earthworm gut	1.8
Unidentified Sequence	T7	Vermicompost	1.1
Unidentified Sequence	OS6	Earthworm gut	1.3
Unidentified Sequence	OS7	Earthworm gut	2.9
Unidentified Sequence	Os3	Earthworm gut	1.5
Unidentified Sequence	Os9	Vermicompost	1.3
Unidentified Sequence	Os10	Vermicompost	1.6
All Others	AOS	Vermicompost	0.96

Interestingly, among eight previously reported strains, three (S8, S10, and S12) showed high cellulolytic and carbohydrate solubilization potential (Table 5.2 (a & b)). As mentioned in the previous section, the N-fixing and P-solubilizing traits of *K. ascorbata* S8 (previously reported as IN2), *Rhizobium sp.* S10 (previously reported as IN4), and *Bacillus sp.* Hussain et al. have reported S12 (previously reported as IN5) (Table 5.4) [21].

Table 5.4: Strain information of all the dominant bacterial taxa

Strain Code (For experimental Purposes)	Organism Name	Seq. Length	Accession Number	Differential Staining	Percentage identity	References
S8	***Kluyvera ascorbata***	**956**	**KU321346**	**Gram Negative**	**100%**	[21]
S10	***Rhizobium sp.***	**989**	**KU321348**	**Gram Negative**	**100%**	[21]
S12	***Bacillus sp.***	**1024**	**KU321350**	**Gram Positive**	**100%**	[21]
C1	***Kosakonia sacchari***	**464**	**MH174457**	**Gram Negative**	**100%**	
C3	***Enterobacter cloacae***	**456**	**MH174458**	**Gram Negative**	**100%**	
C5	***Bacillus alcalophilus***	**NA**	**FAME analysis**	**Gram Positive**	**100%**	

However, their carbohydrate solubilization and cellulose degradation properties have yet to be evaluated. Although plant-originated sugar and polymers are dependable feedstocks for biofuel production, their recalcitrant character is the major obstacle to their solubilization [3]. Therefore, the bacterial isolates' sugar and cellulolytic degrading potential were promising indicators of potential bioethanol-producing organisms.

5.3.3. Ethanol production potential of bacterial strains in different substrates (5% sugar solution and banana peel) and their molecular characterization

Based on the cellulolytic and sugar degradation efficacy of the 22 bacterial strains, their ethanol production potential from different substrates was assessed. However, only six out of 22 could produce ethanol from sugar solution; among which three (*Kosakonia sacchari* C1, *Enterobacter cloacae* C3, and *Bacillus alcalophlius* C5) were from the 14 newly isolated strains (Fig. 5.3a). Ethanol production potential of the bacterial isolates was compared with yeast (*S. cerevisiae* MTCC 170). The ethanol produced by the bacterial isolates and the yeast from

the 5% sugar solution was in the order: C5 >S10 = yeast > C1 > S12 > S8=C3 (P for organism < 0.01; LSD for organism = 5.575; Fig. 5.4a).

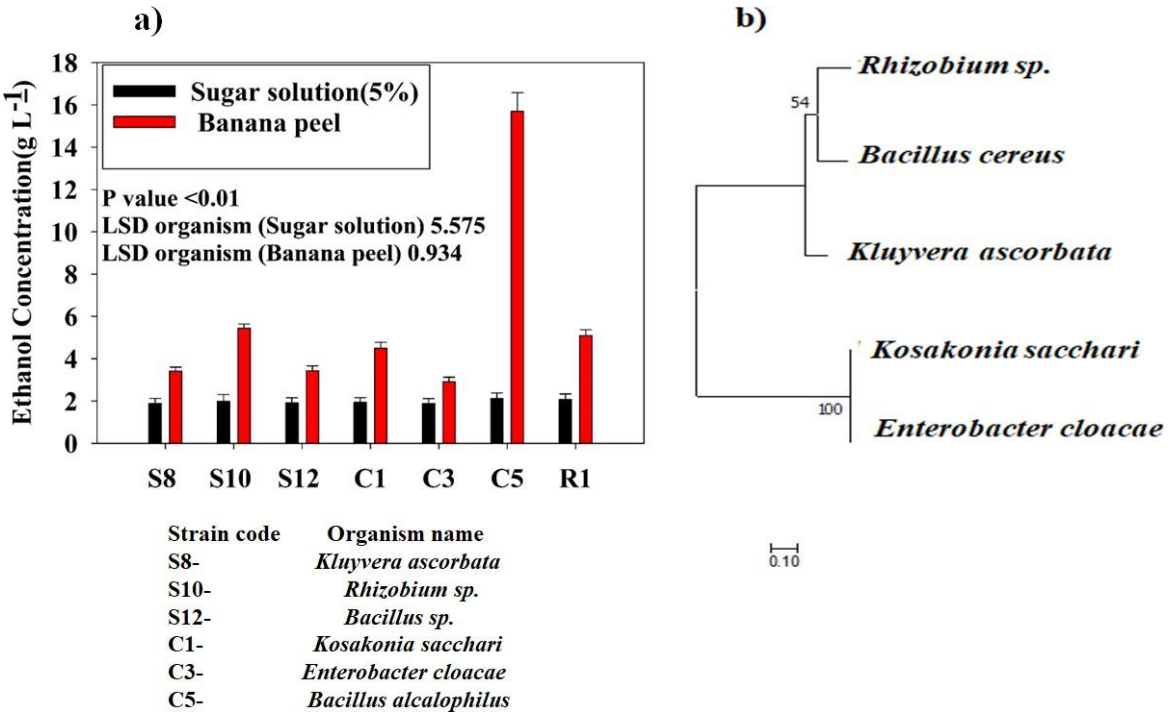

Fig. 5.4: Ethanol production from sugar solution and banana peels by ethanologenic bacterial strains and their characterization. a. Variation in ethanol production by the different bacterial strains. Values represent mean±standard deviation (n=3); b. Phylogenetic relationships of taxa of the bacterial strains isolated from vermicompost and earthworm gut.

It was interesting to note that *Bacillus alcalophlius* C5 strain was most efficient in deriving ethanol from banana peel followed by C1 and S10 (Fig. 5.4a). The gas chromatographic analysis also confirmed high purity (~99%) of the ethanol produced by the bacterial isolates. As such, ethanol produced from banana peel by all the studied strains was significantly greater than that of 5% sugar solution, and the organism-feedstock interaction effect was also significant (P for feedstock & feedstock × organism <0.01). This implied that the ethanol generation performance of bacteria and yeast would undoubtedly fluctuate depending on the feedstock characteristics. These results agreed with the previous finding [51], implying that wasted foods, particularly roughage, could be highly effective for biofuel generation, thereby reducing environmental pollution [52]. At this stage, it was confirmed that six out of 22 isolates were prolific bioethanol

producers; and three among the six strains were yet to be characterized. Therefore, the complete 16s rRNA genes of these three strains were amplified and partially sequenced. The sequencing output and identity of the strains and their accession numbers, as obtained from the NCBI database, are presented in Table 5.4. The DNA sequences of the isolated strains were utilized to create a phylogenetic tree to explore their similarities (Fig. 5.4b). The phylogenetic tree analysis revealed that the *Rhizobium sp.* IN4 and *Bacillus cereus* IP4 were closely related, while *Kluyvera ascorbata* IN2 was distantly linked to *Bacillus* and *Rhizobium*.

Interestingly, a high similarity was detected between *K. Sacchari* C1 and *E. Cloacace* C3. Such high resemblance was because both species originate from the enterobacteria complex. Moreover, according to the phylogenetic tree, all the strains were distantly related to each other. The *B. alcalophilus* C5 could not be included in the phylogenetic analysis because the organism was identified by FAME analysis; thus, a complete sequence was unavailable. As such, the strength of identification through FAME analysis is generally equal to that of the classical DNA sequencing [53].

The previously reported strains of *Bacillus*, *Kluyvera*, and *Rhizobium* were primarily utilized for their plant growth-promoting traits [21]. However, the bio- fuel production potential of *Kluyvera* has never been reported earlier. However, there has yet to be a report on cellulolytic and ethanol production traits of *Rhizobium* strains, a few strains of *Bacillus* (*B. subtilis* WB600, and *B. subtilis* WBN were able to generate ethanol under *in vitro* condition [54]. On the other hand, the cellulolytic potential of *Rhizobium* strains was reported in rhizosphere soil in addition to their N-fixing potential in previous studies [51]. The *K. sacchari*, a well-known human pathogen, is known for its multidimensional plant growth promotion activities [55]. On the other hand, the *Enterobacter* strains have been studied for alcohol generation from lignocellulose-derived sugars and glycerol [56]; yet, their ethanol production potential from biomass under *in vivo* conditions has been estimated for the first time in this investigation.

5.3.4. Sedimentation rate percentage, ethanol tolerance, sugar tolerance, and enzyme activation- mechanistic understanding of bacteria-mediated bioethanol generation

The data on sedimentation rate percentage (SRP), ethanol tolerance, and sugar tolerance is presented in Fig. 5.5a-e

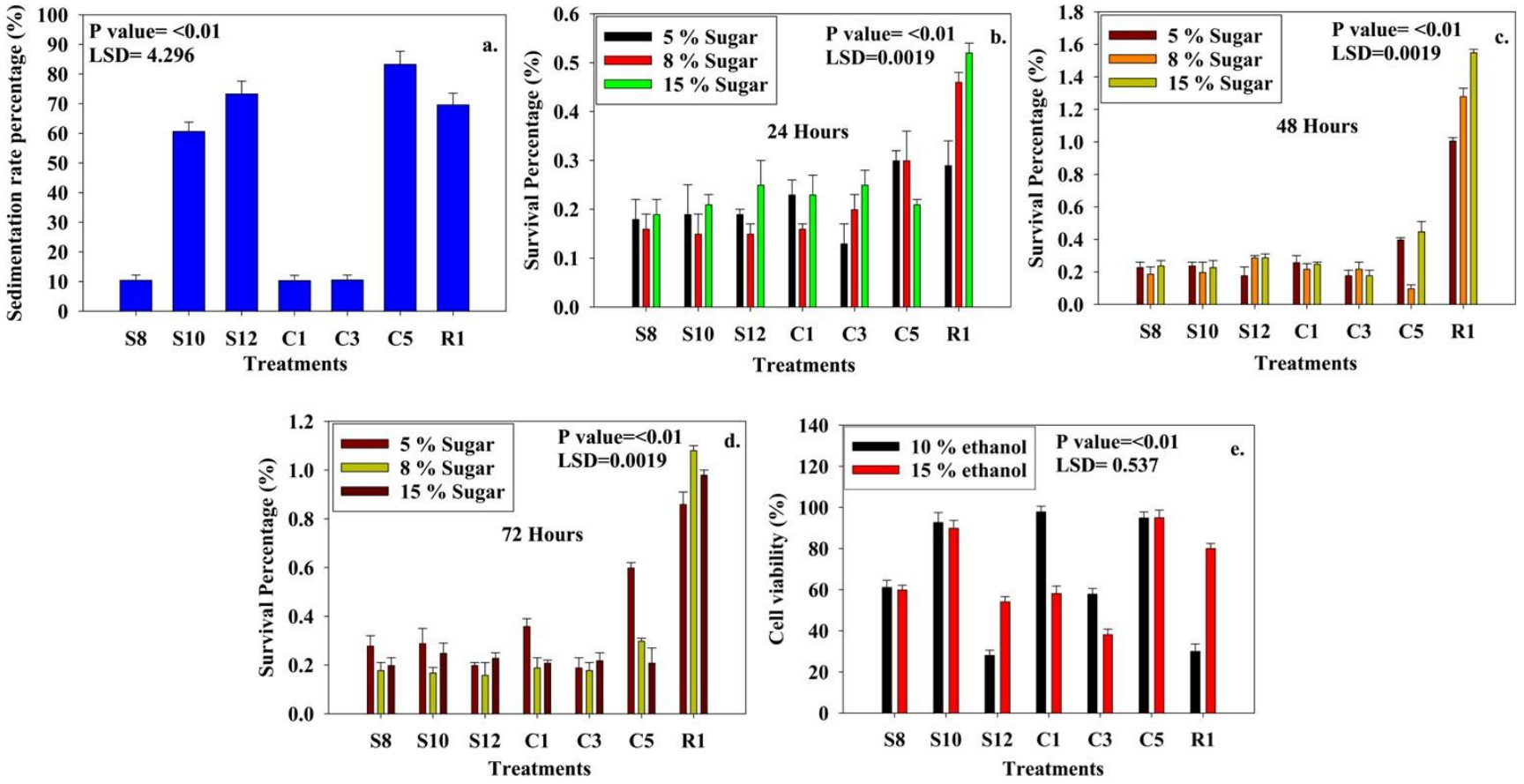

Fig. 5.5: Assessment of tolerance limits to different shock treatments (sugar exposure - at 5%, 8%, and 15 %; ethanol exposure – 10% and 15%) in the bacteria and yeast. a. Variation in microbial sedimentation rate percentage (SRP). Values represent mean±standard deviation (n=3); b. Variation in sugar tolerance potentials of bacteria and yeast at 24 hours. Values represent mean±standard deviation (n=3); c. Variation in sugar tolerance potentials of bacteria and yeast at 48 hours. Values represent mean±standard deviation (n=3); d. Variation in sugar tolerance potentials of bacteria and yeast at 72 hours. Values represent mean±standard deviation (n=3); e. Variation in ethanol tolerance potentials of bacteria and yeast. Values represent mean±standard deviation (n=3).

Although the SRP of C1, C3, and S8 was poor, the rate of sedimentation in LB agar of *Bacillus alcalophlius* C5 was significantly higher than the yeast (*S. cerevisiae* MTCC 170) grown in YPD agar (Fig. 5.5a). The SR of *Bacillus spp.* S12 and *Rhizobium spp.* S10 were either the same or marginally lower than the yeast. The sedimentation vis-à-vis flocculation features of microorganisms indicates the ease of their separation from the medium after completion of the fermentation, which is immensely important for the recovery and reuse of the organisms, which signifies the industrial suitability of microbe-mediated ethanol production process [20].

The tolerance to sugar exposure of the bacterial isolates was generally lower than that of the yeast until 72 hours of incubation (Fig. 5.5b-d). The cell growth of all six bacterial isolates constantly increased over time, while the yeast growth was significantly retarded at 72 hours in a 5% sugar solution (Fig. 5.5b). A similar pattern of yeast cell growth was also evidenced in 8% and 15% sugar solutions (Fig. 5.5c & d). However, the temporal growth patterns of the bacteria marginally varied among the strains in 15% sugar solutions — for example, *Rhizobium sp.* S10 showed a steady increase over time, and activity of the key extracellular enzymes that regulate the microbe-mediated ethanol production process was evaluated in the treated (5% sugar solution) organisms (Table 5.5). Activities of acetyl CoA synthase and alcohol dehydrogenase were significantly greater in *Rhizobium sp.* S10 and *Bacillus alcalophilus* C5 inoculated solutions ($P < 0.01$; LSD: acetyl CoA synthase = 0.447; alcohol dehydrogenase = 0.957). Acetyl CoA synthase catalyzes acetyl CoA synthesis by utilizing acetic acid, thereby arresting the acetate-induced retardation during the fermentation [57]. At the same time, the alcohol dehydrogenase upregulates the conversion of acetyl CoA to ethanol [58].

Table 5.5: Activities of different enzymes by different isolated strains during the experiment (mean±standard deviation)

	Enzyme activities				
Organisms	**Pyruvate decarboxylase (mM NADH min^{-1})**	**Pyruvate kinase (mM NADH min^{-1})**	**Acetyl CoA synthetase (mM NADH min^{-1})**	**Alcohol dehydrogenase (mM NADH min^{-1})**	**Pyruvate dehydrogenase (mM NADH min^{-1})**
K. ascorbata S8	9.48 ± 0.2	10.43 ± 0.4	4.73 ± 0.2	9.1 ± 0.2	6.43 ± 0.3
Rhizobium spp. S10	23.73 ± 1.1	23.7 ± 0.7	9.2 ± 0.5	24.7 ± 0.9	9.44 ± 0.3
B. cereus S12	18.27 ± 0.6	16.45 ± 0.2	7.44 ± 2.9	14.7 ± 0.9	7.17 ± 0.4
K. sacchari C1	18.84± 0.6	21.07 ± 0.7	5.6 ± 0.8	23.2 ± 1.6	8.47 ± 1.3
E. cloacae C3	8.9 ± 0.7	10.3± 0.6	7.57 ± 0.9	8.6 ± 1.1	7.57 ± 0.8
B. alcalophilus C5	28.94 ± 0.5	27.7 ± 0.6	8.44 ± 0.9	26.4 ± 1.6	18.27 ± 0.6
P_{value}	<0.01	<0.01	<0.01	<0.01	<0.01
LSD	0.561	0.479	0.447	0.957	0.604

Interestingly, the ethanol production efficiency of S10 and C5 was remarkably greater than the other strains described in the previous section. Correspondingly, pyruvate decarboxylase, kinase, and dehydrogenase activities were also significantly higher in *Bacillus alcalophilus* C5 inoculated sugar solution, followed by *Rhizobium spp.* S10, *K. sacchari* C1, and *Bacillus cereus* S12 (Table 5.5). Pyruvate, a component of carbohydrate metabolism, is decarboxylated by pyruvate decarboxylase to produce acetaldehyde, which is eventually reduced to ethanol by alcohol dehydrogenase [59]. The pyruvate kinase is known to sustain cellular homeostasis via regulation of energy metabolism, which in turn induces thermo-tolerance to S*accharomyces cerevisiae*, and the pyruvate dehydrogenase complex dramatically enhances the free fatty acids levels in *S. Cerevisiae* [60]. Overall, the results strongly postulate that the high ethanol-producing ability of the bacterial isolates (mainly C5 and S10) from sugar solution and lignocellulosic biomass was due to the efficient release of essential enzymes during fermentation.

5.3.5. Membrane integrity and cellular response of yeast and bacterial to sugar exposure: Understanding the differential defense mechanism using flow cytometry and confocal microscopy

Fig.5.6 represents the results of the FACS (i.e., flow cytometry) analysis of the stained bacteria and yeast cells exposed to sugar solutions of different concentrations. The FACS and confocal microscopic analyses were performed to comprehend the variations in apoptosis-mediated defense mechanisms between yeast and ethanologenic bacterial cells in response to sugar-induced shock. The two most prolific ethanologenic bacterial strains (*Rhizobium sp.* S10 and *Bacillus alcalophilus C5*) were selected for this study. The FACS study demonstrated that incubation of yeast cells with 5 or 15 % sugar causes a dose-dependent increase in apoptosis compared to the untreated cells. In sugar-treated yeast, a higher percentage (77.2 and 89.3 %) of dual positive cells (both Annexin+PI) indicates a robust increase in apoptosis (about two folds more than the control). The shift of cells from early to late (i.e., mature) apoptosis was also evidenced in yeast (Fig. 5.6).

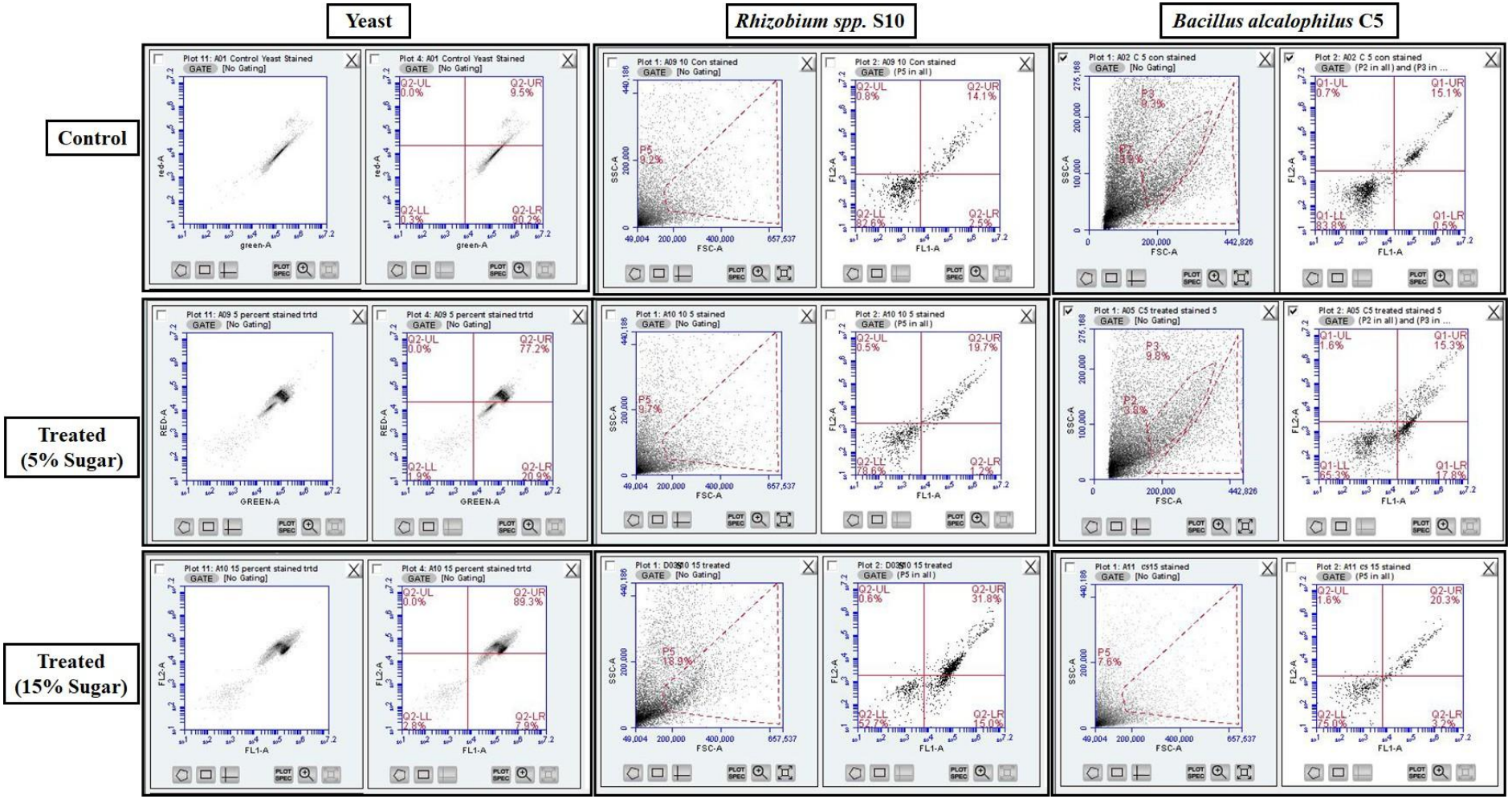

Fig. 5.6: Representation of the fluorescence-activated cell sorting (FACS) analysis of the stained microbial and yeast cells exposed to different concentrations (0% (i.e., control), 5% and 15%) of sugar solutions.

This observation indicates that apoptosis is probably the primary defense mechanism in yeast (*S. cerevisiae*) in response to sugar-induced stress. However, bacterial cells responded differently from yeast to sugar exposure. In *Rhizobium sp.* S10, 5, and 15% sugar exposure mildly induced apoptosis in bacterial cells with a slight exhibition of dose-dependent apoptotic induction. In contrast, in *Bacillus alcalophilus* C5, sugar incubation showed no apoptotic induction compared to the control. These results imply that the apoptotic response of ethanologenic bacteria in a sugar-enriched environment may vary among species, and reverse apoptosis or delayed apoptosis could be the probable defensive adjustment of sugar-exposed bacterial cells. Although evidence of reverse- apoptosis with increased survivability in human cells has been reported [61], such instances have not been found in bacteria. However, delaying apoptosis by the CpG motifs in DNA has been detected in some bacterial species [62]. Hence, studies with more prolonged exposure to varying sugar concentrations may be able to vindicate the present hypotheses.

The results of the confocal microscopy were quite intriguing regarding the differential response between ethanologenic bacteria and yeast to sugar exposure (Fig. 5.7). Briefly, it appears that almost 90% of yeast cells were invaded by the PI at 5 and 15% sugar concentrations, which implies that increasing sugar exposure considerably caused fetal impacts on the fungal (*S. cerevisiae*) cells (Fig. 5.7).

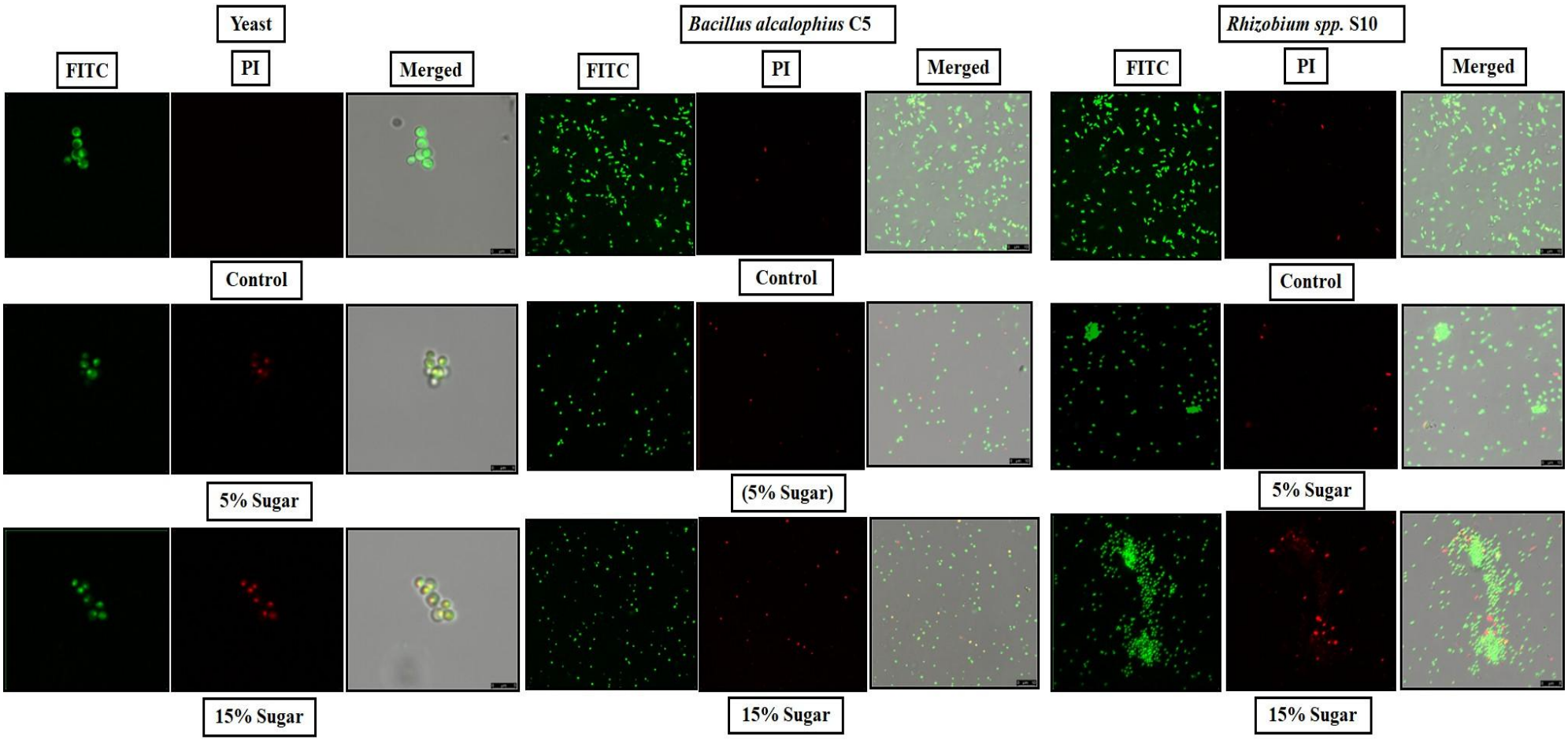

Fig. 5.7: Confocal microscopy-based assessment of cellular integrity of representative bacterial strains and yeast exposed to sugar solutions of different concentrations using specialized stains (green fluorescein-5-isothiocyanate (FITC) and red propidium iodide (PI)). The FITC stains all cells and the PI stains only ruptured cells.

However, careful observation of the images in Fig 5.8 would clarify quite a few yeast cells have remained unstained while repeating the staining process. This may be due to the yeast cells' poor compatibility with the stains owing to their cell wall-induced inhibition. However, this technique has been previously used for observing apoptosis of yeast cells [34]; while bacterial cells have often been studied in a similar manner [39].

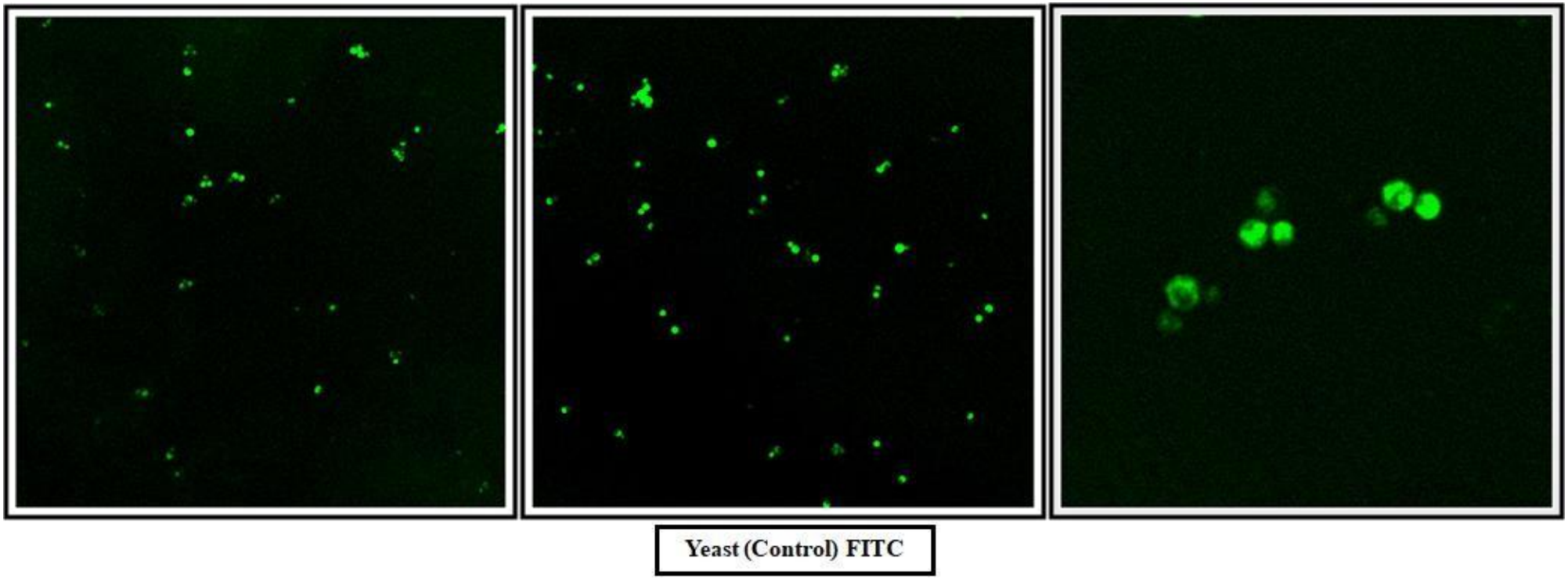

Fig. 5.8: Imaging variation in Yeast (Control) using confocal microscopy.

Nevertheless, the result clearly shows that yeast's cellular wall and membrane integrity might be severely disrupted due to sugar exposure. Interestingly, 5% sugar exposure caused mild damage to the membrane integrity of *B. alcalophilus* C5 and *Rhizobium sp.* S10, but the responses of the two bacterial species to 15% sugar exposure were conspicuously different. The extent of PI invasion was considerably greater in 15% sugar exposed S10 than the C5 (Fig. 5.7), signifying that the membrane integrity of C5 was more robust than S10. Overall, bacterial membrane integrity was more potent than the affected yeast cells.

This observation substantiates the hypothesis that some bacteria, like *B. alcalophilus* C5, have more robust defense mechanisms than the ethanol-producing yeast and other bacteria. The current results also indicate that more robust membrane integrity could be one of the effective defensive strategies in addition to apoptosis in bacterial species in response to sugar-induced stress.

5.3.6. Waste conversion efficacy

Bacteria-mediated bioethanol production from biosolids is well recognized for its ecological compatibility [63]. Therefore, we have computed the WCE for the bacteria-mediated bioethanol generation. We recorded that the production process was highly organism dependent. extraordinarily high WCE for *B. alcalophilus* C5, followed by *Rhizobium sp.* S10 and yeast (*S. cerevisiae* MTCC 170) ($P < 0.01$; LSD = 1.26). The enumerated Waste conversion efficacy (WCE) for bacteria mediated process and bioethanol generation for economic evaluation is presented in Table 5.6.

Table 5.6: Waste-to-wealth conversion efficiency (WCE) for microbe mediated composting systems and microbe-mediated bioethanol generation

Treatments	WCE %
Compost	61.8±1.33
P-Value	<0.01
Bioethanol generation	
C1	68.83±0.50
C3	64.48±0.26
C5	73.05±0.51

S8	59.7±0.23
S10	72.42±0.95
S12	54.72±0.37
R1	69.58±0.33
P-Value	<0.01
LSD	1.26

Interestingly, the WCE was highest for the *B. alcalophilus* C5-based ethanol production process ($P < 0.01$; LSD = 1.26). As such, using ethanol as a substitute for fossil fuels would be highly significant while, it also decreases emissions of air pollutants like particulate matter, CO, and volatile hydrocarbons [64].

5.4. Conclusion

Based on the findings of the NGS-analyses, six multidimensional plant-growth-promoting endophytic bacterial species were isolated from the LCWv system, conducted at the first stage. The phylogenetic analysis of the 16S rRNA sequences of these bacterial isolates reveals their association with the genes identified from NGS-based metagenomic analyses. Moreover, novel, and industrially suitable ethanologenic bacteria were isolated from the stage-2 vermicomposting experiment. Six of 22 efficient sugars and cellulose solubilizing bacterial isolates exhibited high ethanol production ability. Two bacterial strains *Bacillus alcalophilus* C5 and *Rhizobium spp.* S10 strains produced significantly more ethanol (~5-15 g L^{-1}) than the yeast without pre-treatment or externally supplemented enzymes. Additionally, these strains were strongly tolerant to inhibitory factors like ethanol and sugar shocks that assure their industrial applicability. We postulated through enzyme assay that appropriate activation of enzymes like alcohol dehydrogenase was one of the critical attributes that imparted high ethanol-producing capability in C5 and S10. The study with flow cytometry and confocal microscopy revealed that reverse/delayed apoptosis and strong membrane integrity could be the defence strategies in bacteria that facilitate their growth and maintain ethanol production capacity in sugar-enriched conditions. High waste-to-wealth conversion efficiency with a significant benefit-cost ratio strongly substantiates the practical applicability of the identified organisms for bioethanol generation from recalcitrant biowastes. However, technological improvement for the up-scaling of bacteria-mediated ethanol production systems warrants in-depth studies in future.

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