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Optimization of Exopolysaccharide Production by Bacteria Strains Isolated from Brewery Wastewater Sludge collected from a Brewery Company in Uyo, Akwa Ibom State, Nigeria

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ABSTRACT: Bacteria make expolysaccharides (EPS) in two basic forms; as firmly attached capsular-EPS and loosely attached slime-EPS. Hence, the objective of this paper was to investigate the optimization of exopolysaccharide (EPS) production by bacteria strains isolated from brewery wastewater sludge collected from a Brewery Company in Uyo, Akwa Ibom State, Nigeria using random mutagenesis. Bacterial isolates from brewery wastewater sludge samples were screened for EPS production using standard microbiological method. Six (6) best EPS-producing bacterial isolates were selected, identified molecularly as *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Heyndrickxia oleronia*, and *Acinetobacter calcoaceticus*, and used for optimization of EPS production after exposure to UV-irradiation and ethidium bromide (EtBr). In all the isolates, UV-irradiation caused the production of significantly ($P<0.05$) higher EPS yield than EtBr. However, both treatments induced the formation of mutant strains with higher EPS yields than the wild strains. The quantity of EPS produced varied among the mutant strains and ranged from 0.46±0.02 g/100ml in EtBr-mutant strain EPBS.6 to 0.86±0.01 g/100ml in UV-mutant strain EPBS.1, indicating 15.1% to 26.5% yield increase compared to the wild strains with EPS yields of 0.40±0.02 g/100ml to 0.68±0.01 g/100ml. Previous studies have reported *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus* as EPS-producers, but little or no known report was on ground regarding EPS producing capacity of *Heyndrickxia oleronia*, sufficing that it might be a novel EPS-producing bacteria strain. Based on these results, we therefore conclude that the bacteria strains obtained have the potential to produce EPS and the production can be improved through random mutagenesis with UV-irradiation and EtBr. We recommend brewery wastewater sludge as a rich source of novel EPS-producing bacteria and suggest further study on the mechanism of mutagenesis on EPS production by bacteria.

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Bacteria exopolysaccharides (EPS) are long-chain, large-molecular size poly-carbohydrate molecules synthesize in bacteria and exported into the external medium or surrounding (Osemwegie *et al.*, 2020; Jacob *et al.*, 2024). It is made up of sugars as repeating units associated by glycosidic linkages, as well as non-carbohydrate materials such as amino acids, nucleic acids, phospholipids, humic acids, functional groups

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and glycerol (Yi *et al.,* 2021; Andharia *et al.,* 2023). Most bacteria naturally produce and export diverse types of polysaccharides as part of their normal metabolic activity and these polysaccharides may be grouped into three categories as (i) intracellular polysaccharides, which function in cellular metabolism, (ii) structural polysaccharides, which confer structural integrity to the bacteria (iii) extracellular polysaccharides usually exported outside the bacteria cells (Rana *et al.,* 2020; Sharma *et al.,* 2022). EPS-producing bacteria are found in diverse environments, but environment having a medium with high carbon-nitrogen ratio or high amount of organic substances have been claimed suitable habitat for the growth of bacteria with high EPS-producing potentials (López-Ortega *et al.,* 2021; Prete *et al.,* 2021). Such carbon-rich environment includes brewery wastewater sludge, which is used in this study. However, in a hostile environment, bacteria may produce EPS as a means of protection against such environment (López-Ortega *et al.,* 2021). Bacteria EPS have found outstanding applications in many areas of industrial processes and biotechnology as conjugate for drug production, thickening and stabilizing agent in industrial products, emulsifier and surfactant in biological remediation processes, and flocculants in water purification (Banerjee *et al.,* 2021; Siddharth *et al.,* 2021). Due to these wide industrial applications of EPS and its high commercial demands, a search for novel EPS-producing bacteria strains and techniques to enhance overproduction have gained increasing importance in recent times. Several biotechnology approaches have been used over the years to enhance the biosynthesis of metabolites by microorganisms in the industrial fermentation processes (Demirkan *et al.,* 2024). These include strains improvement by mutagenesis, which genetically modifies bacteria strains to achieve higher productivity (Adebayo-Tayo *et al.,* 2017). This method is relatively inexpensive and sustainable to achieve strain improvement (Muzzamal and Latif, 2016; Ishola and Adebayo-Tayo, 2018). Hence, the objective of this paper was to investigate the optimization of exopolysaccharide (EPS) production by bacteria strains isolated from brewery wastewater sludge collected from a Brewery Company in Uyo, Akwa Ibom State, Nigeria using random mutagenesis.

MATERIALS AND METHODS

Isolation and screening of isolates for EPS production: Brewery wastewater sludge samples were collected from a brewery company in Uyo, Akwa Ibom State, Nigeria using standard microbiological procedure. The samples were prepared by serial dilution in normal saline, inoculated into sterile nutrient agar by pour-plating technique and incubated at room temperature for 24h (Jacob *et al.,* 2024). Colonies with characteristic mucoid and ropy appearance were isolated and purified (Nwosu *et al.,* 2019). Pure colonies were further screened for EPS production qualitatively using alcian blue 8GX as described in our previous study (Jacob *et al.,* 2024). Bacterial isolates with the evidence of EPS production were selected and preserved on agar slants in the refrigerator (4^{o}C).

Molecular characterization and identification of isolates: EPS-producing bacterial isolates were identified molecularly using Sanger sequencing method. The genomic DNA of the bacterial isolates was extracted using the extraction kit and procedure from Norgen (model 24700, Norgen Biotek Corp, Canada). PCR amplification of the 16S rRNA region of the extracted genomic DNA was carried out on a real-time thermocycler (ThermoFIsher, Model 9700, USA) with the universal primer sequences 27FAGAGTTTGATCMTGGCTCAG (forward primer sequence) and 1492RTACGGYTACCTTGTTACGACTT (reverse primer sequence) and the amplicons viewed on 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The amplified PCR products were sequenced by the Sanger sequencing method at Inqaba Biotechnical Industries (Pty) Ltd, West Africa. Sequence chromatogram analysis was performed using FinchTV analysis software (version 1.4.0, Geospiza Inc.) and identified using BLASTN analysis and a neighbor-joining phylogenetic tree based on the sequences constructed using MEGA 11.0 software (Moghannem *et al.,* 2018; Tamura *et al.,* 2021).

UV irradiation: Ten milliliter (10 ml) of 24h nutrient broth culture (1 $O.D_{600nm}$) of each EPS-producing bacteria strains was transferred into six screw-caped tubes in duplicates and treated with ultraviolet irradiation of wavelength 254 nm at 10cm apart for the intervals of 20, 40, 60, 80, 100, and 120 minutes (Adebayo-Tayo *et al.,* 2017; Ishola and Adebayo-Tayo, 2018). The UV treated cultures were withdrawn at each interval, wrapped with aluminum foils and incubated at 25^{o}C for 24h (Muzzamal and Latif, 2016). After incubation, serial dilution of the cultures was carried out up to 10^{-8} and 0.1ml aliquots from 10^{-6} dilution was inoculated in duplicates on nutrient agar plate by spread-plating method, incubated at 25^{o}C for 24h, and observed for growth. At least three (3) mutant colonies from plates having 10 - 1% survival rate (equation 1) were selected.

$$\text{Survival rate (\%)} = \frac{\text{No. of colony after exposure}}{\text{No. of colony before exposure}} \text{ X } 100 \quad (1)$$

A parent (wild strain) culture prepared in the same manner, but without exposure to UV light was used as control.

Ethidium bromide (EtBr) treatment: Ten milliliter (10 ml) of 24h nutrient broth culture (1 $O.D_{600nm}$) of each EPS-producing bacteria strains was transferred into test tubes and 1ml of EtBr solution at final concentration of 10mg/ml was aseptically added to each tube and incubated at 25ºC on a rotary shaker (120 rpm) at varying intervals of 20 - 120 minutes (Ishola and Adebayo-Tayo, 2018; Demirkan *et al.*, 2024). Then 1ml aliquots was drawn out aseptically at each time interval, centrifuged at 4000 rpm for 30 minutes, and washed twice with sterile normal saline. The washed cell mass was serially diluted up to 10^{-8} times in sterile normal saline and 0.1 ml aliquots from 10^{-6} dilution was inoculated in duplicates on nutrient agar medium and incubated at 25ºC for 24h. The survival rate of the mutant strains was calculated (Equation 1) and at least three (3) mutant colonies from plates having 10 - 1% survival rate were selected for further studies. A parent (wild strain) culture prepared in the same manner, but without EtBr treatment was used as control.

Growth and EPS production yields by wild and mutant strains: The growth and EPS production yields of the wild and mutant EPS-producing bacteria strains was determined as described in our previous study (Jacob *et al.* (2024). Two percent (2%) of 24h broth culture (1 $O.D_{600nm}$) of each of the wild and mutant EPS-producing bacteria strains was inoculated in 100 ml of nutrient broth medium supplemented with 5% sucrose in 250ml Erlenmeyer flasks and incubated on a rotary shaker (120 rpm) at 30ºC for 48h. Cells were harvested in a centrifuge (4000rpm for 30min), washed twice with normal saline to remove every elements of broth, placed in *Petri*-dishes, dried to constant weight in desiccators overnight at 25ºC, and weighed. To determine EPS yields, the supernatant phase of the centrifuged medium of each wild and mutant strain were mixed with ice-cold absolute ethanol (3:1) and incubated at 4ºC in the refrigerator for 24h to precipitate EPS. The mixtures were centrifuged at 4000 rpm for 15 minutes and the pellet containing slime-EPS collected into Petri dishes, weighed and dried in desiccators overnight at 25ºC. The dried EPS were weighed and preserved as crude EPS at 4ºC in the refrigerator for further studies.

Data analysis: One-way analysis of variance (ANOVA) on IBM SPSS software package (version 23) was used to analyze all the data generated in this study to compare the effect of UV-irradiation and EtBr treatment on EPS production and the EPS yields between the wild-strain (control strains) and the mutant strains. Data were expressed as means ± SE and were separated using the Duncan.

RESULTS AND DISCUSSION

Table 1 shows the morphological characteristics of best six (6) EPS-producing bacteria isolates (EPBS.1 to EPBS.6) out of the 29 isolated from the brewery wastewater samples. The degree of mucoid and ropy characteristics of the selected strains, which is a primary indicator of EPS production, varied among the bacteria isolates. After molecular characterization, the isolates (EPBS.1 to EPBS.6) were identified as *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Heyndrickxia oleronia*, and *Acinetobacter calcoaceticus*, respectively (Plate 1 and Fig.1). Previous studies on EPS production by bacteria have reported *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* strains as EPS-producers (Moghannem *et al.*, 2018, Sahar *et al.*, 2018; Thite *et al.*, 2021). The species of *Bacillus* obtained in this study possess diverse physiological abilities which have enabled them to survive in different environments including brewery wastewater sludge and are known sources of huge microbial metabolites with commercial and biotechnological values (Thite *et al.*, 2021). However, as at the period of this study, little or no known report was on ground regarding the EPS producing capacity of *Heyndrickxia oleronia*, sufficing that it might be a novel EPS-producing organism.

Table 1: Morphological characteristics of the best six (6) EPS-producing bacteria strains isolated from brewery wastewater sludge samples

Isolate code	Size	Shape	Colour	Elevation	Margin	Mucoid consistency	Ropyness	Gram's reaction	Spore formation	Capsule
EPBS.1	Large	Circular	Creamy	Raised	Entire	+	+++	+ rods	Terminal endospore	+
EPBS.2	Large	Irregular	Grey-white	Flat	Undulate	+	+	+ rods	Sub-terminal endospore	+
EPBS.3	Large	irregular	Creamy	Flat	Undulate	+	+	+ rods	Sub-terminal endospore	+
EPBS.4	Small	Circular	Green	Raised	Entire	+	++	- rods	Non-spore forming	-
EPBS.5	Small	Circular	Creamy	Flat	Lobate	+	++	+ rods	Sub-terminal endospore	-
EPBS.6	Small	Circular	Creamy	Raised	Entire	+	+	+ rods	Non-spore forming	-

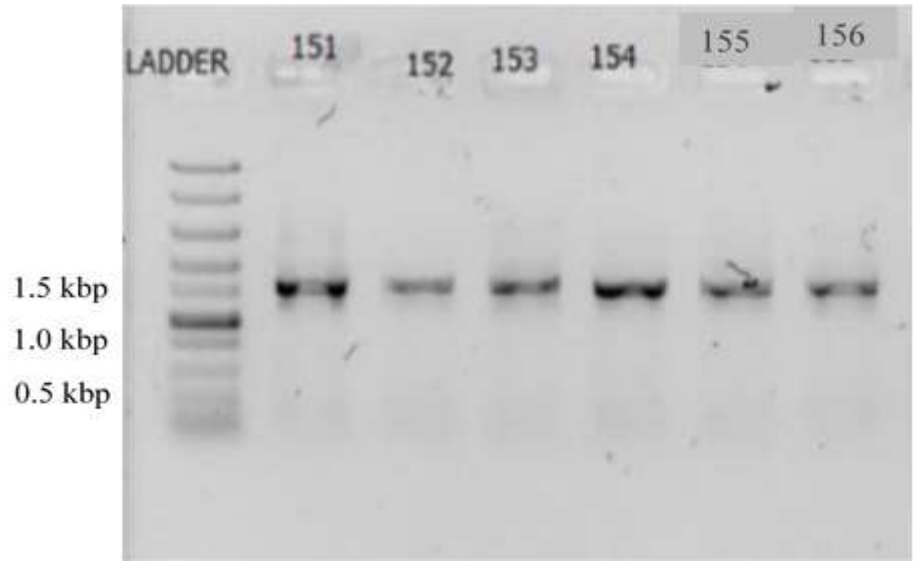

Plate 1: Gel-image showing the 16S rRNA gene amplicons (1.5 kbp) of the EPS-producing bacteria strains [EPBS.1 (151) to EPBS.6 (156)] isolated form brewery wastewater sludge

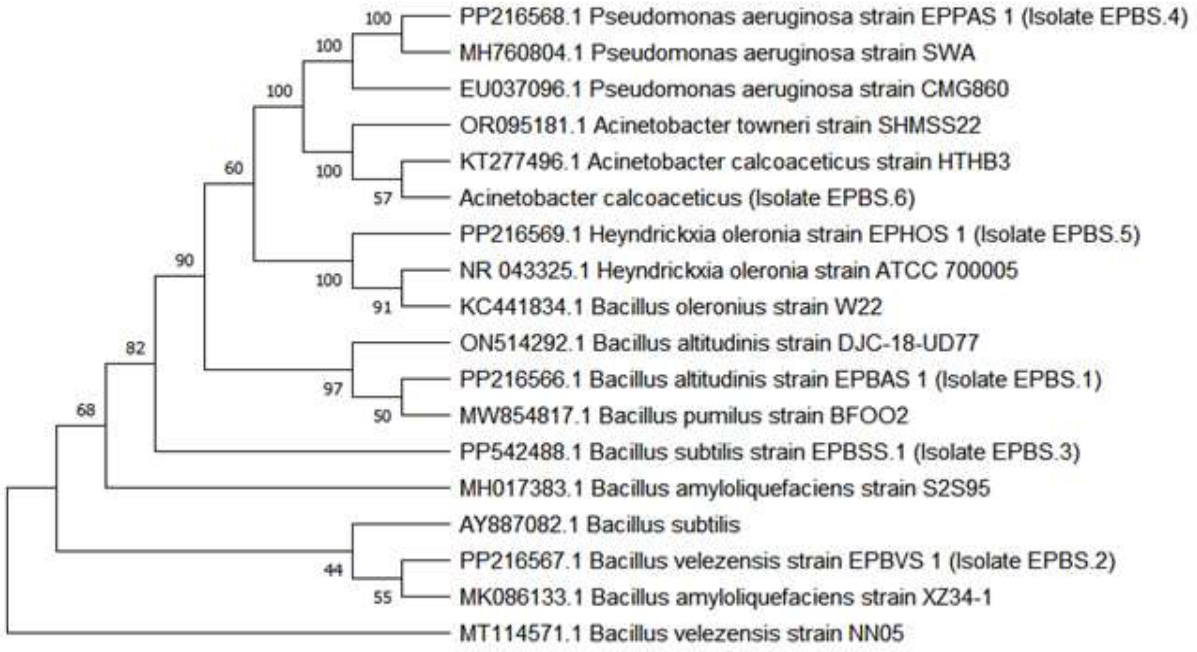

Fig.1: Phylogenetic tree and evolutionary relationship of the 16S rRNA nucleotide sequences of isolates EPBS.1 to EPBS.6 with closely related sequences available in GenBank

The growth (Cfu) and survival rate (%) of the EPS-producing bacteria strains were reduced significantly ($P<0.05$) with exposure to the mutagens as the exposure time was increased and these ranged from $5.0x10^6$±1.0 cfu (5.8%) to $3.0x10^6$±1.1 cfu (2.8%) with UV-irradiation (Table 1) and $9.0x10^6$±2.1 (9.8%) cfu to $7.0x10^6$±1.0 cfu (5.8%) with EtBr treatment (Table 2) both at 120 minutes exposure time. The growth (cfu) of the control (the wild bacteria strains) ranged between 105±5.2 cfu (100%) and 61±5.1 cfu (100%). UV irradiation showed the least survival rate compared to the EtBr treatment. The colony morphology of the exposed bacteria strains were slightly different in size compared to the wild strains. This variation may be due to changes in growth pattern or alteration in the genome components of the bacteria strains caused by exposure to such harmful conditions of UV-light and EtBr (Muzzamal and Latif, 2016; Ishola and Adebayo-Tayo, 2018).

Table 2: Growth (cfu) and survival rate (%) of EPS-producing strains exposed to UV (254nm) irradiation

Exp. time (m)	EPBS.1		EPBS.2		EPBS.3		EPBS.4		EPBS.5		EPBS.6	
	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)
*0	105±5.2	100	92±2.3	100	63±4.2	100	105±2.2	100	86±4.2	100	61±5.1	100
20	82±2.4	78.1	58±31	63.0	43±5.1	68.3	59±1.4	56.2	59±5.1	73.3	41±1.3	67.2
40	73±4.1	69.5	32±5.1	34.8	35±5.1	55.6	41±1.1	39.0	38±5.2	44.2	33±2.1	54.1
60	34±1.8	32.4	21±2.1	22.8	18±2.2	28.6	27±3.4	25.7	21±1.2	24.4	18±1.0	29.5
80	18±1.1	71.1	11±5.3	11.9	11±2.0	17.4	21±5.1	20.0	15±2.1	17.4	11±1.0	18.0
100	9±3.1	8.6	3±1.1	3.3	5±1.2	7.9	10±1.2	9.5	8±1.6	9.3	7±1.1	11.5
120	3±1.1	2.8	3±1.0	3.3	2±1.1	3.2	4±2.0	3.8	5±1.0	5.8	3±1.0	4.9

**0 – Control (Wild EPS-producing strains without UV-irradiation)*

Table 3: Growth (cfu) and survival rate (%) of EPS-producing strains exposed to EtBr (10mg) treatment

Exp. time (m)	EPBS.1		EPBS.2		EPBS.3		EPBS.4		EPBS.5		EPBS.6	
	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)	Growth ($x10^6$Cfu)	Surv. rate (%)
*0	105±5.2	100	92±2.3	100	63±4.2	100	105±2.2	100	86±4.2	100	61±5.1	100
20	93±4.1	88.6	61±5.1	66.3	56±2.3	88.9	55±2.1	52.4	63±5.3	73.3	59±4.2	96.7
40	79±5.3	75.2	47±1.3	51.1	45±2.1	71.4	43±1.3	41.0	51±2.4	59.3	43±1.8	70.5
60	68±2.3	64.8	31±6.2	33.7	32±3.2	50.8	29±3.2	27.6	38±2.1	44.2	34±3.3	55.7
80	34±1.5	32.4	23±2.1	25.0	18±2.1	28.6	23±3.5	21.9	19±2.6	22.1	21±5.1	34.4
100	17±1.0	16.2	15±1.2	16.3	12±1.3	19.0	16±1.2	15.2	13±1.2	15.1	10±2.1	16.4
120	10±1.2	9.5	9±2.1	9.8	6±2.1	9.5	9±1.0	8.6	7±1.0	5.8	4±1.2	6.6

**0 – Control (Wild EPS-producing strains without exposure to EtBr*)

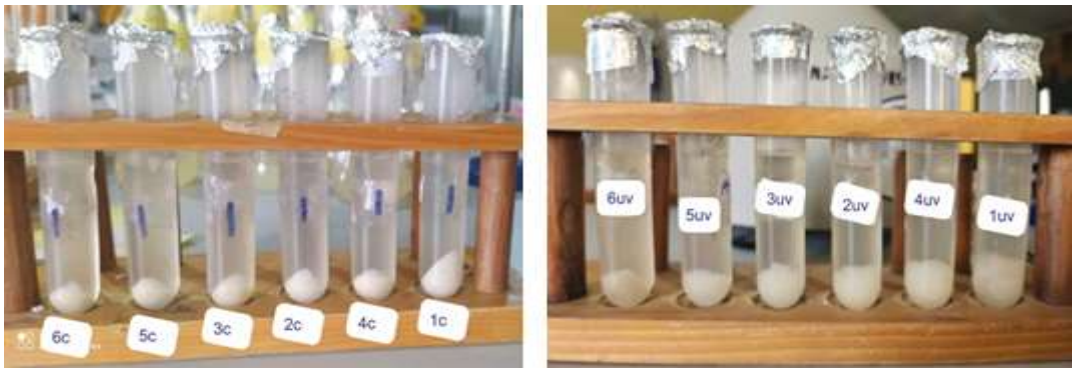

Plate 2: EPS extracts from wild strains (control 1c-6c) and UV-mutant strains (1uv-6uv)

The quantity of EPS produced varied significantly ($P<0.05$) between the wild strains and the mutant strains. EPS yields obtained from the mutant strains ranged from 0.50±0.02 g/100ml to 0.86±0.01 g/100ml after UV-irradiation (Plate 2 and Fig.2) and from 0.46±0.02 g/100ml to 0.81±0.01 g/100ml after treatment with EtBr (Fig.3) compared with the wild strains which ranged from 0.40±0.01 g/100ml to 0.68±0.01 g/100ml. The mutants strains obtained with UV-irradiation showed higher percentage (23.6% to 26.5%) increase in EPS yield than those obtained with EtBr treatment (15.1% to 20.8%) (Fig.4). The observed increase in the quantity of EPS production by the mutant strains suffices improvement in the bacteria strains. This may be linked to certain modifications in the bacterial genome and EPS-biosynthesis machinery by random mutagenesis or altered cell membrane permeability and transporters by exposure to the mutagens, thereby enhancing the external transportation of EPS by the bacteria strains (Ishola and Adebayo-Tayo, 2018; Zhi-Qiang *et al.*, 2023).

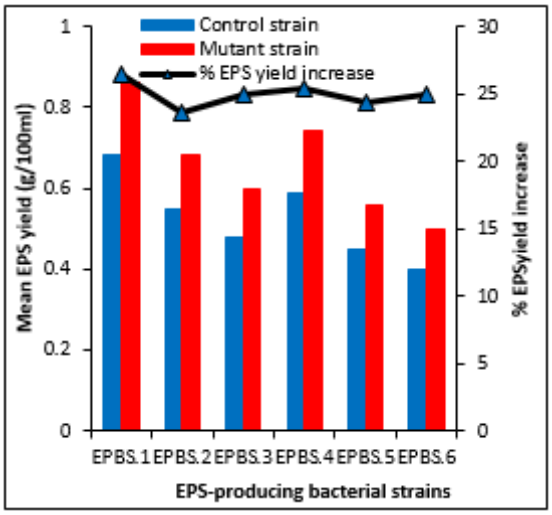

Fig.2: EPS-production yields of wild strains (control strain) and the mutant strains exposed to UV-light (254nm)

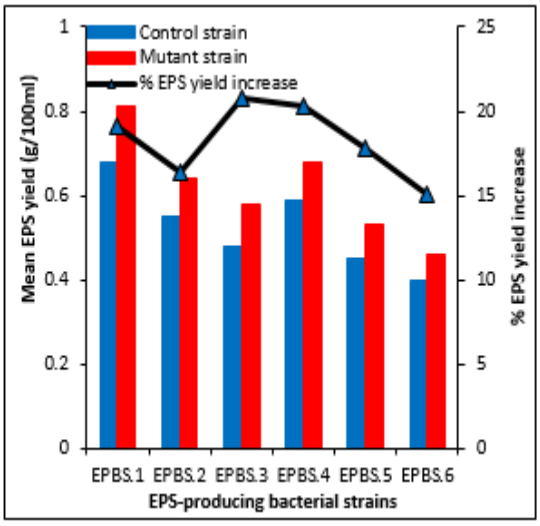

Fig. 3: EPS-production yields of wild strains (control strain) and the mutant strains exposed to EtBr (10mg)

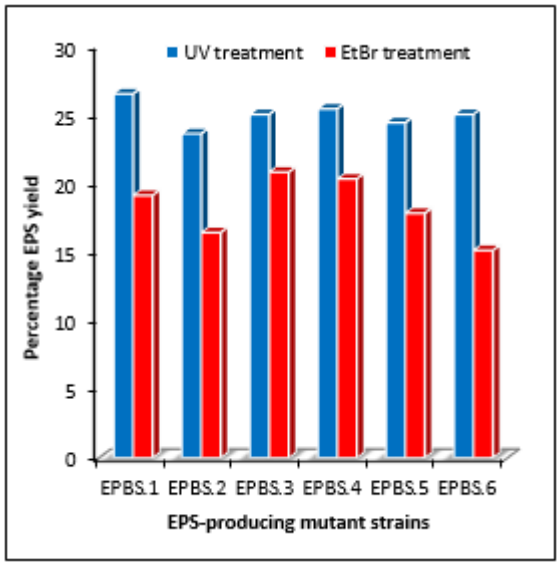

Fig.4: Comparative EPS-production yields (%) of the mutant strains exposed to UV-irradiation (254nm) and EtBr (10mg) treatment

Conclusion: Based on the result of this study, *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Heyndrickxia oleronia*, and *Acinetobacter calcoaceticus* obtained from the brewery wastewater sludge has the potential to produce EPS and the production can be significantly enhanced through random mutagenesis with UV-irradiation and EtBr. Previous studies have reported *Bacillus altitudinis*, *Bacillus velezensis*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* as EPS-producers, however, little or no report regarding *Heyndrickxia oleronia* as EPS producer was available as at the period of this study, sufficing that it might be a novel EPS-producing organism. We therefore recommend brewery wastewater sludge as a rich source of novel EPS-producing bacteria and suggest that future study be focused on the mechanism of mutagenesis on EPS production by bacteria.

Declaration of Conflict of Interest: The authors declare no conflict of interest

Data Availability Statement: Data are available upon request from the corresponding author.

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