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Mutagenesis and Immobilization Effect on Exopolysaccharide Production by *Weissella confusa* and *Lactobacillus delbrueckii*

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Authors' contributions

This work was carried out in collaboration between both authors. Author BCAT designed the study and wrote the protocol. Author ROI carried out the bench work under the supervision of author BCAT and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This work aimed at investigating the effect of mutation and immobilization on EPS production and to characterize the EPS produced by *L. delbrueckii* and *Weissella confusa.*
Study Design: To determine the effect of mutation using UV irradiation and immobilization using different matrixes on EPS production by the strains and to characterize the EPS produced.
Methodology: *Weissella confusa* and *Lactobacillus delbrueckii* was exposed to UV irradiation and the wild and mutants strains was immobilized. The immobilized wild and mutants was used for EPS production. The produced EPS was characterized.
Results: The LAB count that survived the UV irradiation reduced as the exposure time increases. The Wild *L. delbrueckii* produced more EPS (583.72 mg/L) than its mutant strain (581.42 mg/L). However, wild *Weissella confusa* and wild *Lactobacillus delbrueckii* produced more EPS than the mutant strain. Production of EPS by wild and mutant *Weissella confusa* immobilized in Sodium alginate (WWCNA and WWCNA), agar matrix (WWCAA and MWCAA) and polyurethane foam (WWCPF and MWCPF) ranged from 240.25 – 544.28 mg/L with WWCPF producing the highest EPS. Production of EPS by wild and mutant *L. delbrueckii* immobilized in Sodium alginate

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(IWLDNA and IMLDNA), agar matrix (IWWCAA and IMLDAA) and polyurethane foam (WLDPF and MLDPF) ranged from 220.06 – 502.81 mg/L with IWLDPF producing the highest EPS. However, immobilization in agar matrix supported the least EPS production. Characterization of the EPS using FT-IR spectroscopy confirmed the presence of the different degrees of functional groups which indicates that they are polysaccharides, thus confirming the EPS. Un-immobilized *Weissella confusa* had the highest EPS production (558.72 mg/L and 583.61 mg/L) compared to the immobilized strains.

Conclusion: Immobilization of mutant *L. delbrueckii* and *Weissella confusa* using polyurethane foam supported EPS production.

Keywords: Immobilization; mutation; exopolysaccharide; polyurethane foam and sodium alginate.

1. INTRODUCTION

Lactic Acid Bacteria (LAB) have attracted immense commercial interests, for their capacity to secrete a host of exopolysaccharides having industrially useful physicochemical properties [1-2]. They have received great interest as the major group of probiotic bacteria that promote the growth of gut micro flora [3] due to their GRAS (Generally Regarded as Safe) properties. The term exopolysaccharide (EPS) refers to all forms of bacterial polysaccharide, both slime and capsule, found outside the cell wall [4]. Capsular polysaccharides (CPS) are differ in their degree of attachment to the cell surface: CPS are tightly linked, often covalently, to the cell surface and form a capsule around the cell, whereas EPS are secreted into the extracellular matrix or loosely associated with the cell surface are referred to as Slime or ropy exopolysachharide [5]. EPS produced by LAB have found wide applications as food preservatives, flavouring and texturizing agents for centuries [6]. EPS from LAB are also reported to cure irritable bowel disorder, allergies, lactose intolerance, urinary tract infections and to stimulate immunity [7-8]. The wide industrial application of EPS from LAB such as jellying agents, stabilizers, thickening agents, adhesives, flocculants, emulsifiers and flushing agents has been reported [9]. Efforts are still ongoing to improve technological characteristic of dairy food starters, to amend texture and flavor of fermented dairy food and enhance the probiotic and adaptive properties of LAB [7]. Mutagenesis is a cost-effective procedure for reliable short-term strain development. Optimization of Mutagenic procedures can be achieve in terms of type of mutagen and dose, mutagen specificity effects can be taken into account and mutagenesis itself can be enhanced or directed in order to obtain the maximum frequency of desirable mutant types among the isolates to be screened [10]. Mutagenicity and lethality in variety of organisms, including bacteria can be induced by Ultraviolet (UV) light [11]. Mutagenic lesions are formed in the DNA as a result of exposure of cells to UV radiation.

Immobilization of microbial cells has received increasing interest in the field of waste treatment [12]. The immobilized microorganism technology offer lots of advantages such as high biomass, high metabolic activity and strong resistance to toxic chemicals [13-14]. The quantity of EPS produced varies with bacteria species. However, the yield of EPS produced by different LAB is generally low and several attempts have been made to control the fermentation conditions to increase EPS production [15].

This work aimed at investigating the effect of mutation and immobilization on EPS production and to characterize the EPS produced by *L. delbrueckii* and *Weissella confusa* LAB sp.

2. MATERIALS AND METHODS

2.1 Collection of Culture

Lactobacillus delbruckii and *Weissella confusa* were collected from the culture collection of our previous work in the Microbial Physiology Laboratory, Department of Microbiology, University of Ibadan. The isolates were maintained in De Man, Rogosa and Sharpe (MRS) broth [16] and stored at 28°C.

2.2 Mutagenesis Study

2.2.1 Mutation using UV radiation

Mutagenetic study of the *Lactobacillus delbruckii* and *Weissella confusa* was done using ultraviolet radiation according to the method of Sobrun et al. [10]. 10 ml of the broth culture of each isolate was transferred into 7 test tubes. The broth culture was exposed to UV light of wavelength

254 nm at a distance of 15 cm at different time interval of 10, 20, 30, 60, 90, 120 minutes. The inoculated culture were wrapped in aluminum foil and kept in the dark for 6 hours to prevent light penetration which can cause photo reactivation. The cultures were incubated at 37^{o}C for 6 hours. After incubation, the cultures were serially diluted and 0.1 ml of the diluents (10^{-1}, 10^{-2}, and 10^{-3}) was plated on sterile MRS agar. The inoculated plates were incubated at 37°C for 24 hours. Colonies were selected from cultures having 2 - 1% survival rate.

2.3 Immobilization of the Wild and Mutant Selected LAB Strains

The wild type and the selected mutant LAB strains were immobilized using different materials. In polyurethane foam, adsorption method was done [17]. The polyurethane foam with 100- 500 µm porosity was cut into 1 cm^2 pieces and washed with distilled water. One gram of the foam was submerged in 50 ml of the fermentation medium. The medium was inoculated with the culture [18]. The inoculated PUF were properly agitated and stabilized. The reaction vessel was kept on ice for 2 hours for the hardened of the PUF. The foam was removed from the reaction vessel, rinsed with buffer to remove free cells and stored at 4^{o}C in carbon free growth media. The foam was rinsed three times with buffer to remove any free cells before use [19].

In Sodium alginate, the entrapment method of immobilization was used [11]. Sodium alginate (2g) was dissolved in 100 mL NaCl (0.9%). The solution was sterilized and inoculated with 2 mL of wild type and mutant cultures of *Weissella confusa* and *Lactobacillus delbrueckii* respectively. The mixture was added drop wise with stirring to a 0.1 M $CaCl_2$ solution. The gel beads were allowed to stay before filtered off. The beads were then washed in a 0.9% NaCl solution for 20 min.

Agar matrix entrapment of cells was carried out in sterile 4% (w/v) agar saline solution [20]. The LAB strains were cultured in MRS broth for 12 hours and centrifuged at 5000 rpm for 10 minutes to obtain cell pellets. The cell pellets were suspended in sterile saline solution. The suspension was mixed with sterile 4% (w/v) agar-saline solution, allowed to set and cut with a sterile cork borer to obtain cell-entrapped beads. The agar beads were washed successively with distilled water and saline [21].

2.4 Production of EPS by Un-immobilized (um) and Immobilized (im) Wild Type and Mutant Strains of *Weissella confusa* and *Lactobacillus delbrueckii*

The seed culture of unimmobilized strains of *Weissella confusa* and *Lactobacillus delbrueckii* were prepared by culturing in MRS broth at 37°C for 18 hours. The seed cultures were used to inoculate the Exopolysaccharide Selection Medium (mESM) (10% v/v). The inoculated medium was incubated at 37°C for 48 hours. For immobilized strains of *Weissella confusa* and *Lactobacillus delbrueckii*, the mESM was inoculated with 5% (w/v) of the immobilized LAB [21]. The inoculated medium was incubated at 37°C for 48 hours. The EPS produced was purified [22] and quantified using the method described by Dubois et al. [23].

2.5 Characterization of the EPS Using Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR was done according to the method of Bai et al. [24]. The EPS were prepared for infrared analysis by grinding a mixture of 2 mg of the EPS with 200 mg dry KBr, followed by pressing the mixture into a 16 mm diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Shimadzu IR Affinity 1S instrument with a resolution of 4 cm-1 in the 4000-400 cm-1 region.

2.6 Statistical Analysis

The results from this research were statistically subjected to analysis of variance (ANOVA) using SPSS (version 11.0, Chicago, IL). Probability values (P=0.05) were considered significant to indicate difference.

3. RESULTS AND DISCUSSION

3.1 Mutagenetic Study on the Selected EPS-producing *Weissella confusa* and *Lactobacillus delbrueckii*

The effect of UV-radiation on the colony forming unit and viability of the LAB strains is shown in Table 1. Exposure time zero indicate the control which was not expose to UV radiation. The cell count of the control was 1882 and 1314 CFU/mL at 10^{-5} dilution which corresponds to 100% survival. Exposure of the isolates for 120 second, leads to a reduction in cell count to 19 and 0.9

CFU/mL which indicate 1% survival for the two isolates. As the exposure time increases, the colony forming units and the survival rate decreases for the two isolates (Fig. 1). This is in conformity with the observations of Sobrun et al. [10] where survival rates of 1% were achieved at UV exposure times of 10s, 25s and 30s using a radiation intensity of 25W and wavelength 302 nm at a distance of 30m. The low survival rates observed at shorter exposure times could be due to the difference in wavelengths of UV light used to induce mutations. Wavelength 302 nm falls within the UV-B category while wavelength 254nm used in this present study falls within the UV-A category and UV-A radiation is known to be 10000 times less mutagenic than UV-B [25]. The present study is also similar to the work of Patel and Goyal [26] in which it was observed that UV radiation generated mutants with approximately 1% survival rate after UV exposure for 30s, 60s and 90s. Ultraviolet irradiation can increase the energy of the inner electron in the atom and can make the inner electron into the active molecule.

3.2 EPS Production by the Mutant Strains

Production of EPS by the wild and mutant *L. delbrueckii* and *W. confusa* strain is shown in Fig. 2. EPS production ranged from 549.16 – 581.42 mg/L. The highest was produced by wild *Lactobacillus delbrueckii* (WLDYG2) while the least was produced by wild *Weissella confusa* (WWCFF1). There was a significant difference ((P=.05) in the EPS production by the wild and mutant strains. This implies that the genetic modifications that occurred in the LAB as a result of random mutagenesis had led to the development of strains with improved characteristics. Sobrun et al. [9] reported that ultraviolet irradiation can increase the energy of the inner electron in the atom and can make the inner electron into the active molecule. The energy of UV is about 3 - 5 ER though the energy is very weak, the penetrability of UV cannot cause ionization. the UV can change the structure of DNA; for example, DNA strand breakage, cross linking of intramolecular and intermolecular in the DNA, cross linking of nucleic acid and protein, hydration of cytosine and uracil, formation of pyrimidine dimers thereby leading to the death of the bacteria [27-28].

3.3 EPS Production by Wild, Mutant, Immobilized and Un-immobilized *Weissella confusa*

EPS Production by wild, mutant, immobilized and un-immobilized *Weissella confusa* is shown in Fig. 3. EPS produced by mutant

Table 1. Effect of UV-irradiation on the colony forming unit and survivability of the *Weissella confuse* (WCFF1) and *Lactobacillus delbrueckii* (LDYG2)

Exp. time (s)	Dilution	*Weissella confusa* (WCFF1)			*Lactobacillus delbrueckii* (LDYG2)		
		Colony forming unit	Survival rate (%)	Average Survival Rate (%)	Colony forming unit	Survival rate (%)	Average Survival Rate (%)
0	10^{-5}	1882	100	100	1314	100	100
	10^{-7}	1407	100		1070	100	
	10^{-9}	970	100		973	100	
10	10^{-5}	1882	100	97.4	912	69.4	63.7
	10^{-7}	1392	98.9		664	62	
	10^{-9}	905	93.2		581	59.7	
20	10^{-5}	1500	79.7	78.4	519	39.5	30.8
	10^{-7}	1101	78.2		326	30.5	
	10^{-9}	750	77.3		218	22.4	
30	10^{-5}	750	50.5	41.7	205	15.6	11.3
	10^{-7}	610	43.4		112	10.5	
	10^{-9}	302	31.1		76	7.8	
90	10^{-5}	47	2.5	2.3	91	6.9	4.5
	10^{-7}	31	2.2		45	4.2	
	10^{-9}	20	2.1		24	2.5	
120	10^{-5}	19	1.8	1	0.9	0.9	1
	10^{-7}	14	1		0.9	0.9	
	10^{-9}	8	1		0		

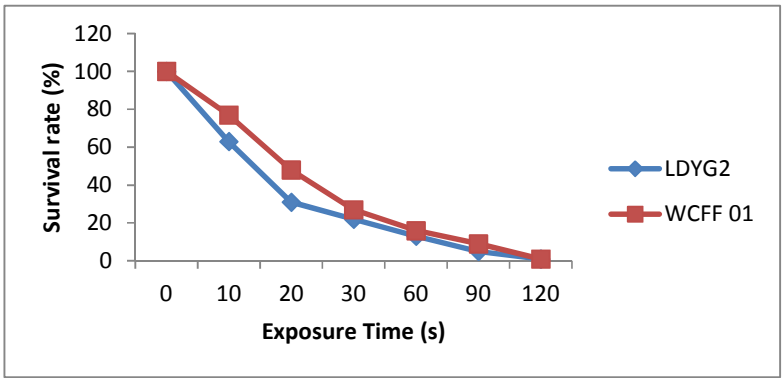

Fig. 1. Survival rates of isolates after exposure to UV irradiation
Key: WCFF1: Weissella confusa, LDYG2: Lactobacillus delbrueckii.

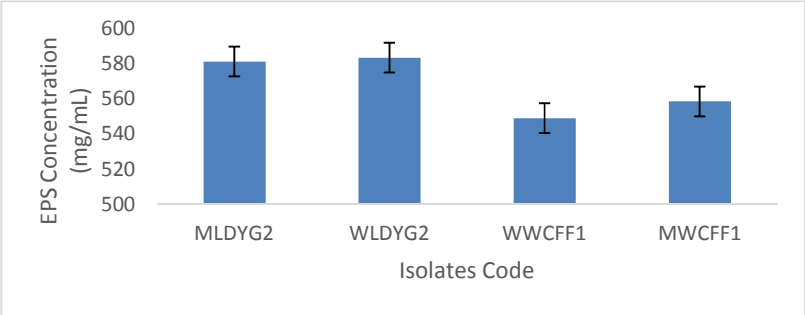

Fig. 2. Production of EPS by the wild and Mutants *Lactobacillus delbrueckii* and *Weissella confusa*
MLDYG2: Mutant Lactobacillus delbrueckii, WLDYG2: Wild Lactobacillus delbrueckii WWCFF1: Wild Weissella confusa, MWCFF1: Mutant Weissella confusa

Weissella confusa immobilized in sodium alginate, agar-agar and polyurethane foam ranged from 240.25^{b} – 308.30^{a} mg/L, 278.14^{b} – 342.39^{a} mg/L and 418.39^{b} – 544.28^{a} mg/L respectively. Wild *Weissella confusa* immobilized in polyurethane foam (IWWCPF) had the highest EPS production while mutant *Weissella confusa* immobilized in sodium alginate (IMWCNA) had the lowest EPS production. Production of EPS by un-immobilized *Weissella confusa* ranged from 549.72^{b} – 558.72^{a}. Un-immobilized wild *Weissella confusa* (UWWC) had the highest production.

3.4 EPS Production by Wild, Mutant and Immobilized *Lactobacillus delbrueckii*

EPS production by wild and mutant *Lactobacillus delbrueckii* immobilized in sodium alginate, agar-agar and polyurethane foam is shown in Fig. 4. EPS production by wild and mutant *Lactobacillus delbrueckii* immobilized in sodium alginate, agar-agar and polyurethane foam ranged from 220.06^{b} – 351.62^{a} mg/L, 312.84^{a} – 384.57^{b} mg/L and 402.60^{b} – 562.81^{a} mg/L. Wild *Lactobacillus delbrueckii* immobilized in polyurethane foam (IWLDPF) had the highest EPS production while mutant *Lactobacillus delbrueckii* immobilized in Sodium alginate had the least production. For un-immobilized *Lactobacillus delbrueckii,* EPS production ranged from 581.42^{b} – 583.72^{a}. Un-immobilized mutant *Lactobacillus delbrueckii* had the highest production. Generally, the highest EPS production was observed in wild *Lactobacillus delbrueckii* immobilized using different matrices. Un-immobilized wild *W. confusa* UWWC and *L. delbrueckii* UWLD produced highest EPS than its immobilized strains. The increase in EPS production by the un-immobilized LAB strains could be due to the fact that competition between cells may be reduced when immobilized than when they are free in the medium as fewer cells are encapsulated within each unit of the immobilization matrix [12]. It was however

observed that of all the immobilized technique used for the research work, only polyurethane foam (PUF) was more favourable matrix for immobilized cells for enhanced EPS production. This could be as a result of high rates of sorption of positive charge and hydrophobic character of the polyurethane foam which allow interaction with most microbial cell surfaces [29]. Immobilized cell technology has been applied in a wide variety of research applications. For instance, the high potential of *Lactobacillus rhamnosus* RW-9595M for EPS production and the importance of immobilized cell technology were emphasized [30-31]. Prevention of feedback inhibition could account for higher yields of EPS by immobilized cells because as the EPS is being produced, the immobilization matrix separates the cells from the products formed, thus, inhibition of further EPS synthesis is prevented. These observations are in agreement with the work of El-Gizawy et al. [32] where the production of EPS was found to have been enhanced by microencapsulation due to an increase in the viable count of the *Lactobacillus bulgaricus* strain used. Ismail and Nampothiri [33] also studied EPS production by encapsulated *L. bulgaricus* and found that encapsulated cells gave higher EPS production than free cells. It also agrees with the findings of Goranov et al. [34] whereby immobilized *Lactobacillus rhamnosus* ATCC 11979 cells synthesized significantly higher amounts of lactic acid than free cells because the gel matrix used for encapsulation of the cells protected them from the low pH of the fermentation medium. Thus the cells remained viable and were able to synthesize lactic acid for a longer period.

3.5 Characterization of the EPS Produced by the Strains Using Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR was used to determine the functional groups present in the EPS produced by the isolates. The spectra produced by the EPS of both wild type (Fig. 5a-c) and mutant strains (Fig. 5b-d) showed similarities in having absorption peaks around 3000 cm^{-1}-3400 cm^{-1} which corresponds to the hydroxyl (OH) stretching peak which indicate that the compound is likely to be aromatic or unsaturated. The absorption band at 2828.04 cm^{-1}, 2935.76 cm^{-1}, 2928.04 cm^{-1} and 2929.97 cm^{-1} for WWC, MWC, WLD and MLD respectively which correspond to C-H stretch of the methyl group while the phenyl ring substitution overtones were observed at 2362.88 cm^{-1} (WWC), 2360.95 cm^{-1} (MWC and MLD) and 2364.81 (WLD). The stretching of C=O group was indicated by the absorption band

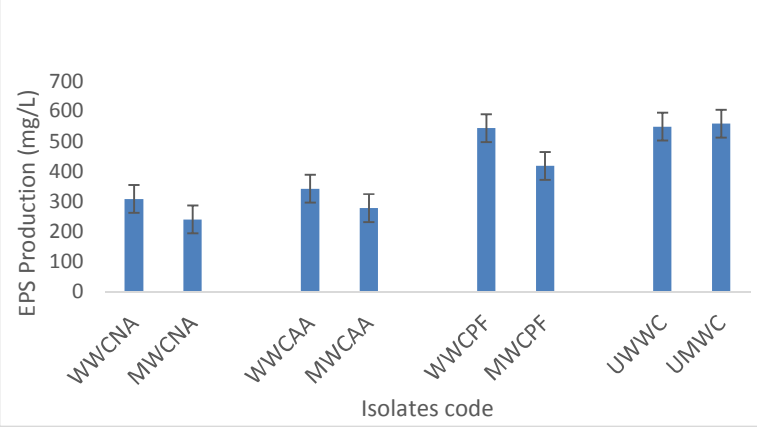

Fig. 3. Production of EPS by the wild, mutant, immobilized and un-immobilized strain of *Weissella confusa*

Key: WWCNA: Wild Weissella confusa immobilized in Sodium alginate
MWCNA: Mutant Weissella confusa immobilized in Sodium alginate
WWCAA: Wild Weissella confusa immobilized in agar agar
MWCAA: Mutant Weissella confusa immobilized in agar agar
WWCPF: Wild Weissella confusa immobilized in polyurethane foam
MWCPF: Mutant Weissella confusa immobilized in polyurethane foam
UWWC; Un-immobilized Wild Weissella confusa
UMWC: Un-immobilized Mutant Weissella confusa

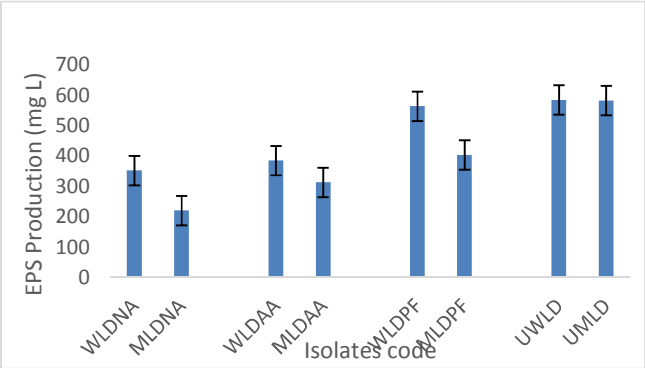

Fig. 4. Production of EPS by the wild, mutant, immobilized and un-immobilized strain of *Lactobacillus delbrueckii*

Key: WLDNA: Wild Lactobacillus delbrueckii immobilized in Sodium alginate
MLDNA: Mutant Lactobacillus delbrueckii immobilized in Sodium alginate
WLDAA: Wild Lactobacillus delbrueckii immobilized in agar agar
MLDAA: Mutant Lactobacillus delbrueckii immobilized in agar agar
WLDPF: Wild Lactobacillus delbrueckii immobilized in polyurethane foam
MLDPF: Mutant Lactobacillus delbrueckii immobilized in polyurethane foam
UWLD; Un-immobilized Wild Lactobacillus delbrueckii
UMLD: Un-immobilized Mutant Lactobacillus delbrueckii

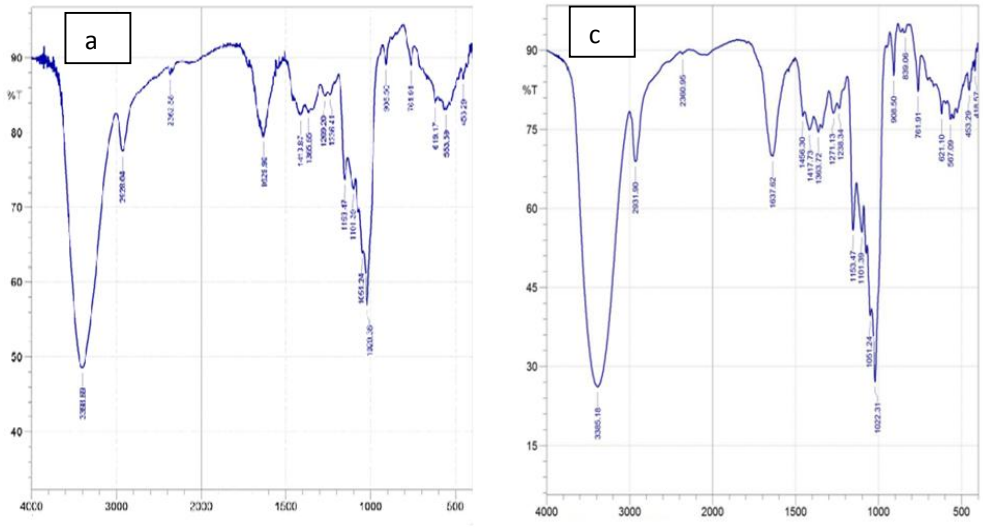

Fig. 5a-c. FTIR Spectrum of EPS from (a) Wild *Weissella confusa* and (b) Mutant *Weissella confusa*

at 1629.9 cm^{-1}, 1639.55 cm^{-1} (MWC), 1637.62 cm^{-1} (WLD) and 1641.48 cm^{-1} (MWD) indicates an aromatic ring stretch. The peaks at 1413.87 cm^{-1} – 1365.65 cm^{-1} could be assigned to >C=O stretch of the COO^- groups and C-O bond from COO^- groups. C-H and O-H deformation was observed at 1363.72 cm^{-1}, an aromatic secondary amine CN stretch occurred at 1269.2 cm^{-1} and the peak between 1269.20 and 1236.41 cm^{-1} was the organic phosphates (P=O) stretch. The 1051.24 cm^{-1} – 1101.39 cm^{-1} stretch represents C-O-C vibrations in the glucopyranose ring. A sharp absorption band at 1020.38 cm^{-1} represents C-O and CC stretching. The band at 908.50 cm^{-1} is associated with the aromatic phosphate (P-O-C) stretch. The peaks appearing between 761.91 and 453.29 cm^{-1} represent alcohol (OH) bends. EPS produced by both mutants and wild strains differs by having one peak less in the region of 1413.87 cm^{-1}– 1236.41 cm^{-1} and 908.50 cm^{-1} - 399.28 cm^{-1} which implies that there is lesser OH bonding in

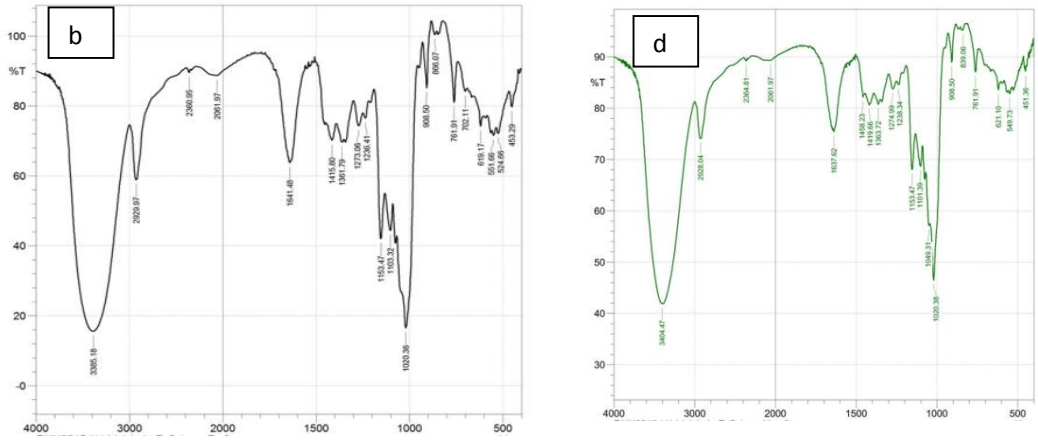

Fig. 5b-d. FTIR spectrum of EPS from (c) Wild *Lactobacillus delbruekii* and Mutant *Lactobacillus delbrueckii*

the mutants' EPS as these two regions are characteristic of alcohol bends. The presence of functional groups and bands in the characterization of EPS produced by wild and mutant *Weissella confusa* and *Lactobacillus delbrueckii* using Fourier Transform Infrared Spectroscopy (FT-IR) revealed the presence of the hydroxyl, methyl and methylene groups as well as stretches of C-C, C-O-C and C-O of alcohol groups in carbohydrates.

This is in line with the work of Wang et al. [35] who reported that FTIR has been a potent and very useful tool for observing structural and functional groups changes in EPS. Absorption of around 3250–3440 cm^{-1} revealed that EPS contains rounded trait typical of hydroxyl groups which propose that the substance is polysaccharide [36].

This work is also in line with the work of Wang et al. [37] who observed that the bands in the region of 1,641.48cm^{-1} were assigned to the stretching vibration of the carboxyl group (C=O) and due to the associated water which indicates the presence of organic substances such as the ring stretching of mannose or galactose . It is also similar to the work of Zhang et al. [38] and Abdhul et al. [39] in which it was observed that the relatively weak absorption peak at 1, 548.15 cm^{-1} might be ascribed to the N-H while the absorption peak at 1385.02 cm−1 was possibly due to the symmetric stretching of the COO− group.

4. CONCLUSION

In conclusion, mutation and immobilization has profound effect on EPS production by *Weissella confusa* and *Lactobacillus delbrueckii*. Immobilization using Polyurethane foam as matrix supported high EPS production than immobilization using agar-agar and sodium alginate as matrixes. Characterization of the EPS using FT-IR analysis showed the presence of functional groups which confirmed that the EPS is polysaccharides.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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