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A Biosensing Technique through a Coadhesion Study between *Sacchromyces cerevisiae* and *Lactobacillus plantarum*

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ABSTRACT

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Primary and secondary microbial adhesion onto solid surfaces has been the onset of development of a mature biofilm in aqueous environments that is predominantly the mode of bacterial contamination and spread of diseases. Adhesion and co-adhesion assays are therefore useful in understanding the adhesive interactions between microorganism and its surfaces. Various factors influence cell adhesion and biofilm formation, depending on aqueous medium and the type of microorganism in place. Similarly, factors such as ionic strength, pH and temperature are vital factors that influence cell growth and surface attachment. Different methods are available for testing adhesion and coadhesion assays such as macroscopic methods, microscopic methods, steady state and kinetic turbidometric methods, mathematical methods and slide based. However, out of these, parallel plate flow chambers (PPFC) are reportedly convenient and easy to use.

Introduction

Earlier studies on microbial adhesion were aimed at understanding the adhesion phenomenon of microbial cells onto solid surfaces such as a glass slide. Cell adhesion phenomenon is influenced by hydrodynamics and is also dependent on the sheer strength of the cells to withstand high fluid shear force i.e. cell retention to surfaces (Busscher *et al.*, 2001). Hydrodynamic shear assay techniques are used to investigate the adhesion phenomenon of cells on solid surfaces that react invariably different under the influence

of turbulent or laminar flows (Bakker *et al.*, 2002). From a clinical context, microbial adhesion on surgical instruments and implants becomes a menace in hospitals and microbiological laboratories where aseptic conditions are mandatory (Vo-Dinh and Cullum, 2000). Such microbial presence may aid in the transmission of pathogens (Katsikogianni and Missirlis, 2004) and distribution of harmful bacteria. Coadhesion is a phenomenon where two different microorganism pair up and one aids in the adhesion and attachment of the other microorganism. An adhesive interaction

between yeast and bacterial strain is one such combination for studying coadhesion behaviour. Flow chamber experiments (Sharma *et al.*, 2005) that provide information about microbial surface behaviour have been a vital source of information. This has been useful in the field of medicine, where knowledge of microbial adhesion aids in limiting pathogenic infections (Dunne 2002).

Busscher *et al* (1997), studied the adhesive interaction between yeast and bacteria on silicone rubber within a PPFC their study investigated coadhesion and interaction of different bacterial strains with yeast (Busscher and Mei, 1997). The study showed that mostly bacterial adhesion was not favouring coadhesion with yeasts. However, a few strains stimulated adhesion of yeasts when a suitable medium of interaction was in place such as change in ionic strength or change in pH of solution. This was observed in this research work too which will be discussed later in the results.

Materials and Methods

Cell culture

(1) *S.cerevisiae* WT Flo11, *S.cerevisiae* SSN6, and *L.plantarum* ATCC 11974: were cultured using a MYGP medium which contained 3g/l of Yeast extract (Sigma-Aldrich, UK), 5g/l of Mycological Peptone (Lab M, International diagnostic group plc idg), 3g/l of Malt extract (Lab M, International diagnostic group plc idg), 10g/l of Glucose (Sigma-Aldrich, UK) for liquid broth and for solid media 20g/l of agar (London Analytical and Bacterial Media Ltd., UK) was added with the MYGP medium (I. Campbell and J. H. Duffus, 1988).

Flow setup

A rectangular parallel plate flow chamber (Figure 1) was fabricated in-house and flow

cell experiments were carried out with suitable hydrodynamic conditions (Busscher and Van Der Mei 2006). Flow of cell suspension into the rectangular PPFC was regulated using a peristaltic pump (Ismatec, Germany) at different flow rates, 0.05, 0.5, 1, 2, 3, 5, 8, 11, 13, 15 and 18 ml/min respectively.

The flow rate was regulated using an external peristaltic pump (Ismatec, Germany) at a rate of 0.1-30 ml/min through a tubing (Ismaprene tubes) with a diameter of 2.06 mm (inner diameter). The suspended cells in the buffer solution were carried into the flow chamber through the inlet and outlet tubings connected to the PPFC, a real time monitoring system was created by mounting an inverted microscope (Leitz Wetzlar, Germany) on top of the flow chamber. A charge coupled device (ccd) camera was attached to the microscope that captured images with a 10x objective over an area of 0.43 x 0.58 mm, which were then recorded and processed using a image software (Pinnacle studio) in a computer.

Hydrodynamic shear assay

Yeast cells were suspended (70×10^6 cells/ml) in the buffer solution in the flask, using a peristaltic pump, cell suspension was allowed to flow to the flow chamber. *L.plantarum* cells were used (1.3×10^8 cells/ml) along with the yeast strain types.

The flow rate was controlled using the peristaltic pump and it was turned on/off using a switch on the pump. The real time images of the PPFC were recorded using the camera as mentioned in the previous section in the flow set up. All the images were recorded and processed using Image J software for data analysis. Cell suspension from outlet of PPFC was collected in a measuring jar which was used to measure the rise of fluid with increase in fluid flow rate.

Coadhesion study with *L.plantarum* and yeast cells using "In liquid behaviour" method

The experiments were repeated similarly as above but here combination of two cells was used. Firstly *L.plantarum* ATCC 11974 and *S.cerevisiae* WT Flo11 was used as combination I and secondly *L.plantarum* ATCC 11974 and *S.cerevisiae* SSN6 was used as combination II for cell suspension in buffer at different pH values of 5, 7 and 9 at 0.1M NaCl buffer solution. Similarly, as above procedure, cell solution was allowed to spread in flow chamber PPFC and later flow rate was increased gradually to increase fluid shear (flow rates, 0.05, 0.5, 1, 2, 3, 5, 8, 11, 13, 15 and 18 ml/min respectively). This was repeated with both combinations of cells. After completing the experiments glass slides were removed from the PPFC and kept in petri dishes for Cryo SEM imaging.

Coadhesion study with *L.plantarum* and yeast cells using "On surface behaviour" method

A novel style of experimental procedure was attempted for understanding surface behaviour of bacteria and yeast cells in flow chamber. Initially, *L.plantarum* ATCC 11974 was soaked on a glass slide surface for 1 hour with high concentration (1.3×10^8 cells/ml) of cells. After soaking for 1 hour *L.plantarum* ATCC 11974 in glass slide was placed in the flow chamber (PPFC).

Initially yeast *S.cerevisiae* WT Flo11 (combination I: *L.plantarum* + *S.cerevisiae* WT Flo11) was suspended in NaCl solution at 0.1M concentration at pH 5 was allowed to flow inside the PPFC with flow rate of 2.0 ml/min and cells were allowed to adhere to the glass surface of the chamber for 2 minutes. Later flow rate was increased to the following flow rates 1.5, 7.5, 18 and 30 ml/min

respectively, with the time interval of 2 minutes each. Similarly, the same procedures were repeated with pH.7 and 9 for the cell suspension combination I (*L.plantarum* + *S.cerevisiae* WT Flo11). Exactly same procedure was repeated for the cell suspension, combination II (*L.plantarum* + *S.cerevisiae* SSN6). After completing each experiment, glass slides were retrieved from the PPFC and kept on petri dishes for Cryo SEM imaging. At the end of each reading cells were counted and images were taken for the above set of experiments. The recorded images were used for plotting graph and analysing results.

Shear equation

Shear rate (s^{-1}) of the microorganism adhering to the surface of substrate within the PPFC was calculated. Increase in fluid flow in the chamber, increased the shear rate, for a laminar flow profile the shear force acts parallel to the surface of the PPFC and depends on the viscosity of the liquid medium. Wall shear rate and Reynolds number (Re) calculations for rectangular PPFC were done using the following equations, here σ is wall shear rate in (s^{-1}), Q is volumetric flow rate in ($m^3.s^{-1}$), and ρ is fluid density in ($Kg.m^{-3}$), w_o and h_o is the width and height of the PPFC in (m) and η is absolute viscosity in ($Kg.m^{-1}.s^{-1}$) using the following equations (Busscher and Van Der Mei, 2006):

$$\sigma = \frac{3 * Q}{2 * (\frac{h_o}{2})^2 * w_o} \quad \dots (1)$$

$$Re = \frac{\rho * Q}{(w_o + h_o) * \eta} \quad \dots (2)$$

Results and Discussion

L.plantarum was used along with the two yeast cell types for studying coadhesion phenomenon. From the coadhesion study, it

was observed that, *L.plantarum* that was loosely adhering to glass surface due to the effect of shear force (Sharma *et al.*, 2005), was able to adhere well in combination with yeast cells. The experiments were conducted with 0.1M NaCl buffer solution with pH 5, 7 and 9. Both the yeast cells were able to co-adhere with the bacteria and were able to stick to the surface against high fluid shear. All experiments were repeated for 3 times and the average values of the 3 repeats were used to determine the coadhesion of bacteria and yeast cells.

In this research, it was found that combination I: *L.plantarum* + *S.cerevisiae* WT Flo11 showed better coadhesion in comparison with combination II: *L.plantarum* + *S.cerevisiae* SSN6. The coadhesion data results for the total number of cells attached during the coadhesion process on the glass surface are as shown in table 1 and 2.

From table 1 of coadhesion results, we can see that *L.plantarum* had 140 cells/mm² and *S.cerevisiae* WT Flo11 had 118 cells/mm² adhered on the glass surface with cell buffer at pH5 achieved at the lowest flow rate of 0.05 ml/min. For a similar flow rate and at pH7 *L.plantarum* had 94 cells/mm² and *S.cerevisiae* WT Flo11 had 88 cells/mm²; at pH9 *L.plantarum* had 79 cells/mm² and *S.cerevisiae* WT Flo11 had 84 cells/mm². However, at the highest flow rate of 18ml/min the cell adhesion greatly reduced and from the table 1 we find that *L.plantarum* were 23 cells/mm² and *S.cerevisiae* WT Flo11 were 51 cells/mm²; at pH7 *L.plantarum* were 28 cells/mm² and *S.cerevisiae* WT Flo11 were 35 cells/mm² and at pH9, *L.plantarum* were 30 cells/mm² and *S.cerevisiae* WT Flo11 were 23 cells/mm². This meant that increase in flow rate greatly reduced the bacterial adhesion as compared to the yeast adhesion.

From table 2 of coadhesion results from combination II of *L.plantarum* + *S.cerevisiae*

SSN6 shows a comparison with combination I results of table 1. Here, at pH5 the total cells adhered on the glass slide for *L.plantarum* were 138 cells/mm² and for *S.cerevisiae* SSN6 were 83 cells/mm² at the lowest flow rate of 0.05 ml/min. At a similar flow rate, at pH7, *L.plantarum* were 86 cells/mm² and *S.cerevisiae* SSN6 were 73 cells/mm²; at pH9, *L.plantarum* were 78 cells/mm² and *S.cerevisiae* SSN6 were 75 cells/mm². At the highest flow rate of 18ml/min, the number of cells significantly reduced, at pH5 there were 37 cells/mm² for *L.plantarum* and 48 cells/mm² for *S.cerevisiae* SSN6; at pH7, there were 34 cells/mm² for *L.plantarum* and 35 cells/mm² for *S.cerevisiae* SSN6 and at pH9, there were 33 cells/mm² for *L.plantarum* and 25 cells/mm² for *S.cerevisiae* SSN6.

The results from table 1 and 2 showed similar coadhesion data in terms of adhesion for *L.plantarum* cells but comparing the two yeast types we find that *S.cerevisiae* WT Flo11 had a better surface adhesion to glass than *S.cerevisiae* SSN6 at pH5 (from low to high flow rate). However, the coadhesion results for *S.cerevisiae* WT Flo11 and *S.cerevisiae* SSN6 at pH7 and pH9 were quite similar (from low to high flow rate). This lead to the optimization of the experiment using the on surface behaviour methods as described in methods section and the flow rates were changed to 1.5, 7.5, 18 and 30ml/min respectively. Figure 2 shows the cell attachment between combination I: *L.plantarum* + *S.cerevisiae* WT Flo11 and combination II: *L.plantarum* + *S.cerevisiae* SSN6 for the "in liquid" behavior of *L.plantarum* with yeast cells. Table 3 and 4 shows the coadhesion results for the on surface behaviour method for combination I and combination II of cells. Coadhesion experiments with "on surface behavior" produced results that were similar in comparison to that of "in liquid behavior" method experiments; at pH5, *L.plantarum* had 384 cells/mm² and WT Flo11 had 191

cells/mm² (Table 3) at the lowest flow rate of 1.5ml/min; whereas for similar conditions *L.plantarum* and *S.cerevisiae* SSN6, resulted in 382 cells/mm² and 164 cells/mm² (table 4) at the lowest flow rate of 1.5ml/min. This behavior was observed at the highest flow rate of 30ml/min as well for both combinations I and combination II of cells (table 3-4) and was similar for both the pH7 and pH9. In both type of experiments (table 1-4) it was clear that *S.cerevisiae* WT Flo11 adhered more in numbers with *L.plantarum* than *S.cerevisiae* SSN6. This is an important result suggesting that *S.cerevisiae* Flo11 shows selective adhesion to the glass surface (Guillemot *et al.*, 2006) when compared to the *S.cerevisiae* SSN6 strain. For the first time, *S.cerevisiae* WT Flo11 and *L.plantarum* were investigated for coadhesion study so a suitable comparison has been made with previous research works based on similar cell characteristics. Figure 3 shows the cell attachment between combination I: *L.plantarum* + *S.cerevisiae* WT Flo11 and combination II: *L.plantarum* + *S.cerevisiae* SSN6 for the on surface behavior of *L.plantarum* with yeast cells.

The influence of pH (Mozes *et al.*, 1987) significantly contributed towards the coadhesion of *L.plantarum* ATCC 11974 and *S.cerevisiae* yeasts, where pH5 was most suitable. Further, it was concluded that van der Waals interaction described by DLVO and the interaction between the outer cell surface macromolecules and the sample substrate, were important factors that described the microbial adhesion with respect to ionic strength and pH (Skvarla, 1993, Bos *et al.*, 1999, Rijnaarts *et al.*, 1999). For the same reason 0.1 M NaCl at pH5 were found appropriate for coadhesion of *L.plantarum* ATCC 11974 with *S.cerevisiae* yeast strains SSN6/ WT Flo11 showing similar response for ‘‘in liquid’’ and ‘‘on surface’’ methods.

Millsap *et al* (2000) developed a dot assay technique for determining the adhesive

interactions between yeast and bacteria under controlled hydrodynamic conditions using a parallel plate flow chamber. Four different bacterial strains (*Streptococcus gordonii* NCTC 7869, *Streptococcus sanguis* PK 1889, *Actinomyces naeslundii* T14V-J1 and *Staphylococcus aureus* GB 2/1) at two different concentrations were used along with *Candida albicans* ATCC 10261. Polymethylmethacrylate (PMMA) was used in the PPFC as a substratum surface and the microorganism were suspended in a TNMC buffer (In one liter: 1 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM MgCl₂, 1 mM CaCl₂). It was found that on an acrylic surface, the presence of adhering bacteria suppressed adhesion of *C.albicans* ATCC 10261 to various degrees, depending on the bacterial strain involved. Suppression of *C.albicans* ATCC 10261 adhesion was strongest by *A.naeslundii* T14V-J1, while adhering *S.gordonii* NCTC 7869 caused the weakest suppression of yeast adhesion.

When adhering yeasts and bacteria were challenged with the high detachment force of a passing liquid-air interface, the majority of the yeasts detached, while *C.albicans* adhering on the control on the bare PMMA surface formed aggregates. It suggested that the differences in suppression of *C.albicans* ATCC 10261 adhesion shown by the bacterial strains did not appear to be dependent on bacterial size or percentage surface coverage. The largest bacterium, *A. naeslundii* T14V-J1, caused the highest bacterial surface coverage and was responsible for the strongest suppression of *C.albicans* ATCC 10261 adhesion to the PMMA surface in the dot assay. However, the bacterial surface coverages for both *S. gordonii* NCTC 7869 and *S. aureus* GB 2/1 at 1x10⁹ bacteria /ml were comparable to that of *A. naeslundii* T14V-J1 at 3x10⁸ bacteria / ml, and both bacterial strains caused a far weaker suppression of yeast adhesion than *A. naeslundii* T14V-J1. However the yeast strains

and bacteria used in this research work were different but as the above mentioned fact it observed the same that when adhering yeast and bacteria were challenged with high shear force majority of the yeast detached compared to bacteria.

Millsap et al (1998), conducted a study on various methods of adhesive interactions between bacterial strains and yeast which ranged from simple macroscopic methods to flow chamber experiments. One of the samples study with *C.albicans* ATCC 10261 suspended in TNMC buffer in a parallel plate flow chamber onto glass with adhering *S.gordonii* NCTC 7869 showed that the presence of adhering bacteria influences the adhesion of yeast which is in comparison with this research work on *L.plantarum* ATCC 11974 and *S.cerevisiae* yeast strains WT Flo11/ SSN6.

Tallon *et al.*, (2007) studied the agglutination test between yeast and *L.plantarum* which showed coadhesion behavior between yeast and *L.plantarum* . It observed that Mannose-containing polysaccharides (mannans) are major constituents of the cell wall of baker’s

yeast, *S.cerevisiae*. Suggested that some micro-organisms carry adhesins specific for mannose-containing receptors and, therefore, are able to agglutinate yeast cells in a mannose sensitive manner. It was found that *L.plantarum* strains 299V, CBE and Lp80 showed the highest titres of agglutination (32 for strain 299v and 16 for CBE and Lp80), while six other strains (529, 67G-1, BMCM12, IMG9205, T25 and CBFM19) agglutinated *S.cerevisiae* at lower titres (eight and two). The rest of the strains were not able to agglutinate yeast cells.

As was reported by (Adlerberth *et al.*, 1996), *L.plantarum* 299v exhibited great agglutination ability in agreement with the mannose-specific adherence mechanism of these bacteria to human colonic cell line HT-29. Methyl- α -D-mannoside greatly inhibited agglutination of yeast by all strains tested. This confirmed a mannose sensitive agglutination mechanism of *S. cerevisiae* by *L.plantarum* strains. Similar results were observed in this research where *L.plantarum* ATCC 11974 co-adhered well with *S.cerevisiae* WT Flo11 than *S.cerevisiae* SSN6 (Table 1-4).

Table.1 Coadhesion results for *S.cerevisiae* WT Flo11 and *L.plantarum* ATCC 11974 at 0.1M NaCl solution at different pH values (In Liquid behaviour)

Wall Shear rate σ (s ⁻¹)	Flow rate Q (m ³ .s ⁻¹)	Average Cell Count/mm ²					
		pH5		pH7		pH9	
		WT Flo11	<i>L.plantarum</i> ATCC 11974	WT Flo11	<i>L.plantarum</i> ATCC 11974	WT Flo11	<i>L.plantarum</i> ATCC 11974
0.008	8.33E-10	118	140	88	94	84	79
0.04	4.17E-09	115	137	87	91	80	76
0.16	1.67E-08	110	134	85	87	77	71
0.32	3.33E-08	101	128	82	82	71	68
0.48	5E-08	95	111	78	76	68	64
0.88	9.17E-08	89	105	74	70	63	61
1.28	1.33E-07	84	96	68	62	56	59
1.68	1.75E-07	80	77	62	51	49	55
2	2.08E-07	75	65	56	46	41	51
2.4	2.5E-07	70	41	48	38	34	45
2.88	3E-07	51	23	35	28	25	30

Table.2 Coadhesion results for *S.cerevisiae* SSN6 and *L.plantarum* ATCC 11974 at 0.1M NaCl solution at different pH values (in Liquid behaviour)

Wall Shear rate σ (s ⁻¹)	Flow rate Q (m ³ .s ⁻¹)	Average Cell Count/mm ²					
		pH5		pH7		pH9	
		SSN 6	<i>L.plantarum</i> ATCC 11974	SSN6	<i>L.plantarum</i> ATCC 11974	SSN6	<i>L.plantarum</i> ATCC 11974
0.008	8.33E-10	83	138	73	86	75	78
0.04	4.17E-09	81	134	71	85	73	76
0.16	1.67E-08	78	124	69	82	70	73
0.32	3.33E-08	76	114	65	78	67	70
0.48	5E-08	72	106	62	72	65	68
0.88	9.17E-08	68	94	59	66	62	64
1.28	1.33E-07	65	81	56	60	58	60
1.68	1.75E-07	62	77	53	57	54	54
2	2.08E-07	59	63	49	51	45	50
2.4	2.5E-07	57	49	44	46	38	42
2.88	3E-07	48	37	35	34	25	33

Table.3 Coadhesion results for *S.cerevisiae* WT Flo11 and *L.plantarum* ATCC 11974 at 0.1M NaCl solution at different pH values (on Surface behaviour)

Wall Shear rate σ (s ⁻¹)	Flow rate Q (m ³ .s ⁻¹)	Average Cell Count/mm ²					
		pH5		pH7		pH9	
		WT Flo11	<i>L.plantarum</i> ATCC 11974	WT Flo11	<i>L.plantarum</i> ATCC 11974	WT Flo11	<i>L.plantarum</i> ATCC 11974
0.00024	2.5E-08	191	384	171	254	152	237
0.0012	1.25E-07	124	344	108	210	86	198
0.0028	2.92E-07	77	236	78	171	54	155
0.0048	5E-07	28	166	25	133	23	110

Table.4 Coadhesion results for *S.cerevisiae* SSN6 and *L.plantarum* ATCC 11974 at 0.1M NaCl solution at different pH values (on Surface behaviour)

Wall Shear rate σ (s ⁻¹)	Flow rate Q (m ³ .s ⁻¹)	Average Cell Count/mm ²					
		pH5		pH7		pH9	
		SSN6	<i>L.plantarum</i> ATCC 11974	SSN6	<i>L.plantarum</i> ATCC 11974	SSN6	<i>L.plantarum</i> ATCC 11974
0.00024	2.5E-08	164	382	154	259	122	205
0.0012	1.25E-07	107	341	113	204	94	165
0.0028	2.92E-07	61	236	67	159	70	116
0.0048	5E-07	32	168	28	111	42	85

Figure.1 Schematic of the flow chamber with dimensions of the glass slide along with the inlet/outlet diameter; A is top view, B is side view and C is front view

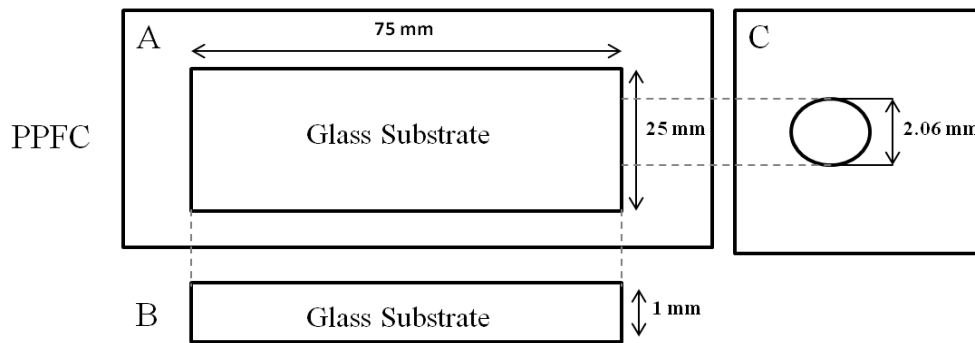


Figure.2 SEM image of *S.cerevisiae* WT Flo11 and *L.plantarum* ATCC 11974 (left); and *S.cerevisiae* SSN6 and *L.plantarum* ATCC 11974 (right), in 0.1M NaCl buffer at pH5 (in liquid behaviour)

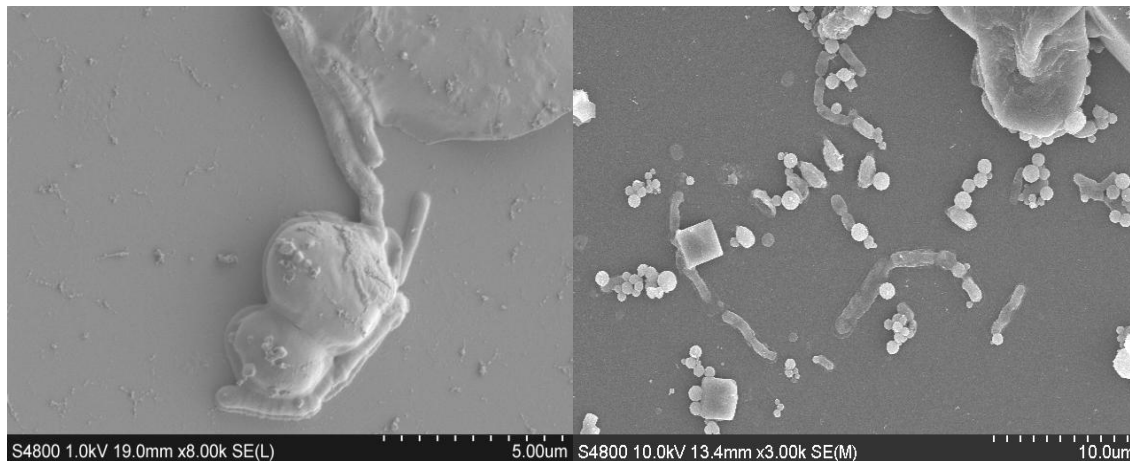
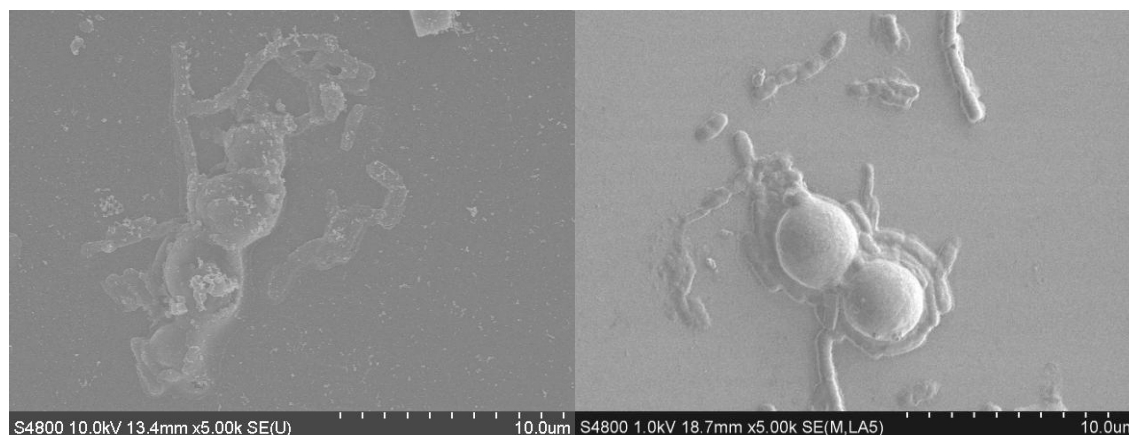


Figure.3 SEM image of *S.cerevisiae* WT Flo11 and *L.plantarum* ATCC 11974 (left); and *S.cerevisiae* SSN6 and *L.plantarum* ATCC 11974 (right), in 0.1M NaCl buffer at pH5 (on surface behaviour)



Microbial adhesion studies are important to understand cell-cell interaction and cell-substratum behaviour, which help in medical applications. Likewise, knowledge of coadhesion behaviour of bacteria in conjunction with yeasts will contribute in developing biosensing models for inhibition and spread of bacterial contamination (Tiago *et al.*, 2018). This research has significantly contributed through coadhesion studies to discern about the cell interaction and behaviour in parallel with another microorganism of a different species. This work is an initiative towards the development of a novel design for biosensor as microorganisms have been part of the biosensing element in the biosensors (Chang *et al.*, 2017) and *S.cerevisiae* and *L.plantarum* have been used initially as sensing elements in biosensors. Previous studies on *S.cerevisiae* and *L.plantarum* provided the basis for this research study and for the first time, *S.cerevisiae* SSN6 and *S.cerevisiae* WT Flo11 were used in combination with *L.plantarum* ATCC 11974 to study their cellular interaction and surface behaviour with glass substrate. This research, therefore, successfully provided experimentation techniques for flow chamber (PPFC) assay and enhanced microscopy techniques (SEM)

for qualitative and quantitative analysis that determined the adhesion and coadhesion factor and showed that pH5 and 0.1M NaCl salt concentration buffer was best suited for microbial adhesion and coadhesion on the glass surface.

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