

Effect of Environmental Variables on Biofilm Formation by Selected Gram-Positive Bacteria

Olubusola A. Odeniyi¹, Olumayowa T. Amoo²

¹Department of Microbiology, University of Ibadan, Ibadan, Nigeria

²Department of Biological Sciences, Wesley University of Science and Technology, Ondo, Nigeria
busolaodeniya@gmail.com, deemayolte@yahoo.co.uk

Abstract: Biofilm formation occurs in many environments where they exert mostly negative effects and examples include plaque formation in teeth, infection of implants. The research compared the biofilm-formation potentials of some Gram-positive bacteria under different environmental conditions. The effect of varied temperature, pH, ultraviolet (UV) light, incubation periods and nutrient composition on the biofilm-forming abilities of vegetative cells of selected Gram-positive bacteria on polystyrene surface was investigated. Supplementation of basal medium with glycerol enhanced biofilm formation in *Bacillus amyloliquefaciens* I58 and *Streptococcus salivarius* C46, recording increases as high as 98.6% and 16.7% respectively, while it reduced biofilm formation in *Bacillus pumilus* I30. Sucrose stimulated biofilm formation in both *B. amyloliquefaciens* I58 and *S. salivarius* C46 while *B. pumilus* I30 showed no increase in their capacity to produce biofilm. *S. salivarius* C46 and *B. pumilus* I30 both produced biofilm with the highest intensities at pH 5, while *B. amyloliquefaciens* I58 formed biofilm with the highest density at pH 6. Increasing concentration of sodium chloride (1 and 2%) added to the basal medium facilitated an increase in the intensity of the biofilm formed by *B. amyloliquefaciens* I58 representing 12.1% and 12.6% increase when compared to the control. Weaker biofilms were formed in *B. pumilus* I30 and *S. salivarius* C46. Ultraviolet (UV) light caused a reduction in the intensity of the biofilm produced by all the isolates, 25.1%, 62.2%, and 75.8% respectively in *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46, boiling the cells resulted in more pronounced reduction in the intensity of the biofilm produced, 29.9%, 89.8%, and 85.9% respectively. Dual species mixture of *B. amyloliquefaciens* I58 with either *B. pumilus* I30 or *S. salivarius* C46 formed stronger biofilms compared to when it was cultivated singly.

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1. Introduction

Biofilms are microbial cells which exist as strongly associated complex communities of single or different bacterial species in a growth and survival polysaccharide supporting matrix. They are found adherent to various surfaces at their solid/liquid interface (Flemming, 1998; Smith, 2005). Examples of biofilm supporting materials include plastics, metals, soil particles, implants (Chmielewski and Frank, 2003), living tissues, piping systems, natural aquatic systems and many others (Giaouris and Nychas, 2006). The characteristics exhibited by microorganisms in biofilms are peculiarly distinct from that of their same species counterparts existing in the suspended or planktonic form. The biofilm matrix is composed of microbial cells, water, organic and inorganic matter such as DNA, nucleic acids, lipids, uronic acids, proteins and polysaccharides (Cheng *et al.*, 2007). The presence of these exopolysaccharides (EPS) protect the microbial community, ensure the structural integrity of the matrix (Leroy *et al.*, 2008), and intercellular communication (Zhang and Fang, 2001; Branda *et al.*, 2005). Biofilm formation starts with the initial

contact between the supporting surface and the microbial cells (Prakash *et al.*, 2003). Under unfavourable conditions, unstable, reversible attachments are created, making cells detach from the support (Ghannoum and O'Toole, 2004). The secondary attachment stage wherein cells strongly attach to the surface begins through the aid of specific adhesions if the environment is conducive (Kumar and Prasad, 2006) and increasingly ages after which some cells detach and may colonise new niches. This work investigated the biofilm-forming characteristics of three gram positive bacteria on a plastic support under diverse medium culturing conditions employing variations of temperature, osmophilic and halophilic content in mono or dual cultures.

2. Materials and Methods

2.1. Microbial Culture Collection

Stock cultures of seven different Gram-positive bacteria (three *Streptococcus* species from decayed tooth samples and four *Bacillus* species from fermented vegetable condiments) were collected from the Department of Microbiology, University of

Ibadan, Nigeria and used in this study to compare their biofilm-forming potentials and the contributions of cultural parameters to biofilm strength. Bacterial cell pellets were harvested from twenty four hour old nutrient broth cultures by centrifugation at 10000 rpm for 15 minutes and stored as concentrated suspension in 5ml sterile water at 4°C (Constantin, 2009).

2.2. Screening for biofilm-forming abilities of bacterial cultures

Cell pellets (equivalent to 10^6) were introduced into 20ml basal medium composed of (g/l): peptone - 7, $MgSO_4$ - 2, $CaCl_2$ - 0.05 and incubated for 24 hours at 30°C. Fresh 1ml aliquots of these culture were transferred into 9ml basal medium from which 100µl of each bacterium culture was transferred into sterile 96-well plastic microtitre plates and incubated for 48 hours at 30°C (O'Toole, 2011).

Cell adherence to wells, indicative of the biofilm formed was quantified according to O'Toole, (2011), where cells were stained with 125µl of 1% crystal violet solution and plates were read at 490nm using a Emax ELISA Reader, Molecular Devices, USA. The concentration of crystal violet in each well is proportional to the number of cells in the biofilm. The bacteria which demonstrated the highest absorbances were selected for further analysis.

2.3. Effect of medium enrichment and temperature on biofilm formation

The basal medium was enriched with 0.5% of either sucrose or glycerol and the incubation temperature and period varied to yield six experimental setups. Each of the selected bacteria were subjected to growth in (a) basal medium only; (b) basal medium supplemented with glycerol; (c) basal medium supplemented with sucrose at 30°C or 37°C respectively over 96 hours incubation period. Screw capped tubes containing 9ml of the 6 different setups were inoculated singly with 1ml cell suspensions of selected bacterial strains. Samples, (100µl), from each culture setup were introduced in triplicates into the microtitre plate wells and incubated as described over 4 days. The plates were read at 24 hours intervals to quantify biofilm formation in response to media composition and temperature. The bacteria were also grown in basal medium supplemented with 1- 6% of either NaCl or sucrose (Elhariry, 2008) over 4 days. Biofilm formation under these conditions was quantified (O'Toole, 2011).

2.4. Influence of incubation temperatures and incubation periods on biofilm production

The bacteria were cultured at different incubation temperatures (25°C, 30°C, 35°C, and 37°C) over a period of 4 days (Constantin, 2009; Rode *et al.*, 2007). Biofilm formation was examined

and the adherent bacterial cells inside the microtitre plate were then quantified (O'Toole, 2011).

2.5. Influence of pH on the production of biofilm

Buffered basal medium was adjusted between pH 5.0 and 8.0 and 9ml of each sterile broth was inoculated with 1ml bacterial suspension. From the resulting culture, 100µl was introduced into the wells of 96-well microtitre plates and incubated over a period of 4 days (Rode *et al.*, 2007).

2.6. Exposure of bacterial cells to ultraviolet light and heat treatment

The influence of a 10 minute exposure to ultraviolet radiation (254 nm) and heat treatment (100°C) on bacterial biofilm-forming ability was studied (Parker *et al.*, 2001) at the optimum incubation temperature for each tested bacterium over a period of 4 days.

2.7. Determination of cell adhesion ability of bacterial isolates to incubation surfaces after radiation and heat treatment

The wells of sterile microtitre plate were filled with 230 µl of phosphate buffer (0.1M, pH 6). A quantity of 20µl of each treated cell stock was introduced into the wells. The microtitre plate was incubated for 2 hours at 37°C (Faille *et al.*, 2002). Thereafter the wells were washed with sterile water and with 100 µl of 2% Tween 80. The detached cells were inoculated by pour plating on nutrient agar and colonies counted after 24 hours (Faille *et al.*, 2002).

2.8. Quantification of biofilm formation by bacterial test isolates singly and as dual species mixtures

Bacteria were cultivated singly and as dual species mixtures for 4 days over a temperature range of 25°C, 30°C, 35°C, and 37°C (Constantin, 2009). Biofilm formation was examined at the end of each incubation period.

2.9. Statistical Analysis

All analysis were carried out in triplicate and the results subjected to statistical analysis using Analysis Of Variance (ANOVA) with a 95% confidence level using Microsoft office excel 2007.

3. Results and Discussion

The seven different bacterial isolates, three from oral C46, C34, and C17 and four from fermented condiment sources I58, I30, I57, and I23 were screened for their biofilm-forming capacities, the results of which are shown in Table 1. The adhesive properties determined using the microtitre-plate assay with crystal violet was highest in *B. amyloliquefaciens* I58, *B. pumilus* I30, and *S. salivarius* C46 respectively and these were therefore selected for further studies. The same assay method was used by Pitts *et al.* (2003) to differentiate strains

of *Staphylococcus epidermidis* and *Vibrio* species through their adhesive properties.

Table 1. Screening for biofilm-formation potential in selected bacterial Isolates (OC, oral cavity; FC, fermented condiment)

Source	Code	Name of Organism	Biofilm formation (Absorbance)
OC	C46	<i>Streptococcus salivarius</i>	0.245
OC	C34	<i>Streptococcus pneumoniae</i>	0.196
OC	C17	<i>Streptococcus mutans</i>	0.139
FC	I58	<i>Bacillus amyloliquefaciens</i>	0.290
FC	I30	<i>Bacillus pumilus</i>	0.313
FC	I57	<i>Bacillus subtilis</i>	0.270
FC	I23	<i>Bacillus licheniformis</i>	0.270

In basal medium at 30°C, *B. pumilus* I30 formed biofilm with the highest intensity over the entire incubation period, closely followed by *S. salivarius* C46 whose biofilm production peaked after 48 hours of incubation (Figure 1). While *B. amyloliquefaciens* I58 formed the weakest biofilm under these conditions, it was observed that biofilm formation increased with increasing hours of incubation.

Although, there was a significant difference in the intensity of the biofilm formed by these organisms; incubation periods did not exert a significant difference on biofilm formation by these bacteria. It was observed that when these organisms were cultivated in basal medium at 37°C, there was significant difference in the density of the biofilm formed over the incubation periods. Constantin (2009) also supported this in his report which showed that incubation periods had a pronounced effect on the production of biofilm by some species of *Bacillus*. A temperature of 37°C enhanced the formation of biofilm by both *B. amyloliquefaciens* I58 and *S. salivarius* C46 more than what was observed when they were cultivated at 30°C in basal medium. Elhariry (2008) also reported that both temperatures (30°C and 37°C) supported biofilm production in *Bacillus* species grown in tryptic soy broth.

B. amyloliquefaciens I58 formed biofilm with higher intensity at 30°C than at 37°C in basal medium supplemented with glycerol, specifically after 72 hours of incubation (Figure 2).

B. pumilus I30 also produced a stronger biofilm (0.145) at a temperature of 30°C than what was

observed at 37°C when glycerol was added to the basal medium. In the case of *S. salivarius* C46, better biofilm production was recorded at a temperature of 37°C after a 96 hour incubation period.

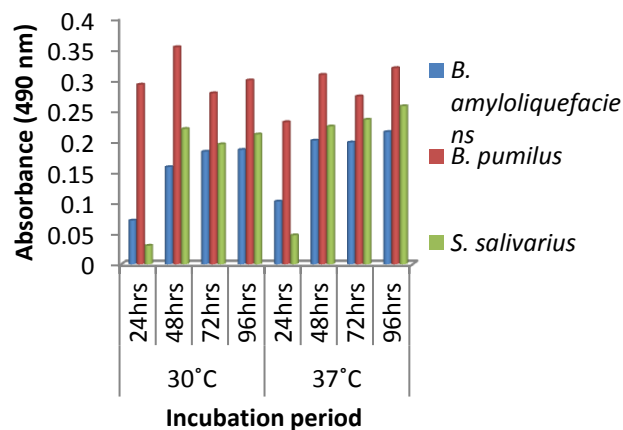


Figure 1. Biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 in basal medium at 30°C and 37°C over 96 hours

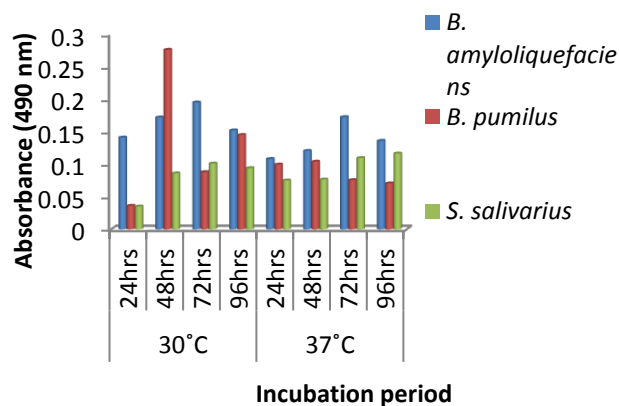


Figure 2. Biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 in glycerol supplemented basal medium at 30°C and 37°C

Glycerol's enhancement of biofilm formation in *B. amyloliquefaciens* I58 at 30°C over the first 72 hours of incubation resulted in percentage increases of 98.6%, 8.9%, and 6.6% respectively. Although, the intensity of the biofilm formed decreased over time until an increase was not recorded at all at 96 hours which showed 18.3% decrease in biofilm formation compared to when only basal medium was used as the growth medium. *S. salivarius* C46 also gave a percentage increase of 16.7% in biofilm production after 24 hours, but there was no increase in biofilm formation from 48 hours to 96 hours. At 30°C, biofilm formation by *B. pumilus* I30 was not stimulated, however percentage decrease in the

strength of the biofilm formed were recorded with the highest value recorded after 24 hours (87.7%). Brugnoli *et al.* (2007); Herrera *et al.* (2007); and Spiers *et al.* (2003) reported the dispersal and detachment of biofilm cells as the biofilm gets older because some bacteria stop producing extracellular polymeric substances (EPS) and get released. Nutrient depletion or nutrient levels has also been implicated in the reduction of biofilm density.

Constantin (2009) reported that the supplementation of basal medium with glycerol at 37°C slightly favoured formation of biofilm by *B. subtilis*, *B. cereus* and *Ps. fluorescens* over a period of 2 and 9 days respectively. Glycerol limited biofilm formation in *B. pumilus* I30 throughout the hours of incubation.

Sucrose elicited an increase in the intensity of the biofilm formed by *B. amyloliquefaciens* I58, over the first 72 hours of incubation which reduced slightly at 96 hours (Figure 3). An increase in biofilm formation by *S. salivarius* C46 was only observed after 24 hours. However, in the case of *B. pumilus* I30 supplementing the basal medium with sucrose culminated in the formation of biofilm with reduced densities over the entire period of incubation.

Sucrose triggered an increase in the strength of the biofilm produced by *B. amyloliquefaciens* I58 at least up to 72 hours of incubation. An increase in the strength of the biofilm formed was also elicited in *S. salivarius* C46 only after 24 hours. The reverse was the case for *B. pumilus* I30 whose capacity for biofilm production reduced after sucrose supplementation. The highest percentage decrease (66.2%) in biofilm production was recorded after 96 hours. This however does not conform to the work of Adetunji and Odetokun (2012) who recorded an increase in the strength of the biofilm formed by another Gram-positive organism, *Listeria monocytogenes*, when cultivated in Tryptic Soy Broth supplemented with 0.5% to 1% glucose. *B. pumilus* I30 produced weaker biofilms and the highest percentage decrease in biofilm formation was 59.3% which was observed after 24 hours.

However, this is in contrast with the work of Hola *et al.* (2006) who established that all the strains of *S. epidermidis* cultivated in Brain Heart Infusion medium supplemented with 4% and 9% glucose showed a better and richer growth of the biofilm layer at a temperature of 37°C in the nutrient-richer environment. Significant differences were recorded in the biofilm produced by *B. amyloliquefaciens* I58 in basal medium, basal medium supplemented with glycerol, and basal medium supplemented with sucrose at 30°C and 37°C over a 96 hour incubation period. Significant differences were also observed in the biofilm formed by *B. pumilus* I30 in basal

medium, basal medium supplemented with glycerol, and basal medium supplemented with sucrose at 30°C and 37°C. This conforms to the work of Hola *et al.* (2006) who reported that there were significant differences in the mass of the biofilm formed by strains of *S. epidermidis* after supplementing the Brain Heart Infusion medium with glucose. Though, the periods of incubation did not significantly affect biofilm formation by *B. pumilus* I30.

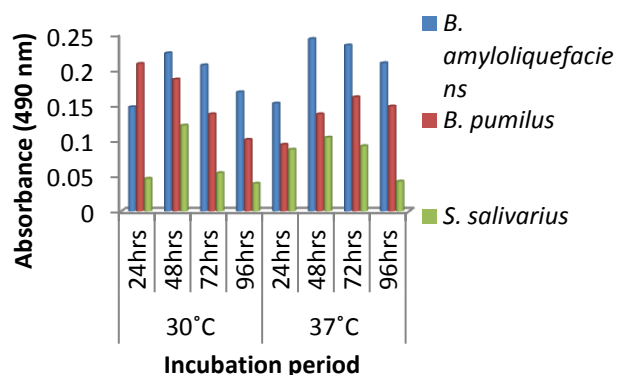


Figure 3. Biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 in sucrose supplemented basal medium at 30°C and 37°C

The pattern of biofilm formation under optimum cultivation conditions is shown in Figure 4. At optimum pH (6.0 and 5.0), a gradual increase was observed in the intensity of the biofilm produced by both *B. amyloliquefaciens* I58 and *S. salivarius* C46 over 96 hours incubation period, but the strength of the biofilm formed by *B. pumilus* I30 peaked after 72 hours and thereafter decreased.

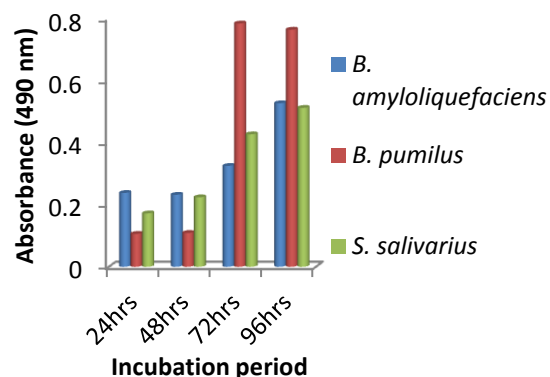


Figure 4. Comparison of biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 under their optimum cultivation conditions and pH

It was observed that increasing additions of sodium chloride concentrations (1-6%) to the basal medium triggered increased biofilm formation in *B. amyloliquefaciens* I58 up to a 3% sodium chloride concentration but further increase in the concentration of sodium chloride from 3% to 6% did not favour biofilm production (Figure 5a).

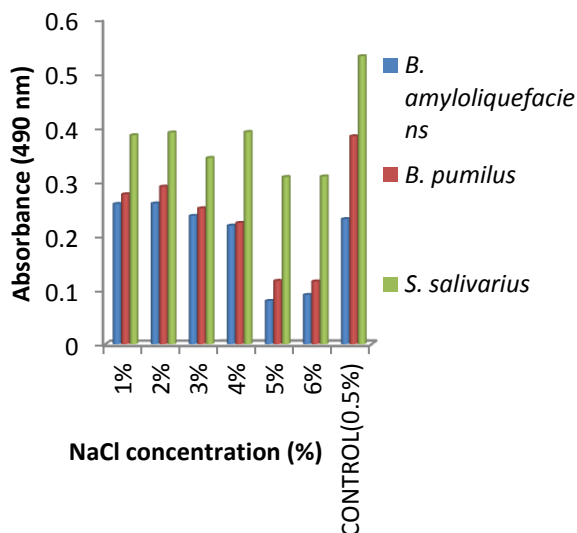


Figure 5a. Effect of increasing NaCl concentration on biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46

Elhariry (2008) recorded enhancement in biofilm formation by *Bacillus* species when the sodium chloride concentration in Tryptic Soy Broth was increased up to 2.5%, however subsequent increases (from 2.5% to 7.5%) resulted in sharp reductions in the strength of the biofilm formed. Pan *et al.* (2000) also recorded significant increases in the density of *Listeria monocytogenes* biofilm formed when cultivated in tryptic soy broth containing 0.6% yeast extract and supplemented with 2% to 5% sodium chloride. However, there was a drastic reduction in the density of the biofilm produced by *B. pumilus* I30 and *S. salivarius* C46, with the highest percentage decrease recorded as 69.8% and 41.9% respectively. Increasing concentrations (2.5%-10% NaCl) in supplemented growth media caused a reduction in biofilm formation by Enterobacteria species (Csonka and Epstein, 1996; Ngwai *et al.*, 2006). This reduction could be as a result of lower water activity (a_w), thereby disrupting normal cellular activities in the growth medium.

Increasing concentration of sucrose used elicited an increase in the density of the biofilm produced by *B. amyloliquefaciens* I58 (Figure 5b) up to 2% sucrose concentration (6.6% and 5.7% increase

compared to the control); while a reduction was observed in the strength of the biofilm formed by *B. pumilus* I30 and *S. salivarius* C46. This is in conformity to the work of Pan *et al.* (2000) who reported that the addition of sucrose stimulated the production of higher-density biofilms in almost all the strains of *L. monocytogenes* tested. Increased glucose concentration in a growth medium improved the growth of *Bacillus* cells in biofilm, demonstrating a link between nutrient availability and biofilm development (Kjelberg *et al.*, 1983).

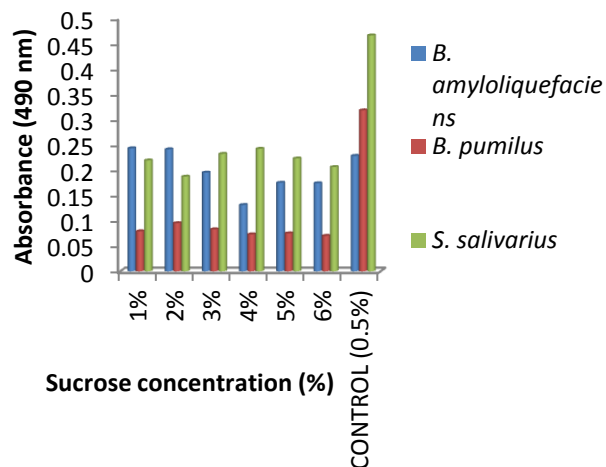


Figure 5b. Effect of varied sucrose concentration on biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46

Exposure of the organisms to ultraviolet light and subjecting them to heat treatment caused a significant reduction in the intensity of the biofilm produced by *B. pumilus* I30 and *S. salivarius* C46, while only a minimal decrease was observed in the strength of the biofilm formed by *B. amyloliquefaciens* I58 (Figure 6a). However, boiling the cells had a much more pronounced effect on the intensity of the biofilm produced when compared to their exposure to ultraviolet (UV) light. This is quite evident in the percentage decrease recorded for biofilm formation: *B. amyloliquefaciens* I58 (boiling: 29.9%, UV: 25.1%), *B. pumilus* I30 (boiling: 89.8%, UV: 62.2%), *S. salivarius* C46 (boiling: 85.9%, UV: 75.8%). Chmielewski and Frank (2003) also reported that heat and ultraviolet light treatments had similar effects in decreasing the intensity of the biofilm formed by some species of *Bacillus*. On the contrary, Elhariry (2008) indicated that ultraviolet light treatment and boiling did not significantly affect biofilm formation in three *Bacillus* species.

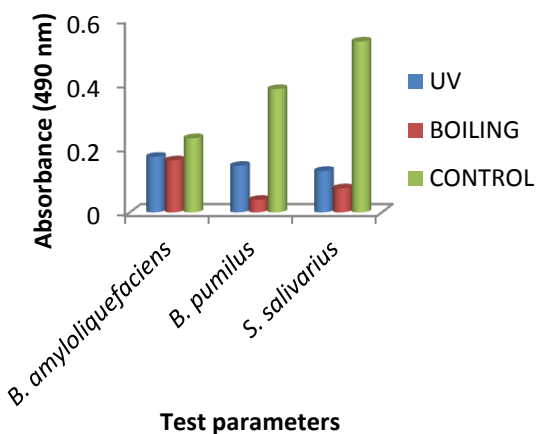


Figure 6a. Effect of boiling and ultraviolet (UV) radiation on biofilm production abilities of *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46

The three isolates were found to possess active attachment characteristics and exhibited more than 64.9% adherence after both boiling and ultraviolet light treatments (Figure 6b). *B. pumilus* I30 and *B. amyloliquefaciens* I58 were least affected by boiling, demonstrating 4.7% reduction in adherence logarithm cfu/ml. Ultraviolet light treatment caused a higher reduction in the amount of adhered cells in both *B. amyloliquefaciens* I58 (29.7%) and *B. pumilus* I30 (35.1%). However, in *S. salivarius* C46, boiling caused more reduction in cell adherence (10.1%) than ultraviolet light treatment (6.24%). Kumar and Anand (1998) reported that attachment of bacterial cells to surfaces is facilitated by cell surface characteristics such as adhesion proteins, surface charge, capsules, pili and flagella. Bower *et al.* (1996) also reported bacterial adhesion to surfaces.

There was significant difference in the density of the biofilm produced by *B. amyloliquefaciens* I58, *S. epidermidis* I30, and *S. salivarius* C46 in basal medium when cultivated as single species (Figure 7a). There was however, no significant difference in biofilm formation across the different incubation temperatures enlisted in the tests.

A stronger biofilm was formed when *B. amyloliquefaciens* I58 was cultivated as a dual species mixture with *B. pumilus* I30 (Figure 7b), compared to the weaker (mono species) biofilm it formed when it was cultivated singly.

Co-cultivation of *B. amyloliquefaciens* I58 with *S. salivarius* C46 also enhanced biofilm formation than when *B. amyloliquefaciens* I58 was cultivated as single species. This agrees with the work of Wen *et al.* (2010) which reported that most dual species bacterial mixtures cultivated formed stronger

biofilms compared to when the organisms were grown singly.

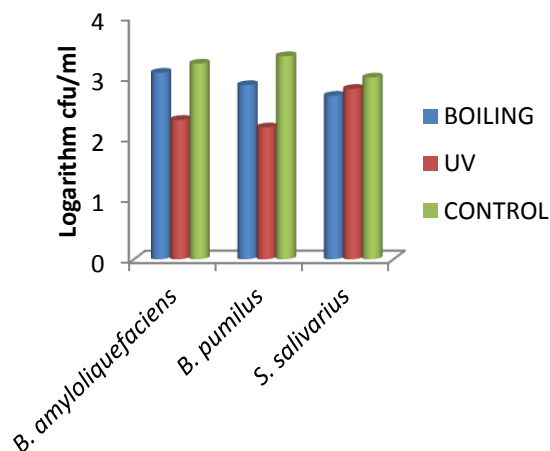


Figure 6b. Adhesion of cells of *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 to incubation surface

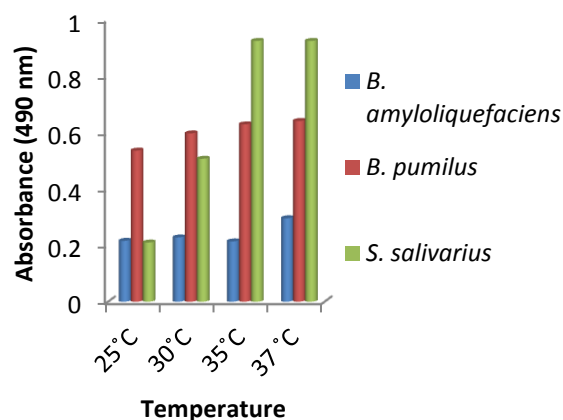


Figure 7a. Biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 cultivated singly in basal medium at different incubation temperatures under optimized parameters

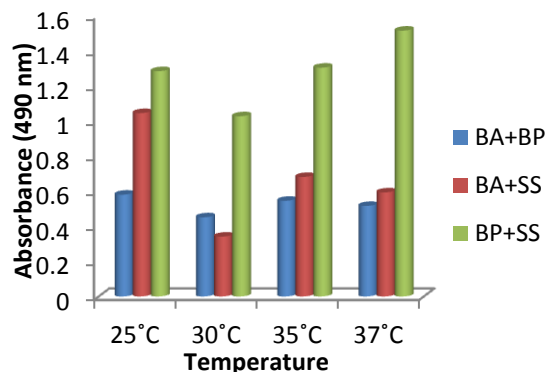


Figure 7b. Biofilm formation by *B. amyloliquefaciens* I58, *B. pumilus* I30 and *S. salivarius* C46 cultivated

in basal medium as dual species mixtures at different incubation temperatures (BA, *B. amyloliquefaciens* I58; BP, *B. pumilus* I30; SS, *S. salivarius* C46)

The increase in the strength of the biofilm formed could be attributed to the synergy created between the organisms when they were cultivated together. Dual species biofilm have a higher metabolic activity than single species biofilms, a phenomenon probably related to the distinct cell densities. In addition, other phenotypic characteristics such as: increased biofilm porosity, growth kinetics and mass transfer efficiency could favour nutrient consumption and increase their metabolic activity (Melo and Vieira, 1999).

B. pumilus I30 only produced a stronger biofilm when it was cultivated as a dual species mixture with *S. salivarius* C46 than when cultivated singly. However, *S. salivarius* C46 produced biofilms of higher intensities when cultivated as dual species mixture with *B. amyloliquefaciens* I58 at 25°C, and also when cultivated with *B. pumilus* I30 as dual species mixture at 25°C and 37°C respectively compared to when it was cultivated as a single specie.

The difference in the thickness of the biofilm formed when *B. amyloliquefaciens* I58, *B. pumilus* I30, and *S. salivarius* C46 were cultivated in basal medium as dual species mixtures was statistically significant, significant difference was also recorded in the biofilm formed over the different ranges of temperature.

A significant difference was observed in the intensity of the biofilm formed by *B. amyloliquefaciens* I58, cultivated singly and as dual species with *B. pumilus* I30 or *S. salivarius* C46. There was also a significant difference in the biofilm produced by *B. pumilus* I30 grown singly and as dual species with *B. amyloliquefaciens* I58 or *S. salivarius* C46. Furthermore, biofilm production by *S. salivarius* C46 cultivated singly and as dual species with *B. amyloliquefaciens* I58 or *B. pumilus* I30 was statistically significant.

4. Conclusion

The Gram-positive bacterial strains; *B. amyloliquefaciens* I58, *B. pumilus* I30, and *S. salivarius* C46 formed strong biofilms when cultivated on plastic microtitre plates and exhibited distinctly different physiological characteristics in biofilm formation. Biofilm formation was however more boosted in *B. amyloliquefaciens* I58. The cells formed stronger biofilms when cultivated as dual species mixtures than when cultivated singly due to the synergistic interactions that were created between them. Variations in incubation periods did not

significantly affect biofilm production. However, temperature, media composition, ultraviolet light, pH of the growth medium, and cultivation of the organisms as dual species all contributed to the quantity of the biofilm formed.

Corresponding Author:

Mr. Olumayowa T. Amoo
Department of Biological Sciences
Wesley University of Science and Technology,
Ondo, Nigeria
Telephone: +234 8030601517
E-mail: deemayolte@yahoo.co.uk

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