CHAPTER 3

METHODOLOGY

3.1 Research Design

This study was conducted in five distinct experimental phases to investigate the impact of various vat polymerization techniques on the physicomechanical and biological properties of the 3D-printed denture base. In the first phase of the experiment, three different vat polymerization techniques, Stereolithography (SLA), Digital Light Processing (DLP), and Liquid Crystal Display (LCD) were used to prepare the 3D-printed denture base specimens. While the printing procedures differed among all groups, the post-processing procedure remained consistent for all specimens to eliminate potential external influences. Then, in the second phase, the mechanical properties of the 3D-printed denture base specimens were assessed, focusing on flexural strength and modulus, fracture toughness, and surface hardness. Following this, the third phase involved an examination of physical properties, encompassing water sorption, solubility, degree of conversion, and surface morphology. Moving forward, the fourth phase delved into the biological properties, including an assessment of fungal adhesion and cell cytotoxicity. Finally, in the fifth and concluding phase, the collected results from the experiments underwent comprehensive data analysis to determine the significance of the findings. The study's design and progression are succinctly summarized and illustrated in (Figure 3.1).

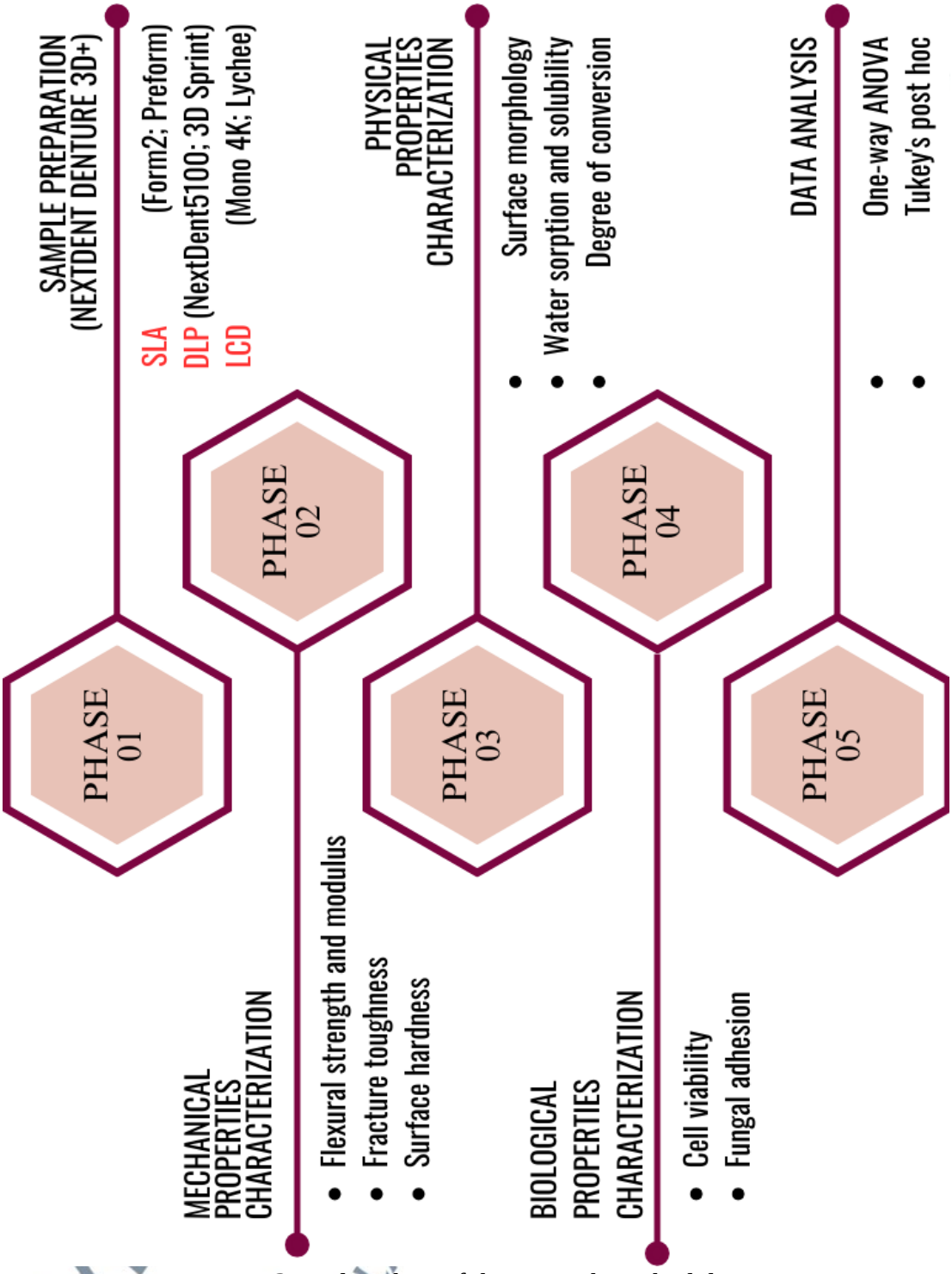

Figure 3.1: Flowchart of the research methodology

3.2 Phase 1: Sample Preparation

3.2.1 Resin Material

Commercially available and Medical Device Authority (MDA) approved 3D printing denture base resin (Denture 3D+; NextDent, 3D Systems, The Netherlands) was selected as the 3D printing resin material in this study. According to the material safety data sheet of the resin material, the composition of the resin was described as follows: Ethoxylated bisphenol A dimethacrylate ($\geq 75\%$); 7,7,9(or 7,9,9-trimethyl-4,13-dioxo-3,14-dioxa-5,12-diazahexadecane- 1,16-diyl bismethacrylate ($10 - 20\%$); 2-hydroxyethyl methacrylate ($5 - 10\%$); Silicon dioxide ($5 - 10\%$); diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide ($1 - 5\%$) and Titanium dioxide ($< 0.1\%$).

3.2.2 3D Printing and Post-Processing

To examine the impact of various vat polymerization techniques, three 3D printers were chosen, each representing SLA, DLP, and LCD methods. The (Form 2; FormLabs, Somerville, USA) was selected to represent the SLA method, (NextDent 5100; NextDent, 3D Systems, The Netherlands) to represent the DLP, and (Mono 4K; Anycubic, Shenzhen, China) to represent the LCD. The 3D printers used in this study and their printing techniques are listed in Table 3.1.

Table 3.1: List of 3D printers used in this study.

Printing technique	3D Printer	Abbreviation	Manufacturer
Digital Light Processing (DLP)	NextDent 5100	DLP	NextDent B.V., Soesterberg, The Netherlands
Stereolithography (SLA)	Form 2	SLA	Formlabs Inc., Somerville, MA, USA
Liquid Crystal Display (LCD)	Mono 4K	LCD	Shenzhen Anycubic Technology Co., Ltd. Shenzhen, China

The testing specimens were initially designed using CAD software (Fusion 360; Autodesk, San Francisco, USA) and saved in Standard Tessellation Language (.stl) format. Subsequently, the 3D design files were processed with the respective slicers for each 3D printer. All specimens were printed at a 45° angle to the build platform with a layer height of 50 microns (Figure 3.2). For the DLP system, the default setting for the 3D printing denture base resin was utilised in the proprietary slicer (3D Sprint; 3D Systems, The Netherlands). However, the SLA and LCD systems lacked the pre-set settings for the specific denture base resin. Furthermore, the SLA slicer did not allow for adjustable printing parameters. Consequently, the default setting for its own proprietary dental resin was used in the SLA slicer (Preform; FormLabs, Somerville, USA). In contrast, the LCD open-source slicer (Lychee Slicer; Mango 3D, USA) offered flexibility in adjusting printing parameters such as exposure time, lifting speed, and light-off duration. Therefore, a custom setting was optimised for the LCD system, employing a trial-and-error approach with varying exposure times, lifting speeds, and light-off durations to achieve shorter printing times while maintaining excellent print quality and high dimensional accuracy.

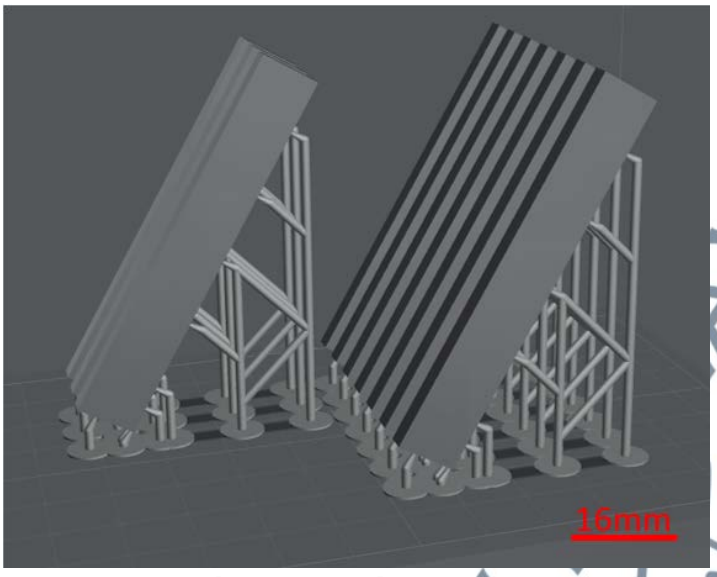

Figure 3.2: Specimens placement and orientation in 3D slicer software

After the 3D printing process was done, the specimens were carefully removed from the build plate using a metal scraper and underwent post-rinsing and post-curing procedures. During the post-rinsing phase, the specimens were immersed in a 99.9% isopropyl alcohol (IPA) ultrasonic bath for 5 minutes. Subsequently, support structures were removed using a snipper, and the specimens were air-dried for 10 minutes. Then, the specimens received 30 minutes of post-curing treatment in a UV light chamber (LC-3DPrintbox; NextDent, 3D Systems, USA), adhering to the manufacturer's recommendations. All the 3D printed specimens were finished using consecutively graded silicon carbide sanding paper (500, 1000, and 1200 grit) and rinsed with running tap water to remove any scarring from the support structure. The printing parameters and post-processing procedures are illustrated in Table 3.2 and Table 3.3.

Table 3.2: Printing parameters and post-processing procedures

Group	Layer Thickness	Wavelength (nm) || Light Intensity (mW/cm^2)	Post-Washing Procedure	Post-Washing Duration	Post-Curing Station	Post-Curing Duration
DLP	50 microns	405 || 1.4	Ultrasonicate in 99.9% IPA	5 minutes	LC-D Print Box	30 minutes
SLA	50 microns	405 || 1.176	Ultrasonicate in 99.9% IPA	5 minutes	LC-D Print Box	30 minutes
LCD	50 microns	405 || 1.09	Ultrasonicate in 99.9% IPA	5 minutes	LC-D Print Box	30 minutes

Table 3.3: Optimised printing parameters for LCD printer

Exposure time	Light-off duration	Lift distance	Lift speed	Retract speed
2 seconds	1 second	5mm	50 mm/s	120mm/s

3.3 Phase 2: Mechanical Properties Characterisation

3.3.1 Flexural Strength and Modulus

The flexural strength and modulus of the specimens were determined through a 3-point bending test. These specimens were fabricated following the dimensions specified in the International Standard Organization (ISO) 20795-1 for denture base polymer, measuring $(65 \times 10 \times 3.3)$ *mm* (*ISO 20795-1. Dentistry-Base Polymers-Part 1: Denture Base Polymers International Organization for Standardization; 2nd Ed. Geneva, Switzerland. 2013*, 2013). Utilizing the Universal Testing Machine (UTM) (SLBL-5kN; Shimadzu, Kyoto, Japan) and the specific 3-point bending fixture (Figure 3.3), the test was conducted. To release the residual monomers within the denture base resin, all specimens were immersed in distilled water at (37 ± 1) °C for (50 ± 2) hours before testing. The specimens were placed in the middle of the fixture with a span width

of 50 mm, and a constant load of 5 kN was applied at a displacement rate of 5 mm/min until mechanical failure occurred. Each group was comprised of 10 specimens for testing ($n = 10$), resulting in a total of 30 specimens for the 3-point bending test. The maximum load experienced by each specimen was recorded, and the ultimate flexural strength ("σ") was computed in MPa using the provided formula (3.1).

$$\sigma = \frac{3F\ell}{2bh^2} \tag{3.1}$$

where F is the maximum force exerted on the specimen, in newtons, ℓ is the span width, b is the width of the specimen, and h is the height of the specimen. Furthermore, the material's capacity to withstand bending forces was further assessed by determining its flexural modulus through the following formula (3.2).

$$E = \frac{F}{d} \cdot \frac{\ell^3}{4bh^3} \tag{3.2}$$

where F/d is the gradient of load against the deflection curve at the linear straight-line portion (with the maximum slope) of the curve.

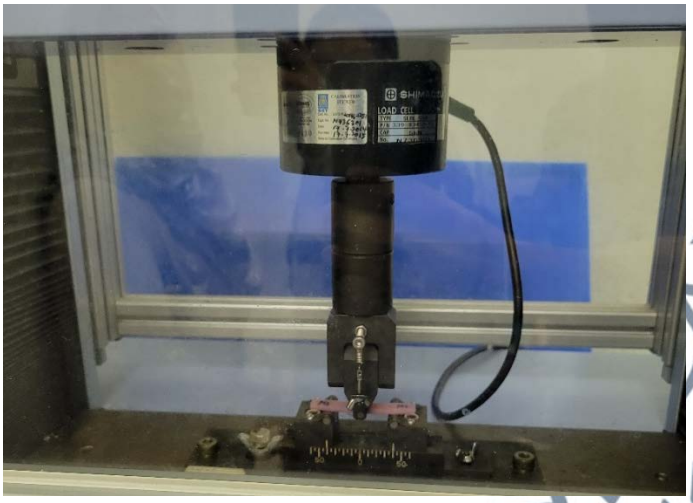

Figure 3.3 :Flexural test on Universal Testing Machine (UTM)

3.3.2 Fracture Toughness

The fracture toughness was assessed using a similar three-point bending test method, though it involved distinct specimens and a different machine-rig setup. These specimens were designed and constructed with dimensions of (39mm x 8mm x 4mm) and featured a (3.0 ± 0.2) mm pre-crack at their centres, conforming to ISO 20795-1 standards (*ISO 20795-1. Dentistry-Base Polymers-Part 1: Denture Base Polymers International Organization for Standardization; 2nd Ed. Geneva, Switzerland. 2013,* 2013). Before testing, these specimens were submerged in distilled water for (7 days ± 2 hours) at (37 ± 1) °C and subsequently conditioned in water at (23 ± 1) °C for (60 ± 15) minutes before testing. During the test, the specimens were positioned in the middle on a fixture with a span width of 32mm, and a consistent load of 5kN was applied at a displacement rate of 1mm/min (Figure 3.4) $(n = 10)$. The maximum force was recorded to calculate the fracture toughness, K_{max} of the denture base material, using the formula provided below (3.3).

$$K_{max} = \frac{f P_m l_t}{b_t {h_t}^{3/2}} \times \sqrt{10^{-3}}$$

$$f = 3x^{\frac{1}{2}} \cdot \frac{[1.99 - x(1-x)(2.15 - 3.93 + 2.7x^2)]}{\left[2(1+2x)(1-x)^{\frac{3}{2}}\right]}, x = \frac{a}{h_t}$$

(3.3)

Where P_m is the maximum force exerted on the specimen, in newtons, b_t is the width of the specimen, ht is the height of the specimen, l_t is the span width, and a is the length of the pre-crack.

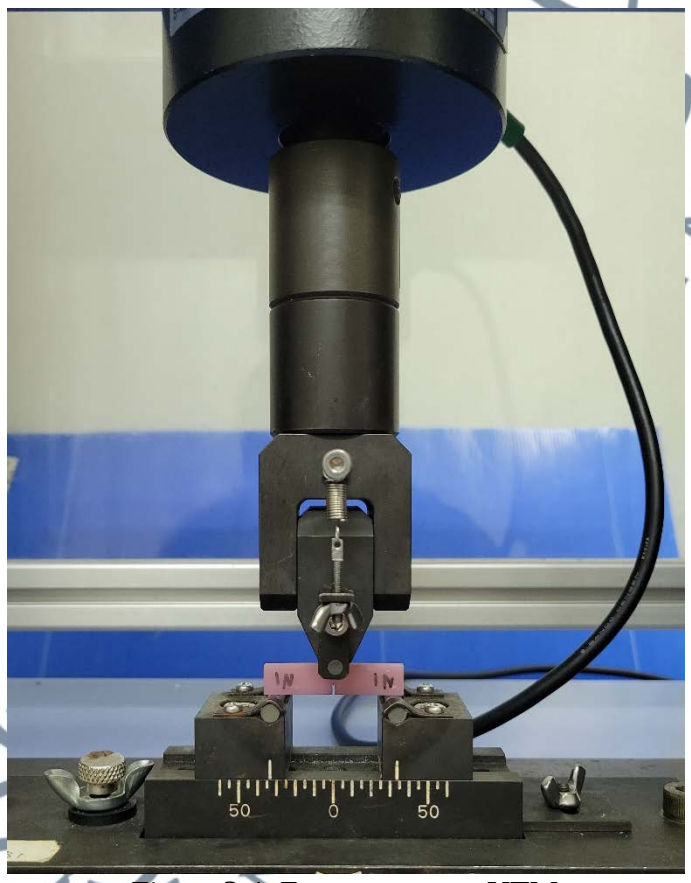

Figure 3.4: Fracture test on UTM

3.3.3 Surface Hardness

Rectangular-shaped specimens with dimensions of $(40 \times 10 \times 4)mm$ were prepared to characterize the surface hardness of the denture base resin. The surface hardness of the specimens was measured and reported as Vickers hardness number (HV)

using a microhardness tester (HMV-2; Shimadzu, Kyoto, Japan). A pyramid-shaped diamond indenter with specified angles was forced into the surface under a load force of 100 gf and a dwell time of 30 seconds to create a diamond-shaped indentation on the specimen. The size of this indentation, measured in (HV), served as an indicator of the specimen's surface hardness. Ten indentations were conducted on each specimen with a 1mm separation between each successive indent (Figure 3.5). The mean value of the ten readings of each specimen was calculated and recorded. Ten specimens were prepared for each testing group ($n = 10$), and a total of thirty specimens were prepared for the surface hardness test.

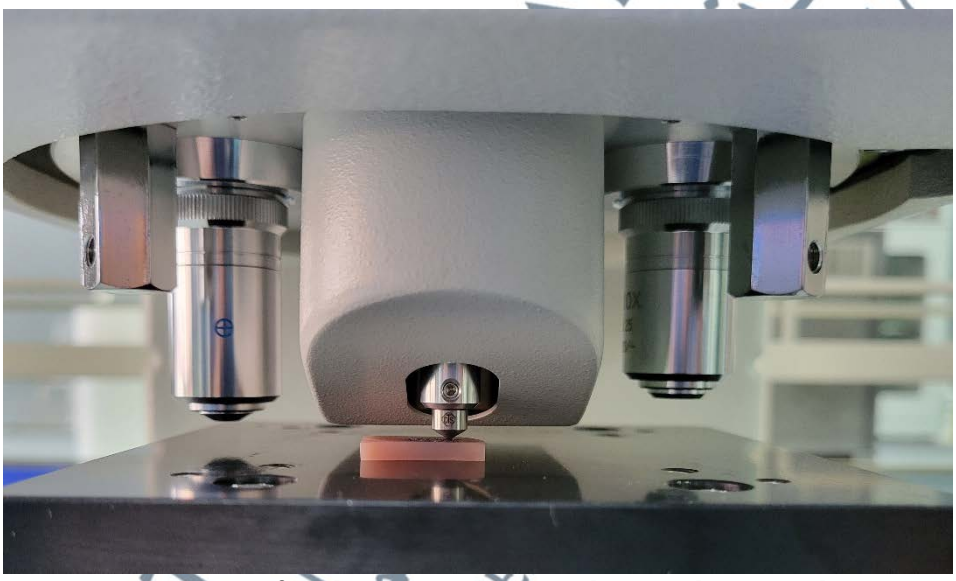

Figure 3.5: Surface hardness test on Vicker Hardness machine

3.4 Phase 3: Physical Properties Characterization

3.4.1 Surface Morphology

The surface morphology of the 3D-printed denture base specimens was studied using an optical microscope (DM750; Leica, Wetzlar, Germany) with a magnification of 40x (Figure 3.6). To further analyse the surface morphology of the 3D-printed

specimens, their surfaces were documented both before and after sanding and polishing (Altarazi et al., 2022).

Figure 3.6: Surface morphology examined under optical microscope with 40x magnification

3.4.2 Water Sorption and Solubility

To assess the water sorption and solubility of denture base resin created via different vat polymerization techniques, specimens were prepared with 15 mm in diameter and 1 mm in thickness. For the water immersion test, five specimens were 3D-printed for each group ($n = 5$), totalling 15 specimens. Initially, these specimens were placed in a desiccator with dried silica gel and kept in an oven at (37 ± 1) °C for (23 ± 1) hours (Figure 4). Subsequently, they were transferred to another desiccator with silica gel at (23 ± 2) °C for an additional hour. After each drying cycle, their mass was cautiously measured using a precise analytical balance (ME103E; Mettler Toledo, Greinfensee, Switzerland) with a 0.1-milligram accuracy. This drying process was repeated until the measured mass (m_1) remained constant, with fluctuations not exceeding 0.2 mg between consecutive weighings. At this stage, the specimens' mean diameter and thickness were determined using a high-precision digital calliper (ABS

Digital Calliper; Mitutoyo, Tokyo, Japan) and the volume (V) was calculated. Next, the specimens were placed in a container filled with distilled water, ensuring complete immersion at (37 ± 1) °C for $(7\ days \pm 2\ hours)$. After the immersion period, the specimens were dried and weighed. This wetting and drying cycle was repeated until a consistent mass (m_2) was achieved. Finally, the specimens were returned to the desiccator, as mentioned in the drying cycle (Figure 3.7) and repeated all over again until they reached their ultimate constant mass (m_3). Using the formula below (3.4), the water sorption (w_{sp}) and solubility (w_{sl}) were calculated in $\mu g/mm^3$.

$$w_{sp} = \frac{m_2 - m_3}{V}, \qquad w_{sl} = \frac{m_1 - m_3}{V}$$

(3.4)

Where m_1 is the mass of the dried conditioned specimen, m_2 is the mass of the specimens after being immersed in distilled water, m_3 is the mass of the reconditioned specimen, and V is the volume of the specimen.

Figure 3.7: Specimens drying in independent desiccators in an oven at (37 ± 1) °C

3.4.3 Degree of Conversion

The degree of conversion (DC) refers to a critical measurement of how completely the liquid resin material used in the printing process has converted into a solid polymer (Aati, Akram, et al., 2022). It is a fundamental parameter because it directly influences the mechanical, physical, and biocompatibility properties of the 3D-printed denture base. Fourier Transform Infrared (FTIR) spectroscopy (Nicolet iS50, Thermo Scientific, Massachusetts, USA) was employed to determine the DC of the 3D-printed specimens. The FTIR spectra were recorded over a wavelength range spanning from 4000 to 400 cm^{-1}, utilising a resolution of 4 cm^{-1} and averaging 16 scans, all conducted at room temperature. Before the analysis, a background spectrum was generated to calibrate the instrument accurately, according to the manufacturer's instructions.

The examination process involved two scans of the 3D-printed resin material. Firstly, in its liquid state, serving as the baseline record, and secondly, after all the post-processing procedures (Figure 3.8). The calculation of DC was determined by evaluating the difference in the peak ratio of double carbon bonds at two distinct frequencies. Specifically, the stretch of aliphatic frequency at 1637 cm^{-1} was compared against the reference aromatic frequency at 1608 cm^{-1} (3.5).

$$DC\ (\%) = 100 \times \left[1 - \frac{\left(\frac{A1637}{A1608}\right) Peak\ after\ polymerization}{\left(\frac{A1637}{A1608}\right) Peak\ before\ polymerization}\right] \quad (3.5)$$

Figure 3.8: Examining the transmittance of the specimens using Fourier Transform Infrared (FTIR) spectroscopy

3.5 Phase 4: Biological Properties Characterization

3.5.1 Cell Viability

Primary human gingival fibroblasts (HGFs; ATCC, USA) derived from gingiva were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, California, USA), 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma Aldrich, St. Louis, USA). The cells were cultured in a T75 cm^2 flask and placed in a carbon dioxide (CO_2) incubator at 37ºC with 5% CO_2, 95% air atmosphere, and 100% relative humidity. The cell culture medium was replenished every 2 days until the monolayer cultures reached $80 - 90\%$ confluency.

Once confluent, the culture medium was removed, and the flask was washed and rinsed with phosphate-buffered saline (PBS) for 1 minute. Then, the PBS was removed and added with 1.5mL of trypsin followed by 5 minutes of incubation. Thereafter, the

cell was detached from the wall of the flask by gently tapping it. Next, an additional 3 mL of cell culture medium was added to the cell solution and centrifuged at 2000 rpm for 5 minutes. Subsequently, the cell pellets were collected and diluted with fresh cell culture medium. Lastly, the cell solution was seeded into a 12-well plate with a seeding density of 100,000 cells/well and incubated for (24 ± 2) hours.

The cell viability of the HGFs on 3D-printed dentures was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay with direct contact method according to ISO 10993-5 and 10993-12 (*ISO 10993-5: 2009 Evaluation of Medical Devices — Part 5: Tests for in Vitro Cytotoxicity*, 2009; *ISO 10993-12: 2012 Evaluation of Medical Devices — Part 12: Sample Preparation and Reference Materials*, 2012). A total of 36 disc-shaped specimens with a dimension of $(10 \times 2\ mm)$ were 3D-printed and submerged in distilled water for (24 ± 2) hours at (37 ± 1) °C to release any unreacted monomers ($n = 3$). Prior to the cell treatment, the specimens were sterilized with 70% isopropyl alcohol (IPA), followed by rinsing three times with (PBS) and exposed under UV light in the biosafety cabinet for 15 minutes.

Firstly, the earlier cell culture medium was gently discarded, and the sterilised specimens were placed in the 12-well plate respectively, with one of the columns remaining empty and serving as the control group. Then, the 12-well plate was replenished with fresh DMEM medium and made sure the specimens were fully immersed in the medium. Subsequently, the well plate was incubated in 95% air, and 5% CO_2 at 37ºC for (48 ± 2) hours. In the control wells, only the cell culture medium (without 3D-printed specimens) will be added (Figure 3.9). After 48 hours of incubation, the medium and specimens were carefully discarded to avoid scarring the cells and the

adhering cells in each well were rinsed with 1mL of PBS. The PBS was removed after gently rinsing the wells, and 1 mL of MTT solution (0.5mg/ml in PBS) was added into all wells and incubated for another ($2\ hours \pm 15\ minutes$) at (37 ± 1) °C (*ISO 10993-5: 2009 Evaluation of Medical Devices — Part 5: Tests for in Vitro Cytotoxicity*, 2009).

After 2 hours of incubation in a dark environment, the MTT solution was discarded, and 500µl/well of dimethyl sulfoxide (DMSO) was added to dissolve the formazan product. A microplate spectrophotometer (SpectraMax iD3; Molecular Devices, California, USA) was used to measure the optical density of the solution at 570 nm with reference to 630 nm (Figure 3.10). The optical density (OD) reading at 570nm was subtracted with the background absorbance at 630nm to obtain the final OD (Srivastava et al., 2018). Four independent replicate experiments were carried out to ensure the repeatability of testing results. The cell viability was calculated with the formula below (3.6) and expressed in percentage.

$$Cell\ Viability\ (\%) = \left(\frac{OD_{570s} - OD_{630s}}{OD_{570c} - OD_{630c}}\right) \times 100$$

(3.6)

Where OD_{570s} is the measured optical density of the specimens at 570nm, OD_{630s} is the measured optical density of the specimens at 630nm, OD_{570c} is the measured optical density of the control well at 570nm, and OD_{630c} is the measured optical density of the control well at 630nm.

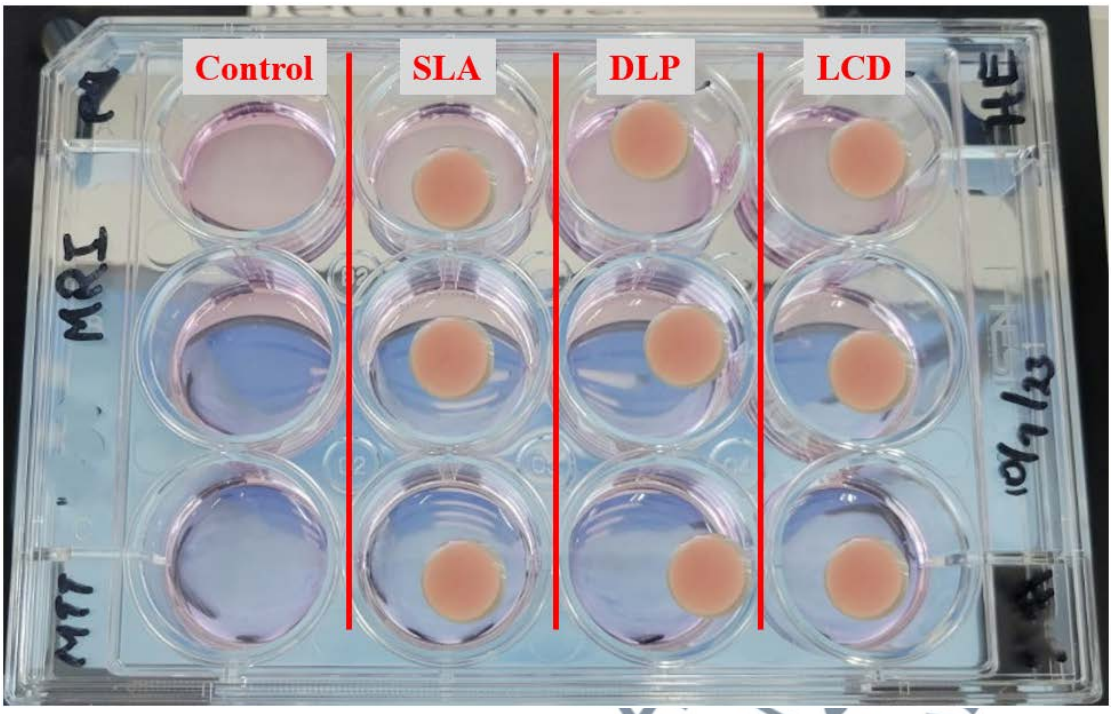

Figure 3.9: Treatment of 3D-printed specimens in HGFs and media. Control wells only contain cells and culture medium.

Figure 3.10: Cell viability test on a microplate reader

3.5.2 Fungal Adherence

3.5.2.1 Colony Forming Units Count

Numerous methods exist in microbiology for quantifying microbial adhesion, each possessing distinct advantages and limitations. The enumeration of Colony Forming Units (CFU) on agar plates is widely acknowledged as the gold standard technique for quantifying bacteria or fungal cells adhered to a specimen (Thomas et al., 2015). Hence, CFU assays were undertaken in this study to evaluate the propensity of the denture base material to attract fungal adhesion. Additionally, fluorescence microscopy was employed to corroborate the findings obtained from the CFU counting.

Similar disc-shaped specimens with a diameter of 10 mm and thickness of 2 mm were 3D-printed to evaluate the fungal adhesion of the denture base material (n=4). *Candida albicans* (*C. albicans*; ATCC, USA) was incubated in 25 ml Brain Heart Infusion (BHI) broth for (24 ± 2) hours at 37°C. After 24 hours of incubation, the fungal suspension was diluted in fresh Brain Heart Infusion (BHI) broth, and its concentration was subsequently adjusted to meet the 3 McFarland standard.

To begin the fungal adherence test, each specimen was carefully positioned in a 12-well plate. Each of the wells of the plate was then subjected to inoculation with 100µL of fungal suspension and 1mL of fresh BHI medium. Subsequently, the well plates were subjected to aerobic incubation at a controlled temperature of 37°C for (24 ± 2) (Han et al., 2022). After this incubation period, the specimens underwent a gentle rinse using phosphate-buffered saline (PBS) to eliminate the non-adherent cells. Following the rinse, the specimens were each carefully transferred to a centrifuge tube containing 2 ml of PBS. To detach the adhered fungus cell from the denture surface, a

sequence of actions was performed, including vortexing and sonication of the specimens. Each procedure was carried out for 5 minutes (Zupancic Cepic et al., 2020).

The suspension containing the adherent cells was then extracted and subsequently serial diluted to a concentration of 2^{-1} with PBS. This precise dilution was essential to prevent excessive cell growth on the agar plate. Next, 100µl of the diluted suspension was extracted and plated onto a BHI agar and incubated for another (24 ± 2) hours at 37°C. After the 24-hour incubation period, the CFU count was determined with the aid of a colony counter (Galaxy 230; Rocker Scientific, Taiwan) (Figure 3.11). The number of colony-forming units per ml (CFU/ml) was calculated using the formula below to quantify the fungus adhesion. The adherence assay was performed in three independent replicates to ensure the repeatability of the testing result (Sampaio-Maia et al., 2012).

$$C = \frac{n_0 \alpha^{-j} \alpha_p{}^{-1}}{V} \quad (3.7)$$

Where C is the concentration of fungal in the suspension, n_0 is the number of colonies grown on the agar plate, α is the dilution factor, j is the number of dilution, α_p, is the percentage of suspension plated to culture, and V is the volume of diluted suspension plated on the agar plate in mL (Ben-David & Davidson, 2014).

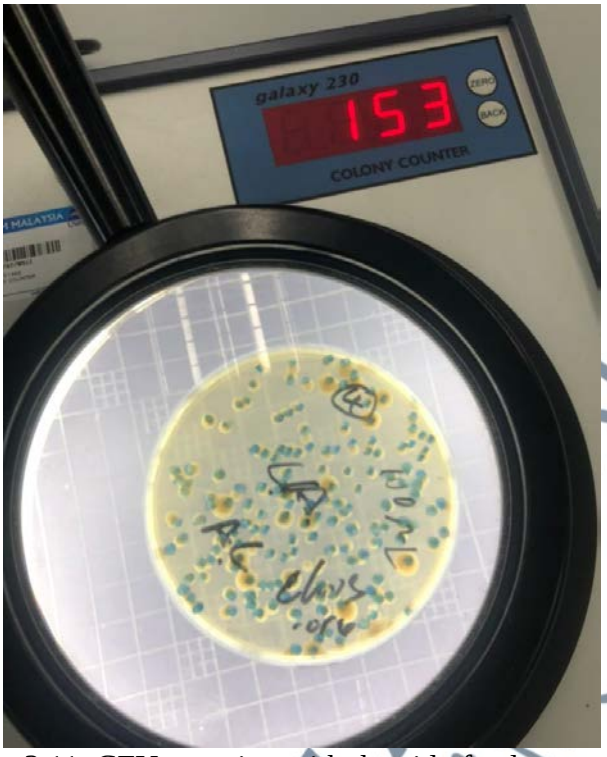

Figure 3.11: CFU counting with the aid of colony counter

3.5.2.2 Fluorescent Microscope

To further investigate the fungal adhesion of the 3D-printed specimens, a qualitative analysis was conducted using fluorescent microscopy. Similarly to the CFU count, the specimens were placed in the 12-well plate and supplied 100µL of fungal suspension and 1mL of fresh BHI medium, respectively. After a 24-hour incubation period of the specimens in the fungal suspension, the specimens were methodically extracted and subjected to two subsequent rinses with PBS. These treated specimens were then carefully transferred to yet another 12-well plate for additional staining procedures. After the requisite rinsing, the specimens were immersed in a 0.03% Acridine Orange (AO) solution for 5 minutes (Al-Fouzan et al., 2017). Following this staining interval, any excess stain was diligently removed through rinsing with distilled water. Subsequently, the specimens were left to air-dry for 10 minutes. Once completely

air-dried, these specimens were thoroughly examined under a fluorescent microscope (BX43; Olympus, Tokyo, Japan) (Figure 3.12).

Figure 3.12: Specimens examined under fluorescent microscope

3.6 Phase 5: Data Analysis

All the data recorded were statistically analysed using SPSS version 22 (IBM, New York, NY, USA). The normality of data distribution was determined using the Shapiro-Wilk test, and the Levene test confirmed the homogeneity of the data. The results were presented as mean ± standard deviation and further analysed with One-way ANOVA to statistically determine the relationship between the different vat polymerization techniques and the tested properties. Following with Tukey's post hoc test for pair-wise comparison according to a significance level set at $p \leq 0.05$. A p-value less than 0.05 was considered statistically significant.