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Effect of Variations in Post-set Temperature and Monomer Concentration on Self-cure Acrylic Surface Candidal Growth: An In-vitro Study

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Abstract **Objectives:** To identify the effect of variations in power-liquid ratio and post-set temperature on water absorption and residual monomer concentration on growth of Candida albicans on self-cure denture base resins. **Methodology:** 60 self-cured acrylic discs (39 x 4mm) were made and divided into 3 groups each having 20 specimens. Group 1 consisted of discs fabricated at a powder-liquid ratio of 5.1:2.8 as recommended by the manufacturer. Groups 2 and 3 constituted specimens with variations in post-set temperatures and powder-liquid ratios respectively. Specimens from group 2 were soaked in four water baths at temperatures of 37°C, 47°C, 57°C and 67°C for 24 hours. Specimens from the temperature-controlled group 3 were fabricated at four different powder-liquid ratios by increasing monomer liquid volume by 10%, 15%, 20% and 25%. The acrylic discs from control groups 1 and 3 were soaked at a constant temperature of 37°C for 24 hours. Residual monomer leeched out from disc into water was analyzed using Ultraviolet spectrophotometer. The variations in water absorption and Candidal growth were recorded. **Results:** Linear regression analysis was used to analyze the results. A moderately positive correlation was calculated for the association between powder-liquid ratio and Candidal growth ($r = 0.67$, $p < 0.001$) suggesting Candidal growth is higher at increased powder-liquid ratios having low monomer content. The correlation between the Candidal growth and the post-set temperatures was found to be moderately negative ($r = -0.41$ $p < 0.001$), indicating a decrease in Candidal cells on increasing post-set soaking temperatures. **Conclusions:** Candidal growth follows a positive linear relation with a decreasing powder-liquid ratio having cells increasing with an increase in liquid monomer content. Candidal growth follows a negative linear relation with post-set temperature soaking with cells decreasing with an increase in temperature.

Keywords: *polymethyl-methacrylate (PMMA), self-cure acrylic resin, residual monomer, candida albicans, water absorption*

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1. Introduction

The oral cavity is considered to be an environment capable of hosting a variety of ecological niches for fungal and microbial colonization [1]. Candida albicans has been termed as a notorious "opportunistic pathogen" with the conversion from commensalism to parasitism and eventually exuberant growth normally being associated with intraoral or systemic changes such as inappropriate oral hygiene and immunosuppression [2]. Additionally, successful Candidal colonization onto the fitting surfaces of acrylic dentures has been observed [3,4]. Dentures made of Poly-methyl-methacrylate (PMMA) have been known to act as reservoirs with Candidal colonization observed mostly on the moist surfaces and sometimes even penetrating into the superficial layers of polymer material [5,6,7]. Literature supports the development of non-specific and denture plaque followed by the sequential colonization by Candida organisms as the most plausible etiology of denture related stomatitis [8,9,10].

Although, multiple factors such as substrate roughness, saliva flow rate, acidity of medium, ionic polarity, release of surfactants etc effect biofilm maturation and eventually the growth of microorganism [11]. The closer the surface free energy of the substrate and the micro-organism, the higher would be the probability of adherence [11,12]. Minagi et al reported a correlation between the surface free energy change and Candidal growth. When a micro-organism and surface make contact being placed in a liquid medium, the interaction energy can be calculated from an assumption that interfaces between micro-organism-liquid and the solid-liquid are replaced by a solid micro-organism interface. If the change in free energy is negative, adhesion is thermodynamically favored and will proceed spontaneously as nature attempts

to minimize free energy. Interestingly, the surface energy of Candida has been reported to be closer to self cure acrylic resin as compared to that for heat cure acrylic resin [9,13,14]. In addition, the presence of porosities and absorption of moisture from the oral environment makes self cure acrylic, in the absence of proper hygiene, a potential habitat encouraging Candidal colonization [15].

Auto-polymerizing or self cure acrylic is used for denture repairs, chair side temporary denture relines, fabrication of obturators, custom trays and orthodontic appliance [16,17]. Upon mixing, activators present in the liquid monomer component immediately start the polymerization reaction by releasing free radicals from initiator break down. The reaction propagates fast resulting in a set polymer with shorter polymer chains and weaker cross linking. A lower average molecular weight of the self cured acrylic chains indicates the possible higher existence of residual monomers. The glass transition temperature (Tg) for self-cure acrylic resin is 90°C as compared to 105°C for heat-cure acrylic [18]. The exposure to a temperature during clinical use higher than the Tg is likely to cause a plasticizing effect on the cross-links with a consequent fall in the stiffness of the system [19]. Additionally, the absorption of external water, both in liquid or vapor form further decreases in the Tg of the resin, which affects the mechanical properties [19].

Physical aging in the glassy state is very slow, whereas it proceeds rapidly at temperatures close to the Tg. This latter is the case of cold-cured resins, in which Tg can be easily approached and even exceeded by the external temperature thereby leading to an earlier development of a unstable form of the product [18].

It has been observed that operators tend to dispense a higher liquid monomer volume without following the prescribed powder-liquid ratio advocated by the manufacturer of the product. An uncalculated mix can not only lead to a semi-cured weaker product with a high residual monomer content but also influence Candidal growth [9]. Additionally, since it has been documented that self-cure acrylic tends to exhibit continued polymerization for the initial 24 hours following initial setting, the influence of post-set temperatures on material properties in particularly Candidal growth remains unknown [15,20]. The objective, therefore, of this in-vitro exercise is to identify the effect of variations in power-liquid ratio and post-set temperature on the growth of Candida albicans in self cure denture base resins.

2. Methodology

a). Preparation of acrylic discs:

Teflon discs having a standard diameter of 39mm and a thickness of 4mm were initially used as templates to create moulds for the fabrication of self-cure acrylic samples [19]. A total of 60 moulds were created and divided into 3 groups each consisting of 20 moulds. Self-cure acrylic powder and liquid (Meadway Self cure/Super cure/Cold Curing Pour Type denture base material, England) was mixed according to the ratio mentioned in Table 1. The powder was dispensed and weighed on a digital weighing scale (Unblock Analytical Balances ATY 224, Shimadzu, Japan) and the liquid volume was measured using a pipette (370710-10,PYREX VISTA,USA). The templates were filled with the mixed resin material and allowed to bench sit for 60 minutes before being carefully removed [16, 20]. Group 1 consisted of 20 discs fabricated at a powder-liquid ratio of 5.1:2.8 as recommended by the manufacturer. Groups 2 and 3 also constituted 20 acrylic specimens each with variations in curing temperatures and powder-liquid ratios respectively. Specimens from group 2 were soaked in four water baths at temperatures of 37°C, 47°C, 57°C and 67°C for 24 hours such that each water bath simultaneously received 5 specimens. Similarly specimens from the temperature-controlled group 3 were fabricated at four different powder-liquid ratios as indicated in Table 1 by increasing monomer liquid volume by 10%, 15%, 20% and 25%. The acrylic discs from control group 1 were soaked at a constant temperature of 37°C. The 60 disks were finally disinfected in 70% alcohol for 15 minutes and washed with sterile distilled water prior to culturing Candida albicans.

Table 1. Powder-liquid ratios and temperature variations among groups

Group 1			Group 2 (Composition-controlled)			Group 3 (Temperature-controlled)		
No. of samples	**Powder(g) /liquid ratio (ml)**	**Temperature**	**No. of samples**	**Powder (g) /liquid ratio (ml)**	**Temperature**	**No. of samples**	**Powder(g)/ liquid ratio(ml)**	**Temperature**
05	5.1 / 2.8	37°C	05	5.1 / 2.8	37°C	05	5.1 / 3.1	47°C
05	5.1 / 2.8	37°C	05	5.1 / 2.8	47°C	05	5.1 / 3.2	47°C
05	5.1 / 2.8	37°C	05	5.1 / 2.8	57°C	05	5.1 / 3.42	47°C
05	5.1 / 2.8	37°C	05	5.1 / 2.8	67°C	05	5.1 / 3.5	47°C

b) Ultraviolet-visible Spectrophotometry

Ultraviolet-visible spectrophotometer (JIR-100, JEOL Co.Ltd, and Tokyo Japan.) was used to detect the residual monomer in all samples. Eight solutions of MMA monomer in distilled water (solvent) were produced with concentration of 5%,4%,3%,2%,1%,0.5%,0.25%, and 0.1% for which UV spectra was obtained. The calibration curve was prepared based on these eight known samples. This served as standard or control curve. The “Beer Lambert equation” was applied and graph was plotted to obtain Figure 1.

The samples were tested on the basis of either being temprature controlled or composition controlled in accordance with Table 1.The water samples were run on UV spectrophotometer after resin samples were removed and values were obtained. These UV spectrophotometer values were compared with the standard calibration graph to obtain results.

In this study Ultraviolet-visible spectrophotometer was used to identify the level of residual monomer that leeches out of the denture sample into the water bath after immersion for 24 hours.

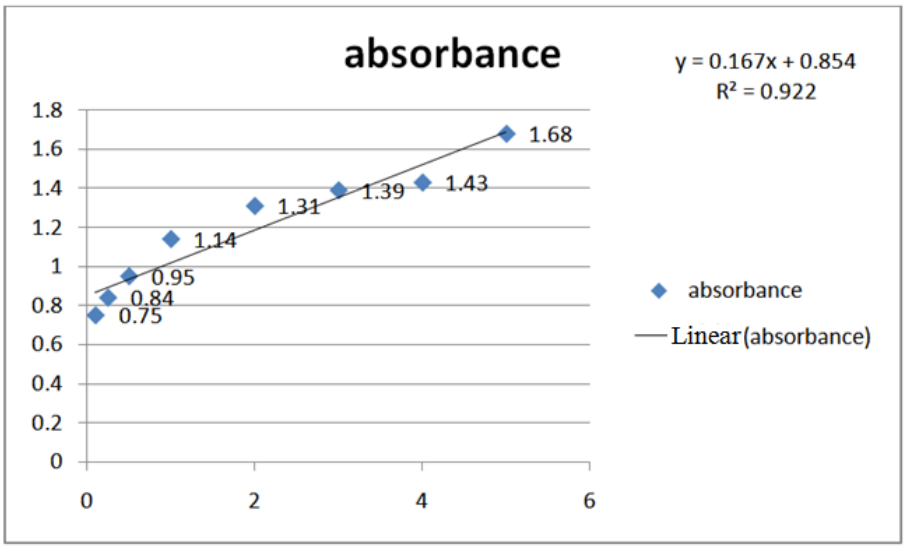

Figure 1. Standard calibration curve

c) Immersion test:

After fabrication, specimens were weighed after every 24 hours until a constant mass was obtained (m1), after which the specimens were soaked in distilled water for 24hours and weighed for a second time (m2). The specimens were reconditioned to a constant mass in the desiccator (m3). The volume of each specimen was calculated (mm3) after the first desiccation. Water absorption (wsp) was calculated using the following equation [21,23]:

$$\mathbf{Wsp = m2 - m3 / V}$$

A scale (SHIMADZU make, model number ATY224 unibloc) with an accuracy of 0.01mg, was used to weigh the powder and the liquid. Small ceramic bowls (100ml) were used to measure the powder and liquid respectively. The weight of these ceramic bowls were subtracted by using the tear function on the scale, which reset the display screen to zero, thereby the weight of only the content of the ceramic bowl was measured. This process was repeated for each individual specimen fabricated. The volume was calculated using the dimension of the acrylic disc to obtain a value of volume in mm3. The amount of water sorption was obtained mathematically by using the formula above which produced values of sorption in µg/mm3

The room temperature was monitored, before commencing the experiment and monitored throughout, using two glass thermometers (N.T. Laboratory Supplies (Pty), 76mm Immersion type). A digital thermometer (TH03 digital hygro-thermometer) was used to indicate a third reading and to monitor humidity.

Figure 2. Preparation of discs for fungal culture

d) Fungal culture:

The Candida strain used in this study was Candida albicans (ATCC 10231) obtained from Islamabad Diagnostic Centre (microbiology department).Candida albicans was incubated on sabourads dextrose agar at 37°C for 48 hours. After which good visible Candidal colonies were seen on the agar. 100mg of Candida was taken from the colonies and diluted in 500ml of distilled water. After dilution, a final fungal suspension of approximately 10(6) Candida albicans per millimeter was prepared. 05 acrylic resin specimens for each group were placed in sterile Petri dish and were covered with 20ml of fungal suspension and incubated at 37°C for 24 hours.

After completion of the incubation period the specimens were removed using sterile forceps to avoid any contamination and washed in sterile phosphate buffered saline (PBS; 10Mn phosphate buffer, 2.7Mn potassium chloride, 137mn sodium chloride, PH 7.4). Fungal cells adhering to acrylic resin surfaces were fixed in formaldehyde for 05 minutes and stained using gram staining technique. The specimens were washed in distilled water and the stained smear was allowed to dry in air.

e) Light Microscopy:

A drop of cedar wood oil was placed over the specimens and the number of Candida cells on each specimen was counted using the calibrated micrometer fixed in the eye piece of microscope at a power of 100x (Olympus-CX31, Japan). Normally two types of micrometers are used to measure an object under a microscope i.e. stage micrometer and ocular micrometer. The ocular micrometer is pre-calibrated using a stage micrometer on the required optical combination before making accurate measurement such that one division of ocular micrometer equals to 3µm using the following equation:

$$100 \text{ div on ocular micrometer}$$

$$= 30 \text{ divisions on stage micrometer} (\text{one div } = 10\mu\text{m})$$

$$= 30 \times 10$$

$$100 \text{ div on ocular micrometer} = 300 \text{ um}$$

$$1 \text{ div on ocular micrometer} = x$$

$$x = 3\ \mu\text{m}$$

Microscopically yeast cells appear blue and show a characteristic budding (Figure 3). A total of 05 random fields were viewed under the microscope for each of the 60 samples. Fields that showed 0 cells were not included in results. The number of Candida cells was counted in the field of vision and mean was taken. Data was entered in SPSS version 17 and analyzed.

3. Results

A total of 60 samples with varying powder-liquid ratios (PLR), post-set temperature (PST) , residual monomer (RM) and absorption were analyzed for Candidal growth. A linear regression model was generated in order to investigate the impact of these four predictors on Candida growth.

Pearson's correlation was employed for evaluating the association between the independent predictor variables (PLR, PST, RM and absorption) and the dependent outcome variable (CG). The CG values for each powder-liquid ratio and post-set temperature (PLR and PST) settings have been illustrated in Table 1.

A moderately positive correlation was calculated for the association between PLR and CG ($r = 0.67$, $p < 0.001$) suggesting Candidal growth to increase with a reduction in monomer content. The correlation between the CG and the PST was found to be moderately negative ($r = -0.41$ $p < 0.001$), indicating an inverse relationship i.e. as the post-set soaking temperature was increased the number of Candidal cells decreased.

Pearson's correlation showed a strong positive correlation between Candidal growth and level of absorbance and for every unit change in absorbance 28 Candidal cells was seen. The maximum absorption was noted at the powder-liquid ratio 5.1: 3.1 which was found to be 3.19 incidentally a high number of Candida cells (256) were also noted on these specimens. Similarly as the absorbance decreased to 1.29 at the ratio 5.1:3.5 the number of Candida cells also declined to 98. A strong negative correlation was observed between Candidal growth and residual monomer content indicating an inverse relationship.

Table 2. Linear Regression Coefficients (95% Confidence Intervals) for the Association of Powder-Liquid Ratio, Post-set Temperature, Residual monomer concentration and Absorption with Candidal Growth

	Candidal Growth
Powder-Liquid Ratio	-50.46 (-77.39 – -23.53)
Post-set Temperature	1.08 (0.67 – 1.49)
Absorption	79.39 (69.44 – 89.15)
Residual monomer	-15.368 (-16.68 –7.65)

Table 3. Means of Candidal growth and absorption

No of cells	Sample size	Control **(Composition ratio 5.1/2.8)**				Composition- controlled **(Composition ratio 5.1/2.8)**				Temperature- controlled **(Temperature at ratio 47°C)**			
Temperature		37°C	37°C	37°C	37°C	37°C	47°C	57°C	67°C	5.1/ 3.1	5.1/3.2	5.1/3.4	5.1/ 3.5
Absorption (µg/mm3)	*20*	*2.81*	*2.79*	*2.80*	*2.84*	2.80	2.50	2.30	3.12	3.19	2.59	2.50	1.29
Standard Deviation		*1.23*	*1.21*	*1.23*	*1.25*	1.04	1.79	0.43	0.52	3.97	3.27	3.22	0.17
Residual monomer concentration	*20*	*0.95*	*0.93*	*0.95*	*0.93*	0.94	1.05	1.18	2.84	1.17	1.24	1.40	1.84
Standard deviation		*1.11*	*1.11*	*1.15*	*1.11*	1.13	1.15	1.22	1.26	2.34	2.42	2.45	2.55
Candidal growth (no of cells)	20	310	310	312	315	310	152	101	153	316	216	111	98
Standard Deviation		8.15	8.15	8.15	8.15	8.15	17.2	13.19	15.6	7.86	4.69	10.3	10.6

The linear regression models for the predictors (PLR, PST, RM and Absorption) of CG have been shown in Table 2. For Candida growth, highly significant associations ($p < 0.001$) were found with powder-liquid ratio [β = -50.46 (-77.39 - -23.53)], post-set temperature [β = 1.08 (0.67 – 1.49)], residual monomer [β = - 15.36(-16.68-7.65)]and absorption [β = 79.30 (69.44 – 89.15). This would suggest that the powder-liquid ratio and post-set temperature directly affect the residual monomer and absorption of the specimen which in turn affects the growth of Candida. As the absorption values increases, an increase in the number of cells of Candida is seen and vice versa. Except for the slight variation at 65°C but the variation was found to be statically insignificant.

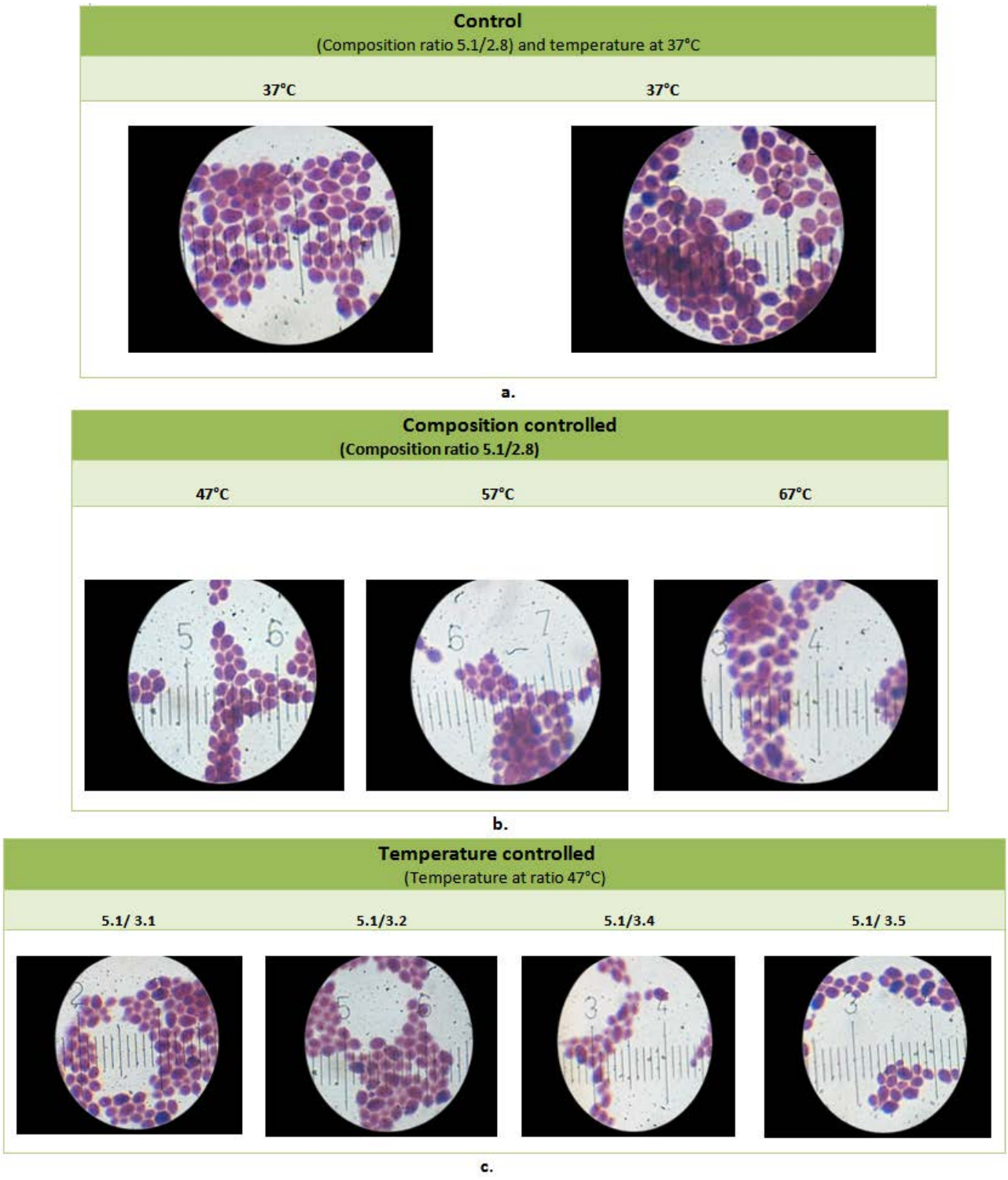

Figure 3. Microscopic views of Candidal cells at 100x in Figure 3(a)(b)(c). Scale is x=3um (x is one unit of ocular micrometer)

4. Discussion

Acrylic dentures have been known to act as potential reservoirs for micro-organisms and depending on environmental cues, such as nutrient availability and mechanical stimuli can cause denture induced stomatitis [24,25]. The process of Candidal adhesion to acrylic resin is however complex. Previous studies have shown that a number of factors including inadequate oral and denture

hygiene, low salivary pH, excess intake of carbohydrates promote colonization. Additionally, one of the important factors affecting adherence of Candida albicans to dentures is hydrophobic interaction [7]. Hydrophobic interaction occurs between cell surface and the substratum. Minagi et al concluded with regards to hydrophobic interaction, there appears to be a higher adherence of Candida to materials which had surface energy closer to that of the organism and thereby hydrophobic interactions are important in the initial attachment of yeast to polymeric surfaces [11,20].

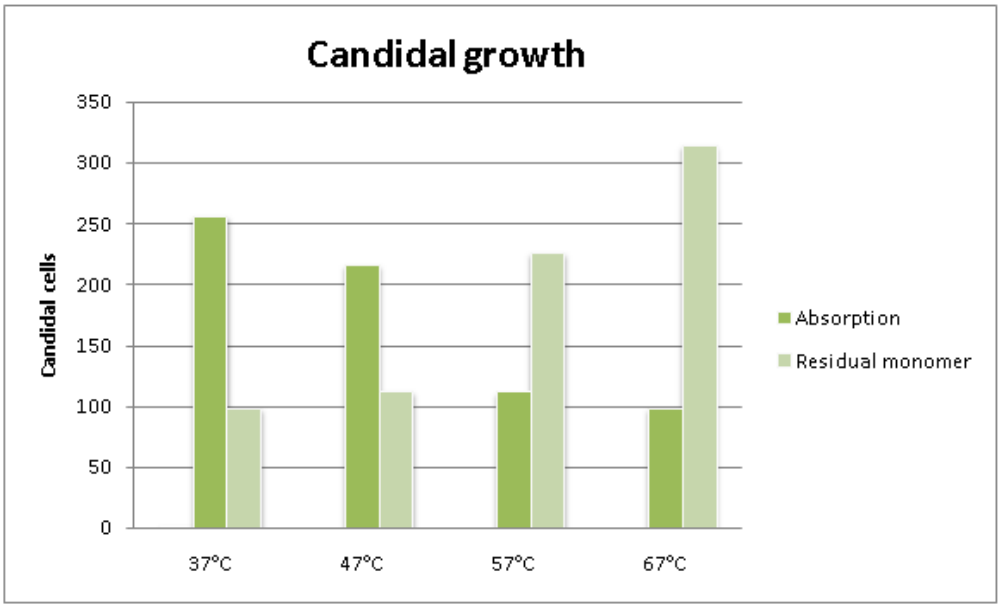

Figure 4. Correlation of Candidal growth with reference to increase in temperature and composition

With the surface free energy of Candida being closer to self-cure auto polymerizing resin as compared to heat cured acrylic, the susceptibility of colonization under unfavorable conditions remains higher in denture repairs, temporary relines and orthodontic appliances fabricated in self-cure resin [12,26]. Irrespective of a number of documented causes active in solitude or in combination, the occurrence of frank infection finally depends on the number of Candidal species colonizing the denture base and the virulence of organism [5,11,14,27]. The objective of this study was to assess the effect of variations in powder-liquid ratios and post set temperatures on Candidal growth on the surface of self-cure acrylic. The adherence of the cells was therefore observed only for the tissue surface of the specimens, keeping in mind of the fact that Candida albicans is mainly seen thriving on surfaces [28]. This has been attributable to the acidic pH prevalent under the fitting surface of the dentures, along with features such as water absorption and permissive rougher surface which aids the surface proliferation of the fungi. The number of organisms was counted and growth correlated with variations in powder-liquid ratios and post set soak temperatures.

The methodology chosen here was based on a study by John F. Miner (1973) with a few modifications. An incubation period of 24 hours was considered for all the specimen groups. Control group specimens had fixed powder-liquid ratios and post set soak temperatures. The selected variations in powder-liquid ratios and post-set soak temperatures in Groups 2 and 3 were identified from literature based on the commonly used values by technicians and operators.

Powder-liquid ratios were prepared with an incremental increase of 10%, 15%, 20% and 25% in liquid. The variations in the powder-liquid ratio of PMMA resins was justified by the common practice of increasing the liquid content to improve flow of the material thereby enhancing the capability of reproducing fine detail.

The absorption and solubility of acrylic resins has been studied in great detail [29,30,31]. The rate and extent of water uptake into polymer networks are controlled by resin polarity, which is in turn dictated by the availability of polar sites available to form hydrogen bonds with water [23]. Water is absorbed into polymer by the polarity of the molecules by means of unsaturated bonds or unbalanced intermolecular forces [23,32]. The contribution of electrostatic forces through the negative-negative charge interactions between Candida Albicans and methyl methacrylate would explain a reduction in Candida cells as the liquid monomer concentration is increased being that methyl methacrylate like Candida is also a negatively charged polymer [15]. Doğan et al. investigated the water absorption of acrylic resins and found that the absorption of resins is a result of ingress of water into the voids of the polymer matrix. These voids are created as a consequence of either air entrapment or leaching of residual monomers due to a continuation of the polymerization reaction [12,33]. The results of this experiment indicate lower water absorption levels with an increase in liquid monomer content in our temperature controlled group. It may be assumed that specimens fabricated with lower powder-liquid ratios presented with a lower mean water absorption levels due to the saturated bonds of the polymer chains containing unfavorable amounts of residual monomer. The residual monomers occupy spaces within the matrix thereby preventing the occurrence of voids capable of being filled by water [34].

Although there appears to be a general trend showing increase in Candida cells with an increased in water absorption, the results obtained from this experiment

indicate that for all groups, water absorption values were statistically insignificant and within the ISO Standard 1567 (1999) specification limit of 32µg/mm^3.

According to Bayraktar et al Immersion of the dental appliance at elevated water temperature ultimately reduces water absorption. There seems to be a correlation between temperature rise and water absorption with our results showing that a temperature rise from 37ºC till 57ºC seems to be associated with a reduction in absorption [22]. The results are in agreement with Bayraktar's observation which suggested that a soaking temperature of below 67ºC shows a lower absorption [21,22]. At higher soaking temperatures an additional polymerization process occurs resulting in lower water absorption values [22,31]. Vallittu et al used high-performance liquid chromatography to detect the level of residual monomer after storage at 22ºC and 37ºC respectively. They found that there was less residual monomer after storage at the higher temperature for both heat and cold-cure resins [12]. However, a temperature rise to 67ºC showed an elevation in absorption values with a corresponding increase in the number of Candida cells observed. It is assumed that at 67ºC the material under investigation may have been subjected to structural changes in the polymer network affecting absorption of moisture. It is assumed that this may have subjected the material to structural changes in the polymer network affecting absorption of moisture. The most plausible explanation for this could be taken from a study conducted by Corcione et al (2013) in which the curing reaction of a cold cure resin was analyzed by thermal analysis as a function of the curing time and sample thickness. Calorimetric analysis were performed to establish the influence of curing time on cross-linking of cold-cure resin. The researchers found an increase in water absorption as it reaches the glass transition temperature [35].

The exact mechanism and nature of chemical alteration of self-cure acrylic resin at or above 67ºC was not considered in this study, however, further work needs to be done to identify an observed change in absorption values.

It seems that surface Candidal growth is favored with an increase in water absorption. Water absorption in turn appears to decrease with an increase in the monomer concentration and post-set soak temperatures. It can be suggested that using the correct powder-liquid ratio and soaking the apparently set self-cure specimens at 57ºC for the first 24 hours might favor in reducing Candidal growth. The results of this study cannot however be considered in isolation. Since, the proliferation of Candidal cells is dependent on a number of variables as mentioned previously, to consider variations in powder-liquid ratios and post-set soak temperatures from an invitro experiment as causative factors of Candida induced infection is unwise. Additionally, residual monomer presence within the set matrix and quantification measurements would have added value in establishing authentic links between monomer use and Candidal growth.

5. Conclusions

Within the limitations of this in-vitro study we can suggest the following conclusions:

1. Even though growth of Candida Albicans is dependent on a number of factors, there seems to be a correlation between powder-liquid ratio and post-set temperature on Candidal proliferation.
2. Candidal growth follows a positive linear relation with an increasing powder-liquid ratio, having cells increasing with a fall in liquid monomer content.
3. Candidal growth follows a negative linear relation with post-set soaking with cells decreasing with an increase in temperature.

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