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Hydration behavior of an experimental tricalcium silicate/tetracalcium phosphate bio-cement in *Streptococcus thermophiles* bacterial solution in comparison with distilled water used as a root canal furcation perforation repair material

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Abstract

Background: The aim of this study is to evaluate an experimental tricalcium silicate phase (C_3S) and tetracalcium phosphate (TTCP) material to be used as a root canal furcation perforation repair. C_3S and TTCP phases were synthesized in nano-size particles by firing the required molar ratios of chemically pure reactants by solid-state reactions at elevated temperatures. The influence of *Streptococcus thermophilus* bacterial medium on the hydration reaction characteristics and morphology of 1:1 composite material of C_3S and TTCP in comparison with distilled water was studied. Setting time, micro-hardness, pH of immersion solution, calcium ion concentration, phosphorous ion concentration, XRD, FTIR, scanning electron microscopy and cytotoxicity of the synthesized composite were investigated, and also, its sealing ability in bacterial media and in distilled water was evaluated.

Results: The results showed that curing of pastes in the bacterial medium did not inhibit the hydration process of the synthesized composite but surface softening due to the great acceleration and encapsulation effects of the highly ionized curing medium resulting in lower micro-hardness values. The dissolution of TTCP phase was also increased in the bacterial medium resulting in precipitation of more hydroxyapatite inside the more porous system of pastes cured in the bacterial solution which was also evident by a non-significant decrease in the sealing ability in bacterial medium.

Conclusions: Mixing of tricalcium silicate (C_3S) and tetracalcium phosphate (TTCP) resulted in a mix that was stable in bacterial medium and could be used for root canal perforation repair.

Keywords: Tricalcium silicate, Tetracalcium phosphate, Calcium silicate hydrate, Hydroxyapatite, Cytotoxicity, Antibacterial, Perforation repair, Root canal

Background

The bioactive calcium phosphate cements, namely tetracalcium phosphate ($Ca_4(PO_4)_2O$) (TTCP), tricalcium phosphate $Ca_3(PO_4)_2$ (TCP), biphasic calcium phosphates (BCP), hydroxyapatite (HA) and tricalcium phosphate (TCP) and hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HA),

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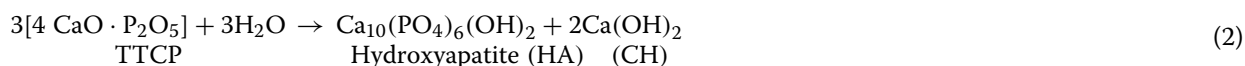
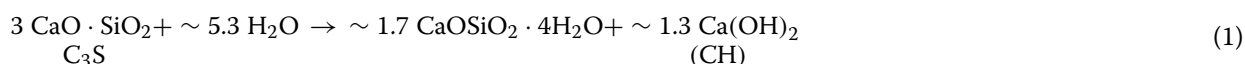
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have been of great interest for medical applications due to their superior properties as bioactive ceramics (Jarcho 1981, De Groot 1983, Hench and Polak 2002). The chemical and physical properties of TTCP render it a suitable biomaterial for dental applications as a filler and sealer for root canal (Sugawara et al. 1995; Chergn et al. 2001; Dickens-Venz et al. 1994). The preparation of TTCP is carried out using CaO–P₂O₅ system at higher temperature above 1300 °C and rapid cooling to a temperature below 1000 °C as the phase is metastable above this temperature (Brown and Epstein 1965, Dickens, et al. 1973; Monma et al. 1986).

Although TTCP material is highly biocompatible, there is a problem concerning using it in dental applications that is plaque adhere resulting in inflammation and root

materials should also be non-resorbable, insoluble in tissue fluids, radio-opaque and dimensionally stable (Buckling and Linck 1987, Huan and Chang 2007, 2008; Laurent et al. 2008, Chen et al. 2009).

The hydration reactions of tetracalcium phosphate are similar to those of tricalcium silicate phase as both materials react with distilled water to form hydrated phases and free lime [Ca(OH)₂] (CH) which are suitable for both medical and dental applications. The free lime liberated during the hydration reactions will result in highly alkaline medium with an increasing pH values that is very useful in destroying bacterial colonies (Brown et al. 1986). The hydration reactions of both materials are represented by the following equations (Martin and Brown 1993a, b; Taylor 1997):



caries (Aranha et al. 2009). Recently, highly pure single phase of tricalcium silicate (C₃S) prepared in the laboratory has been used as a biomaterial for medical and dental applications that differs from Portland cement in mineral trioxide aggregate (MTA) which contains some minor impurities or trace elements during the manufacturing process of Portland cement (Achternbosch et al. 2003, Camilleri 2008, 2011, Monteiro et al. 2008, Schembri et al. 2010).

MTA is the material of choice for sealing root perforations and to perform direct pulp capping as it has several favorable properties including biocompatibility, bioactivity, hydrophilicity, radiopacity, sealing ability and low solubility. High biocompatibility allows for proper healing. This could be evident by the formation of new cementum in cases of root perforations and formation of dentin bridge cases of pulp capping (Faraco and Holland 2001, Tawil et al. 2009). Tricalcium silicate (C₃S) phase has been proved to have better characteristics than MTA such as physicomechanical properties, bioactivity, better injectability and good in vitro degradability. C₃S is considered the main phase present in MTA, and it could also replace MTA in bone repairing as the hardened C₃S pastes can be coated with hydroxyapatite (Martin and Brown 1993a, b). The C₃S cement can adhere to the root canal wall to maintain good sealing ability and better biocompatibility or bioactivity that makes it suitable for use as a root end and posterior restorative biomaterial. These

The word hydration in cement chemistry means complete change of the anhydrous phases used in this investigation upon mixing with distilled water at ambient temperature. When mixing the dry material powder with the desired amount of distilled water, a workable paste is obtained. The stiffening of the paste occurred during the setting process within a short time while hardening of the rigid paste which is responsible for the strength development will be continued by curing of the hardened pastes under distilled water or any other curing medium according to the application field (Taylor 1997). As the composite bio-cement used in this study (C₃S + TTCP) is used in medical and dental applications, their hydration reactions and hydrated products may be affected by the composition of curing medium even if it is physiological or bacterial solution.

Several pathogens have been associated with injuries in the periodontium that are related to endodontic causes; among these are bacteria, virus and fungi. The presence of these pathogens could alter the physical and mechanical properties of the sealing material in contact with them (Jung et al. 2000; Nair 2004; Trope et al. 2006; Ozok et al. 2012).

Thus, the aim of this investigation was to study the influence of *Streptococcus thermophilus* bacteria incubated in nutrient broth media on the hydration reaction characteristics and morphology of the composite material based on tricalcium silicate (C₃S) and tetracalcium phosphate (TTCP) in comparison with distilled water and also the effect of this bacterial medium on

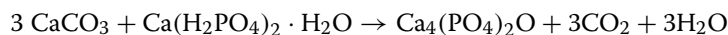
the sealing ability of the materials used. Investigation of setting time, micro-hardness, pH of immersion solution, X-ray diffraction analysis, infrared spectroscopy, scanning electron microscopy and cytotoxicity of the synthesized composite was also carried out, to evaluate ex vivo its sealing ability when used as a furcation perforation repair material.

Methods

Materials preparation and characterization

C_3S phase (≈ 27 nm) was done by firing cubes of molded 3:1 CaO/SiO_2 molar ratio, using ultra-pure limestone and quartz (99.6% SiO_2), with 0.5% boric acid at 1000 °C, and kept for 2 h (Lea 2004). The produced material was ground, and carbon tetrachloride was used for remolding and fired at 1450 °C for another 2 h. This firing process was redone till there was no more reaction. The final produced material was examined if there was free lime.

Commonly, TTCP is prepared using solid-state reaction at high temperature (Brown and chow 1985; Chow and Takagi 1996; Matsuya et al. 1999; Jalota et al. 2005). This occurs by mixing of calcium carbonate ($CaCO_3$) and monocalcium phosphate monohydrate ($Ca(H_2PO_4) \cdot H_2O$) in a molar ratio of 3:1 in the presence of n-heptane for 16 h. The produced slurry was then filtered and dried at room temperature. Following this, it was heated to 1450–1500 °C for 6 to 12 h and quickly quenched to room temperature using this chemical reaction:



The resulting powder was milled in n-heptane for 8 h until particle size of ≈ 34 nm, dried at 100 °C and stored in vacuum to avoid hydration (Sargin et al. 1997). X-ray diffraction (XRD) was used to evaluate the two synthesized powders to check the identity of the synthesized compound. Copper target with wavelength radiation = 1.54-nm was used, X-ray generation of 40-kV and current of 2–5 Ma (Fig. 1). Fine grounding of the final materials was done using agate mill till reaching the desired particle size as shown in Fig. 2, and this was evaluated using transmission electron microscope (TEM) (JEM-1230) at 100 kV.

The synthesized tricalcium silicate (C_3S) and tetracalcium phosphate (TTCP) phases were used to produce the experimental material with the following weight percentages:

- 45 wt% C_3S phase.
- 45 wt% TTCP phase.
- 10 wt% chemically pure bismuth oxide (Bi_2O_3).

The experimental material was dry mixed, homogenized and finely ground for 3 h with the aid of Retsch GmbH PM100, German-milling machine, to pass the above-mentioned 270 mesh standard sieve.

Two types of aqueous solutions, namely distilled water and *Streptococcus thermophilus* bacteria in nutrient broth media (NB media), were used as curing

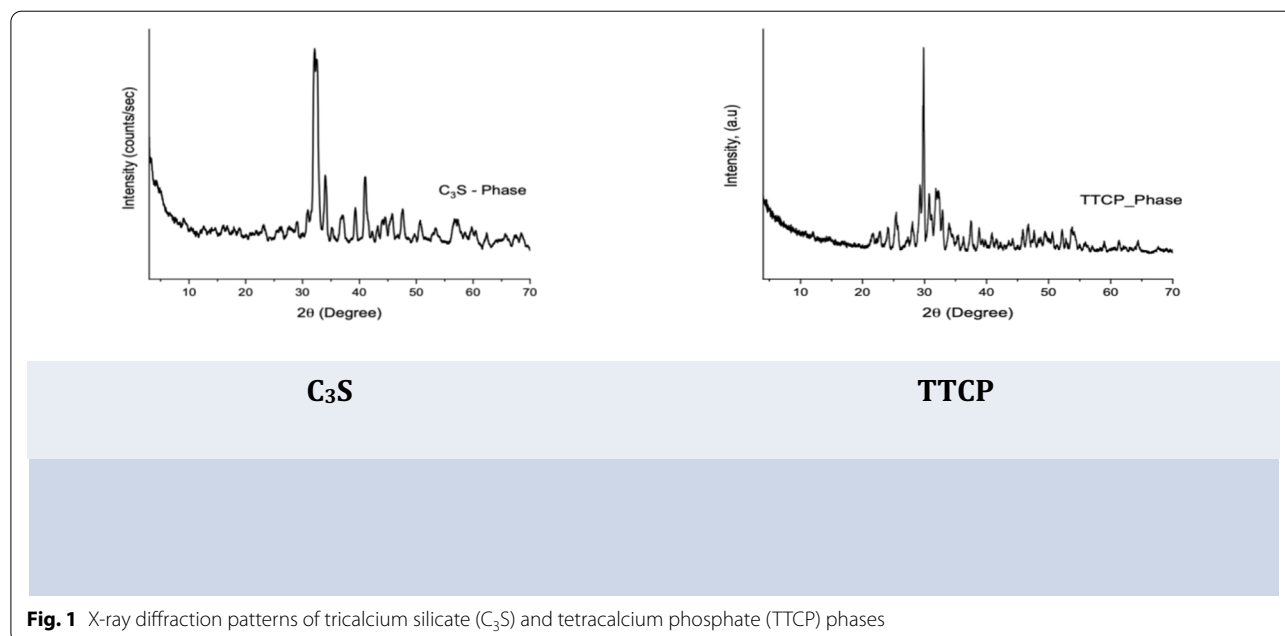


Fig. 1 X-ray diffraction patterns of tricalcium silicate (C_3S) and tetracalcium phosphate (TTCP) phases

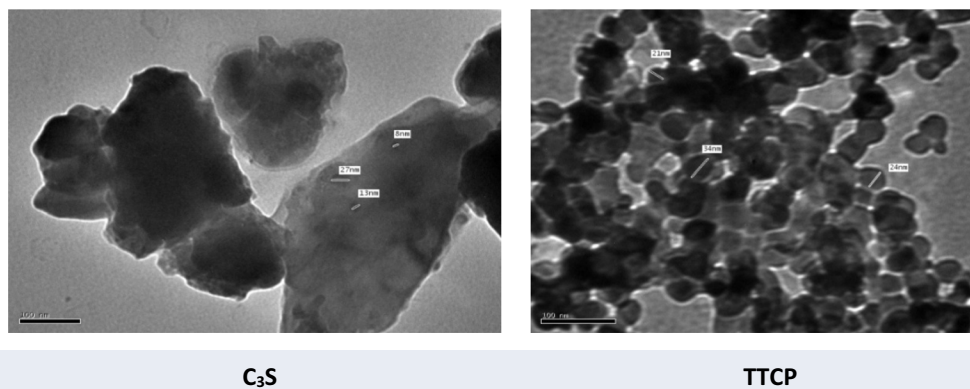


Fig. 2 Transmission electron photomicrographs (TEM) of tricalcium silicate (nano-size C_3S phase: 8–27 nm) and tetracalcium phosphate (nano-size TTCP: 21–34 nm) phases

liquids for the pastes at 37 °C. To prepare a workable paste, the synthesized materials were mixed in a water/powder ratio of 0.25 ml/g. A mold was filled with the paste and compacted until a homogenous specimen was obtained. Curing was done in a 100% humidity chamber with temperature of 37 °C for 24 h immediately after molding. The samples were removed from the mold and cured under distilled water of temperature 37 °C and kept till testing time at 1, 3, 7 and 14 days.

- Chemical composition of NB media (g/L).
- Peptone 10.0.
- Beef extract 10.0.
- Sodium chloride 5.0.
- pH after sterilization 7.3 ± 0.1 .

The incubation of the material was carried out in NB media, and 25 ml of the broth in 100-ml conical flasks was first sterilized and inoculated with 200 μ l of *Streptococcus thermophilus* at 5×10^4 CFU/ml (Greenberg et al. 1985).

Setting time

Vicat apparatus was used to measure the final setting time of the experimental material mixed with a water/powder ratio of 0.25 ml/g. The produced paste was inserted into a disk mold of dimensions: 10 mm diameter and 2 mm in thickness. A Gilmore needle (with a flat end diameter of 2 mm and weighting 100 g load) was used after 120 s from the start of mixing. The needle was then lowered gently to the flat surface of the experimental material. Each 30-s interval, this procedure was repeated till the indenter could no longer make a complete circular

mark on the surface of the tested material. This measurement was repeated for five samples.

X-ray diffraction

This analysis was done on some samples using Philips X-ray diffractometer PW 1730 with Ni-filtered Cu-K α X-ray radiation ($\lambda = 1.5406$ Å) powered at 40 kV and 30 mA. The diffraction data were recorded in the 2θ range from 5° to 70°, counting for 10 s in steps of $\Delta(2\theta) = 0.01^\circ$.

ATR/FTIR spectroscopy

IR spectra were measured for samples using JASCO model FTIR 6100 spectrometer in the range 4000–400 cm^{-1} with resolution 4 cm^{-1} .

SEM-EDS analysis

Selected samples were investigated for the morphology using scanning electron microscopy (SEM) and also using energy-dispersive X-ray spectra (EDX) (type Inspect S, T810, D8571, FEI Co., Japan) with an accelerating voltage of 30 kV and a magnification from 10 \times to 300,000 \times .

pH of the immersion solution

Specimens mixed with a water/powder ratio of 0.25 ml/g were condensed into a disk of dimensions: 10 mm diameter and 2 mm in thickness. The prepared samples were immediately placed in 20 ml of the two curing media (Nurit et al. 1993; Wang and Sun 2008; Formosaa et al. 2013) and kept at 37 °C in a 100% humidity water bath for the curing periods of 3, 7 and 14 days. The pH values of the solution were measured using a solid-state pH sensor connected to a pH meter (Medika Scientific Jenway bench top pH meter, England).

Determination of calcium and phosphorus ions

The concentration of calcium and phosphorus ions released in the two curing media was measured by Agilent 5100 inductively coupled plasma-optical emission spectrometer (ICP-OES) with synchronous vertical dual view (SVDV). Intensity calibration curve was constructed for each series of measurements. Accuracy of the measurements was confirmed using external reference standards from Merck and standard reference material for trace elements in water. Quality control samples from National Institute of Standards and Technology (NIST) were also used to confirm the instrument reading (APHA 2017).

Micro-hardness

Micro-hardness evaluation was done at 24 h and 28 days. Ten specimens were condensed in a mold of 15 mm diameter and 2 mm thickness and cured for 24 h at 37 ± 1 °C in an incubator. The specimens were then removed from the molds and subjected to micro-hardness test after 24 h using Vickers micro-hardness tester (Nexus 4503, Innova Test, Netherlands, Europe). For each sample, three randomized indentations were made using 100-g load, with a dwell time of 10 s. Calculations were made using Vickers software. The tested specimens were then randomly divided into two groups according to the immersion media, either DW or NB media for 28 days. After the immersion period of 28 days, the specimens were dried in a desiccator. The surface of each specimen was ground using progressive fine grits of silicon carbide paper, from 180-grit to 1200-grit, and then polished using polycrystalline diamond paste. Then, measurement of micro-hardness was performed again as mentioned before (Formosaa et al. 2013).

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan.

Procedure

All procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in DMEM-F12 medium with 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000 µg/ml Streptomycin Sulfate and 25 µg/ml Amphotericin B) and 1% L-glutamine at 37 °C under 5% CO₂.

Cells were batch cultured for 5 days and then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO₂ using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media were aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (µg/ml). After 48 h of incubation, medium was aspirated, and 40 µl MTT salt (2.5 µg/ml) was added to each well and incubated for further four hours at 37 °C under 5% CO₂. To stop the reaction and dissolving the formed crystals, 200 µL of 10% sodium dodecyl sulfate (SDS) in deionized water was added to each well and incubated overnight at 37 °C. A positive control which composed of 100 µg/ml was used as a known cytotoxic natural agent which gives 100% lethality under the same conditions.

A microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) was used to measure the absorbance at 595 nm and a reference wavelength of 620 nm. The percentage of change in viability was calculated using the following formula:

$$\left(\frac{\text{Reading of extract}}{\text{Reading of negative control}} - 1 \right) \times 100$$

Cytotoxicity test

Cell culture

1. Preparation of sample.

Black plastic sample was soaked in alone with media for 48 h in refrigerator, and then, the media were added to the cell.

2. Cytotoxic effect on human normal fibroblast cell line (BJ1).

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT

A probit analysis was carried for IC₅₀ and IC₉₀ determination using SPSS 11 program (Bassyouni et al 2014).

Sealing ability test

Forty teeth were selected for sealing ability study. The teeth were painted with a single coat of nail polish to prevent dye penetration into the dentinal tubules or lateral canals especially in the furcation area.

Access opening was prepared, and pulp tissue was removed from the pulp chamber using excavators. Canal orifices were enlarged halfway through the root using Gates Glidden drills sizes 2 and 3 and then sealed with Cavit cement to prevent any leakage through the canal orifices. Furcation perforation was made in the center of

the pulp chamber floor using # 2 long-shank round bur under efficient water coolant. All preparations were then rinsed with saline and dried with compressed air to be free of any debris.

The teeth were divided into two groups according to the evaluation period (1 week and 1 month). Each group was further subdivided into two subgroups according to the medium (thermophilus bacteria and distilled water). In all groups, the perforation site was repaired using the experimental material. A moist cotton pellets were passively placed between the roots in the furcation areas of the teeth during the repair of the perforations to simulate the moist clinical field. The material was then condensed into the perforation site using condenser of the proper size until the perforation site was totally obliterated. The pulp chambers and the access cavities of the teeth were filled using Cavit. The teeth were then placed either bacterial medium or distilled water according to the group.

At the end of each evaluation period, the samples were submerged in 2% methylene blue dye for 72 h at room temperature after each evaluation period. After removal from the dye, the samples were thoroughly washed with tap water and the teeth were then split using a surgical chisel and a mallet.

The sections were examined and photographed for linear extent of dye penetration using Canon digital camera connected to a Zeiss stereomicroscope (Technival 2) with magnification (25×). In each sample, the highest point of dye penetration was measured and the percentage of linear extent of dye penetration from the apical end of the repair material to the pulp chamber floor in relation to the total length of perforation was calculated. The degree of dye penetration, in each microscopic field, at the area of perforation was measured using the image analysis software (Image J).

Results

Setting time and micro-hardness

The setting time of the synthesized material was carried out according to “Setting time” section by using the workable paste prepared at a water/powder ratio of 0.22 ml/g. Mixing the dry material powder with distilled water in such proportion results in a workable paste by which final setting time is determined. The average final setting time was found to be 22 min at 37 °C. In this investigation, the micro-hardness was considered as a mechanical property for the influence of *Streptococcus thermophilus* microbe incubated in nutrient broth media (NB) on the hydraulic behavior of the hardened pastes at the different curing periods in comparison with those cured in distilled water. Figure 3 represents the measured hardness values of pastes cured under DW and NB media for different curing ages. Figure 3 reveals that the hardness values of the samples cured under distilled water increase from 1 to 14 days. The samples cured in *Streptococcus thermophilus* microbe incubated in nutrient broth media showed lower hardness values than those cured under distilled water at all hydration periods. The hardness Vickers values of samples cured in bacterial solution increase from 1 to 3 days followed by a decrease for 7- and 28-day samples that may be due to the adverse effect of the *Streptococcus thermophilus* microbe incubated in nutrient broth media on the mechanical properties of the prepared pastes.

pH of the curing media

Figure 4 illustrates the variation in pH values of the two immersing media with the curing periods. It is clear from Fig. 4 that the pH values at all curing times are higher for pastes cured in distilled water than those cured in NB medium (*Streptococcus thermophilus* microbe incubated

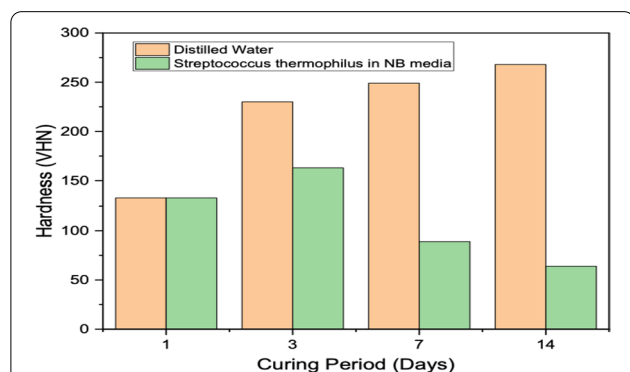


Fig. 3 Micro-hardness data of the hardened pastes cured in distilled water (DW) and *Streptococcus thermophilus* bacteria in nutrient broth media (NB media)

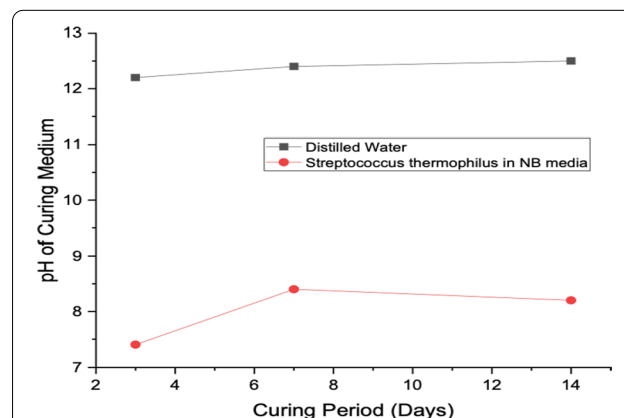
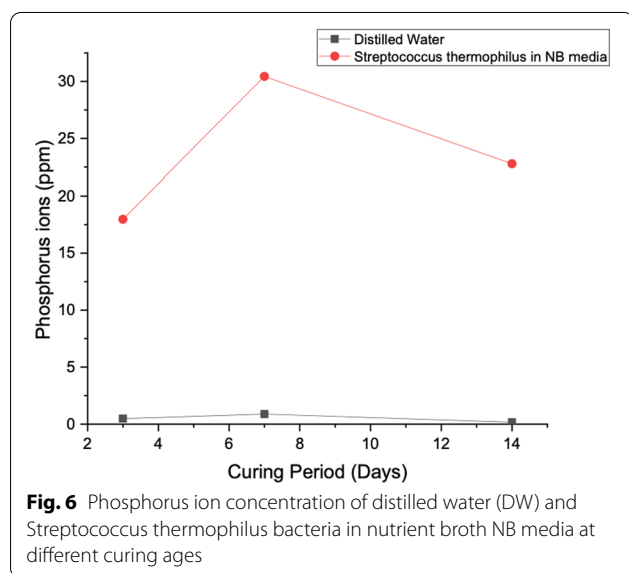
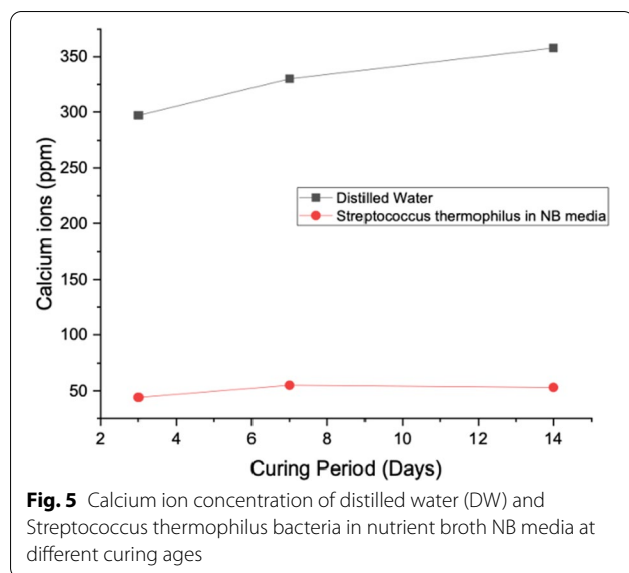


Fig. 4 pH values of distilled water (DW) and *Streptococcus thermophilus* bacteria in nutrient broth NB media at different curing ages



in nutrient broth media microbe). The pH of the samples cured in distilled water showed a slight increase from 3 to 14 days. The pH of the NB bacterial medium increased from the value 7.4 after 3 days to 8.4 at 7 days and then showed slight decrease after 14 days curing medium.

Calcium and phosphorus ions

The data of calcium ion concentrations for both curing media are given in Fig. 5. Figure 5 reveals that the values of calcium ion increase with the hydration period in case of distilled water (DW), while in bacterial solution a little change in calcium ion values was detected. Moreover, the calcium ion concentrations in case of

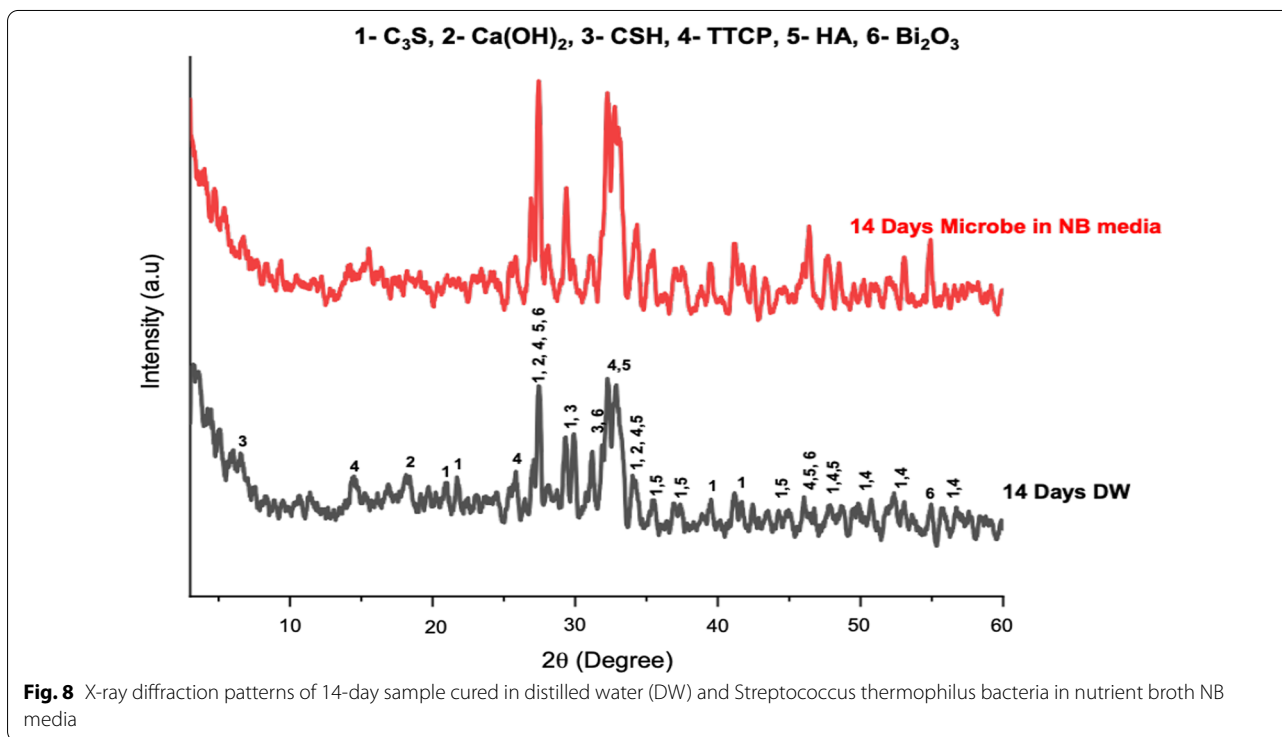
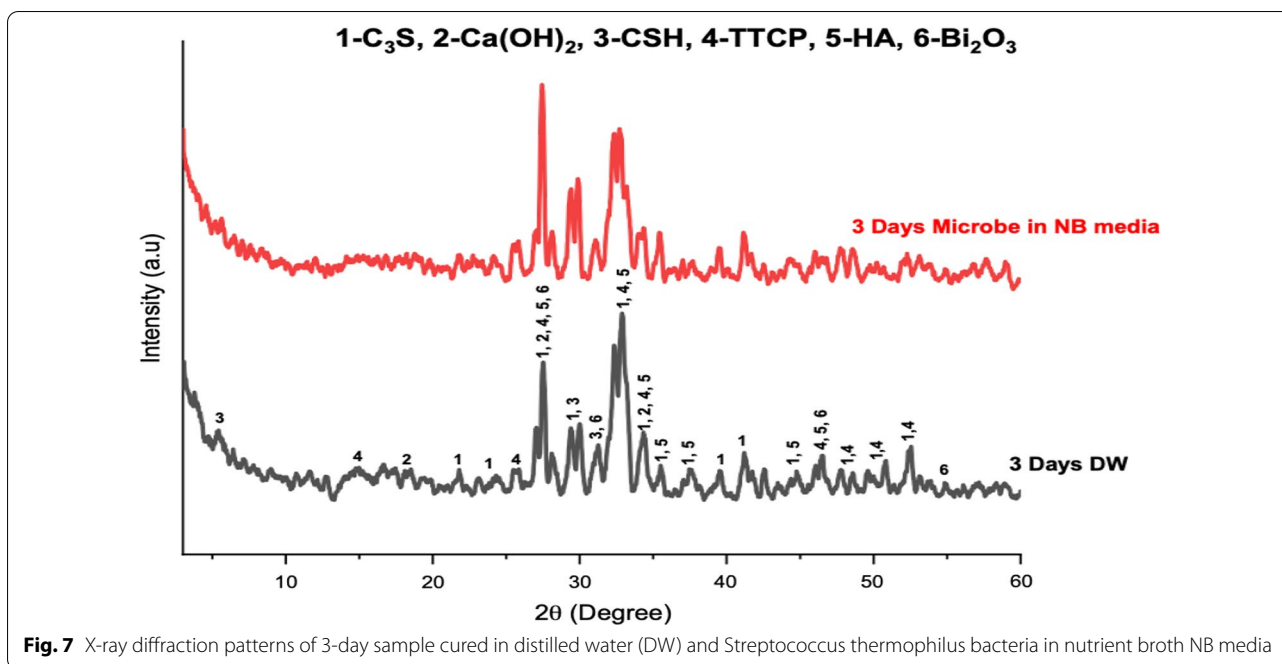
distilled water were higher than those in NB bacterial medium at all curing ages. Figure 6 illustrates the phosphorus ion concentrations in both curing media with the hydration period. The data given in Fig. 6 showed a trace amounts of phosphorus ions in distilled water at all curing ages, while in case of NB medium phosphorus ion concentrations were higher than those determined in distilled water. In case of NB bacterial medium, the phosphorus ion concentrations increased at 7 days and then decreased again after 14 days hydration age.

X-ray diffraction

The X-ray diffraction patterns of samples cured for 3 and 14 days in distilled water and NB bacterial medium are given in Figs. 7 and 8. The XRD patterns illustrate the characteristic peaks of the remaining anhydrous phases (C_3S and TTCP) as well as the hydrated compounds of the composite material. Due to the complex hydration reactions of the hardened samples cured in both distilled water (DW) and Streptococcus thermophilus microbe, an overlapping of the characteristic XRD peaks was found for many hydration products. The XRD data given in Fig. 7 showed that the peak heights of the anhydrous C_3S phase were slightly lower for samples cured in distilled water than those immersed in the NB medium. The characteristic peak of the main calcium silicate hydrate (CSH) could be observed in XRD patterns for both curing media. The XRD peaks of liberated free lime [$Ca(OH)_2$] are found in the pattern of samples in DW, but this peaks are slightly lower for samples in NB medium. The characteristic peak of hydroxyapatite (HA) which is the main hydration product of TTCP phase can be seen in the XRD patterns. The HA peaks heights are slightly larger for samples immersed in the bacterial solution as they may overlap with many other anhydrous phases XRD peaks. The XRD data of the samples immersed in the two different curing media for 14 days illustrated in Fig. 8 reveal the same findings mentioned above for the 3-day sample. However, there is a more increase in the peaks height of the hydrated compounds (CSH and HA) of both C_3S and TTCP phases for samples cured in the bacterial solution than those immersed in distilled water.

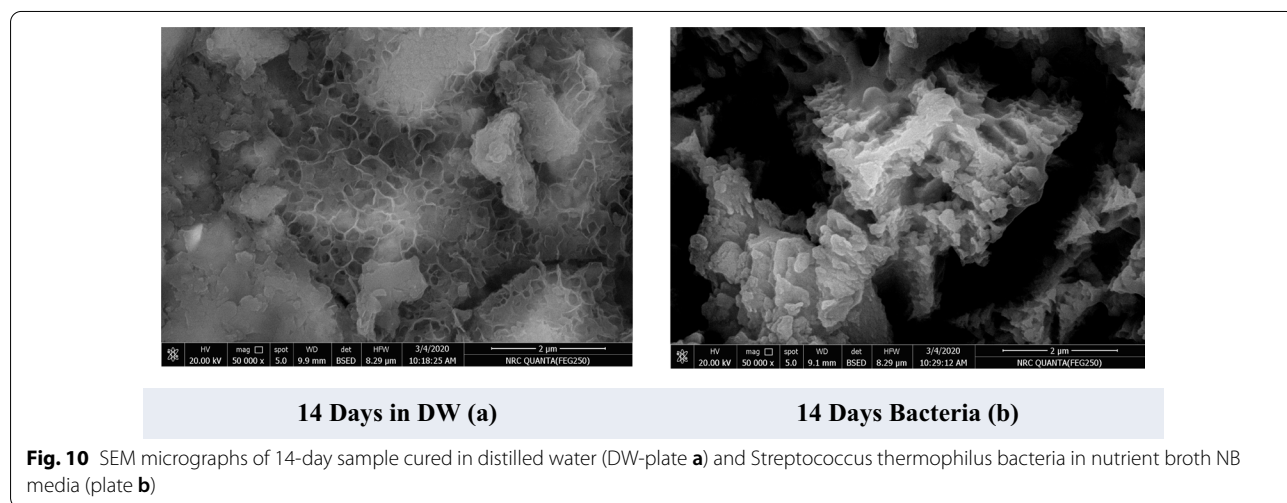
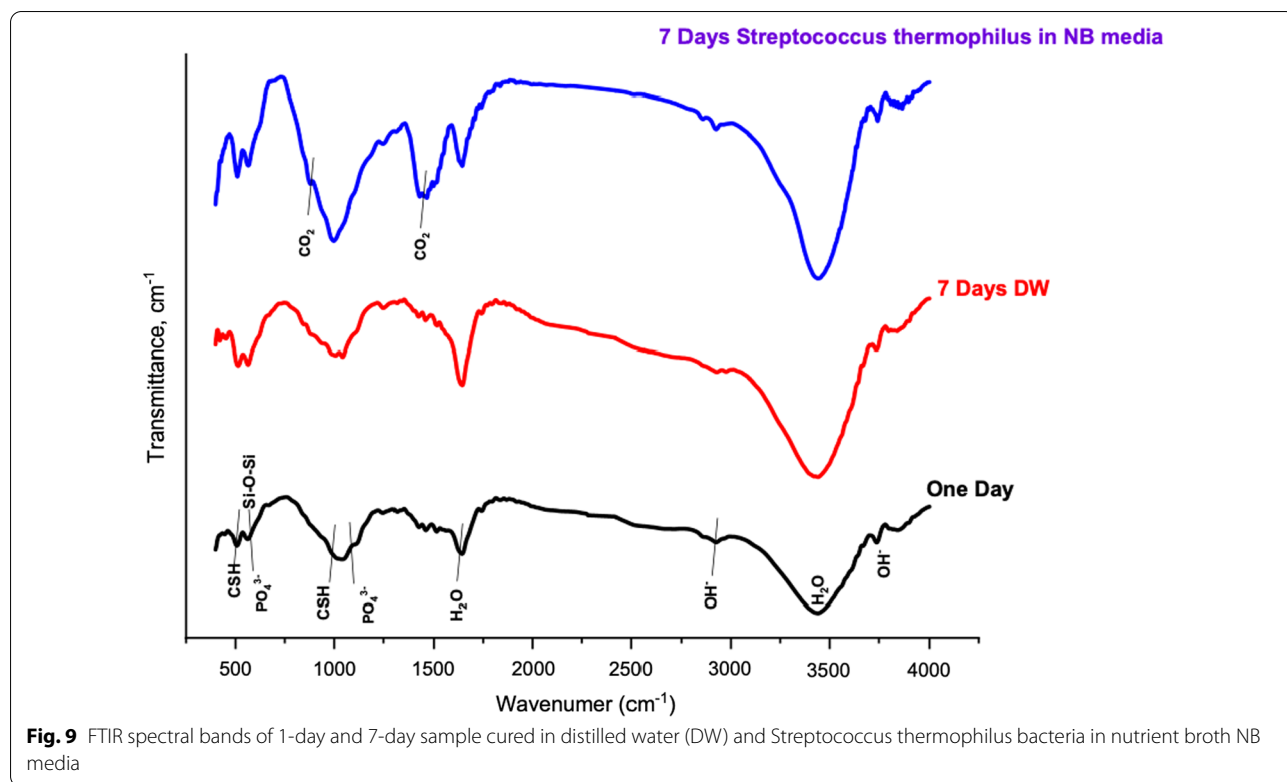
FTIR spectroscopy

To follow up the hydration process and emphasize the XRD results mentioned above, the FTIR spectral analysis was carried out on the samples cured for 7 days in both curing media in comparison with the one-day paste sample. The FTIR spectra given in Fig. 9 indicate the presence of the characteristic IR bands of C–S–H phase at $\sim 445, 815\text{--}950\text{ cm}^{-1}$ that are overlapping with



the IR bands of Si–O–Si \approx 491–667 cm^{-1} and those of $\nu_4\text{PO}_4^{-3}$ at \approx 560–600 (amorphous calcium phosphate) and $\nu_3\text{PO}_4^{-3}$ at \approx 1030 cm^{-1} (antisymmetric stretching). The FTIR spectra of the samples cured in SBF solution illustrated in Fig. 9 reveal a little decrease

in the IR bands characteristic for C–S–H, ν_3 and $\nu_4\text{PO}_4^{-3}$ corresponding to hydroxyapatite (HA), while for samples cured in the bacterial solution, a little increase and broadening of those bands were detected. The characteristic IR bands of aragonite antisymmetric



stretching ν_2 and ν_3 $\text{CO}_3^{2-} \approx 850, 1460$ and 1490 cm^{-1} were sharply found for samples immersed in the bacterial solution, but they showed a very small shoulders for the one- and 7-day samples of distilled water. The FTIR spectra given in Fig. 9 showed the presence of OH^- stretching in $\text{Ca}(\text{OH})_2$ or hydroxyapatite (HA) $\approx 2500\text{--}3700$ and 3650 cm^{-1} that were increased for the samples cured the bacterial solution. The IR bands corresponding to the molecular water incorporated with

all hydrated phases ≈ 1635 and 3365 cm^{-1} were also seen in all IR spectra.

SEM-EDS analysis

The SEM micrographs of the 14-day samples immersed in both curing media are illustrated in Fig. 10a, b. Investigation of the micrographs indicates the presence of fibrous crystals of calcium silicate hydrate (CSH) and platelets of hydroxyapatite (HA) that are embedded in the pores and

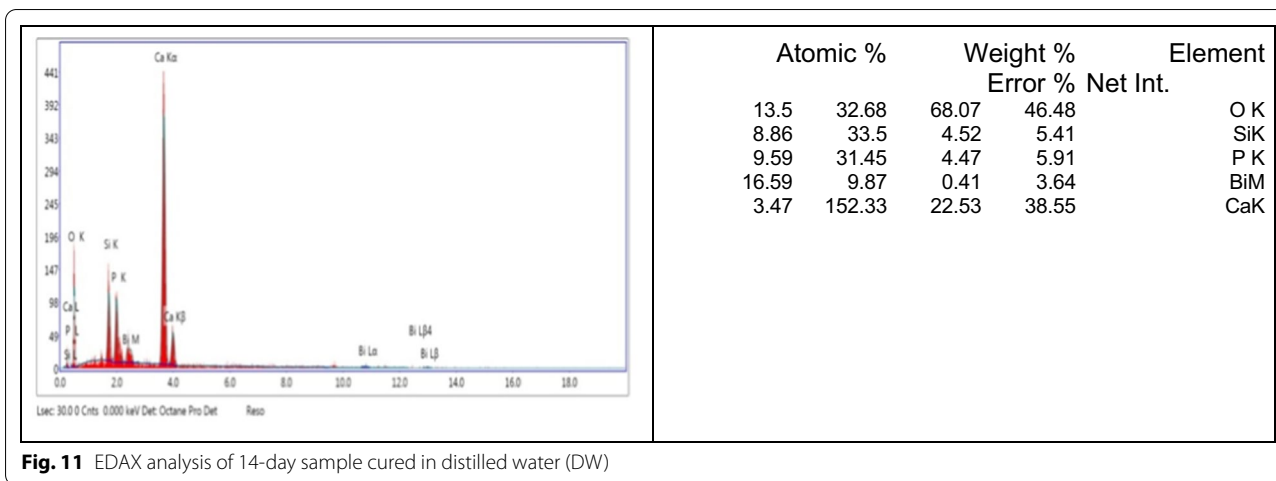


Fig. 11 EDAX analysis of 14-day sample cured in distilled water (DW)

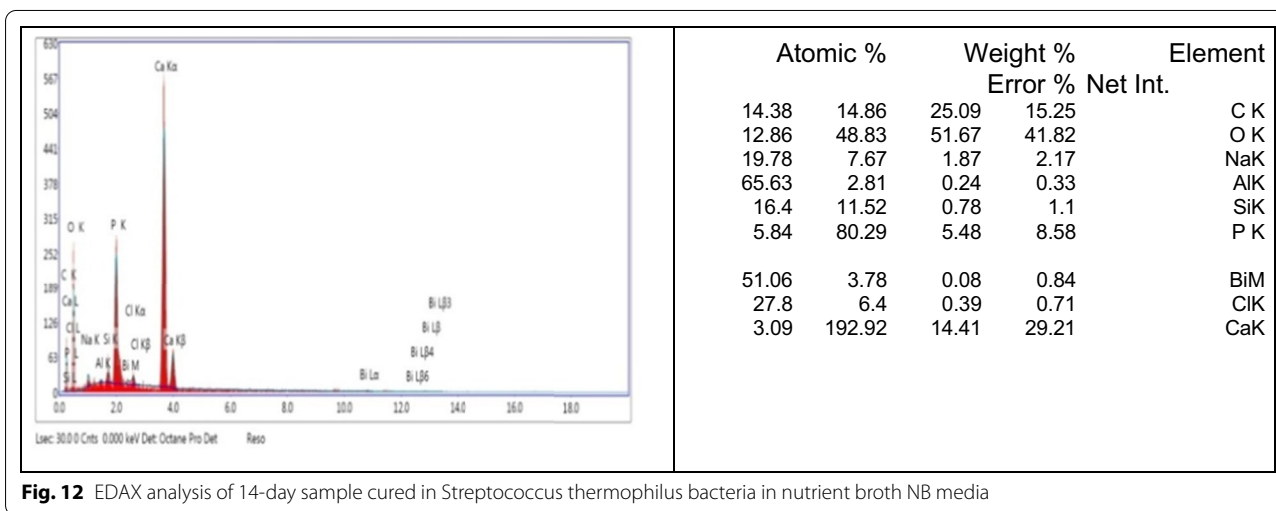


Fig. 12 EDAX analysis of 14-day sample cured in *Streptococcus thermophilus* bacteria in nutrient broth NB media

Table 1 Mean and standard deviation of the sealing ability of the experimental material in both media

	Thermophilus bacteria		Distilled water		P value
	Mean	SD	Mean	SD	
1w	27.2	2.4	21.9	1.6	0.06
1 month	31.4	2.9	21.3	2.6	0.1

deposited over the anhydrous particles. Due to the large amounts of these hydrated compounds, the free lime [Ca(OH)₂] plates could not be clearly detected. Although there is a great similarity in the morphology and crystal shapes of the samples cured in both distilled water (plate a) and bacterial solution (plate b), the micrograph of the sample in DW shows more dense and close structure than those cured in the bacteria (Figs. 11, 12).

Cytotoxicity test

The sample was tested against the normal human epithelial cell line: 1 – BJ1 (normal Skin fibroblast with sample concentration range between (100–0.78 µg/ml) using MTT assay. The cytotoxicity data showed that the synthesized composite was highly safe to normal skin fibroblast cells with cell variability of 91.8% at 100 ppm sample concentration.

Sealing ability

Table 1 shows the mean and standard deviation of the sealing ability results showing that experimental material in distilled water had better sealing ability than in bacterial media; however, this difference was not statistically significant for both evaluation periods.

Discussion

The synthesized bio-composite is mainly composed of C_3S and TTCP phases that undergo hydration process in aqueous medium via similar hydration reactions with distilled water according to the chemical equations mentioned above (Eqs. 1, 2) resulting in a rigid structure having desirable physical and mechanical properties (Brown 1999). The setting process can be explained by the hydration reactions of C_3S that is the main hydraulic component of MTA materials and TTCP phase during the first few minutes after mixing with distilled water. The hardening process of the set pastes begins through precipitation of more hydration products, namely calcium silicate hydrate (CSH), hydroxyapatite (HA) and calcium hydroxide [$Ca(OH)_2$], during immersing the pastes for different curing periods under distilled water or any other curing medium at 37 °C (Camilleri 2008, 2011). Curing of C_3S phase in the incubator at 37 °C may enhance the rate of hydration reactions if it is used in medical or dental applications (Taylor 1997).

Although TTCP phase ($4CaO.P_2O_5$) undergoes hydration reactions (at 37 °C), it can develop a relatively good physical properties but weak mechanical properties (Martin and Brown 1993a; b; Taylor 1997; Lea 2004). The tetracalcium phosphate phase (TTCP) reacts with distilled H_2O [DW] in a slow reaction rate resulting in the formation of the main hydration products that are hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] and free lime [$Ca(OH)_2$] (Martin and Brown 1993a; b; Taylor 1997). Due to the fact that tetracalcium phosphate (TTCP) phase is highly biocompatible material, it was mixed with tricalcium silicate (C_3S) phase in a ratio of 1:1 to improve its biocompatibility and setting properties. To provide adequate radio opacity needed for medical and dental applications, 10% by weight bismuth oxide was added to the formulated bio-cement. The added amount of bismuth oxide did not show any adverse effect on the hydration process of the prepared composite. It was stated that hydration process of calcium silicate and calcium phosphate phases is greatly affected by the chemical composition of the curing media at constant curing temperature (37 °C) (Taylor 1997; Lea 2004). The influence of *Streptococcus thermophilus* bacteria in nutrient broth media (NB media) as an expected bacterial curing medium when using these phases in medical or dental applications in comparison with the normal distilled water (DW) was investigated. The micro-hardness Vickers data given in Fig. 3 showed a continuous increase from 1 up to 14 days for the samples cured in distilled water (DW) due to the formation of calcium silicate hydrate which is responsible for the hydraulic properties. The continuous decrease in hardness values for the samples cured in bacterial solution from 3 to 14 curing days may be attributed

to the adverse effect of the *Streptococcus thermophilus* bacteria present in the hydration medium as the bacterial action may cause softening of the sample surface (Taylor 1997). The chemical composition of the nutrient broth media (NB media) contains 5 g/L (0.5%) of sodium chloride (NaCl) which will be completely ionized to Na^+ and Cl^- in the hydration medium. The hydration reactions of C_3S and TTCP phases are very sensitive to the free ions present in the bacterial solution. It was stated in a previous studies that the presence of low concentration of NaCl in the curing medium may accelerate the rate of hydration reactions as these free ions may enhance the dissolution of the two phases in the hydration medium (Taylor 1997; Roy et al 2000; Lea 2004). The flocculation of hydrophilic particles is enhanced by the action of cations and anions due to their competition with water molecules keeping them apart leading to the promotion of the hydration process (Tay et al. 2007). The sodium chloride (NaCl) salt present in the bacterial medium will accelerate the hydration process through enhancing the dissolution of C_3S and TTCP phases in the curing medium, and there will be an activation effect due to the formed NaOH from the reaction between Na^+ and OH^- present in the hydration medium as sodium hydroxide is considered an activator for the hydration process of the two composite phases (Taylor 1997). Regarding the samples cured in the bacterial solution, there are three factors that may accelerate and activate the hydration process, namely, the free ions (Na^+ and Cl^-) present in the medium, NaOH results from the reaction between Na^+ and free OH^- ions and curing at 37 °C which will be responsible for fast and abnormal hydration process that causes an encapsulation of the anhydrous particles by the formed hydrated compounds on its surface in addition to the surface softening due to the adverse bacterial action which are responsible for the decrease of hardness value of the samples cured under the bacterial NB media (Taylor 1997). According to the hydration reactions (Eqs. 1, 2) of C_3S and TTCP phases, the liberated free lime [$Ca(OH)_2$] is responsible for the increased pH values (alkalinity) of the reaction medium and it is taken as a measure for the hydration process (Brown 1999; Tay et al. 2007). The pH values of samples cured in DW given in Fig. 4 showed higher values than those for the samples cured in the bacterial solution at all hydration ages. Due to the adverse effect of the *Streptococcus thermophilus* bacteria (NB media) on the hydration process of the investigated material, the pH values showed a slight increase from 3- to 7-day samples followed by a decrease again at 14-day samples which emphasize the encapsulation of the anhydrous particles as mentioned above for samples cured in the bacterial solution. The data of Ca^{2+} ion concentration given in Fig. 5 are in agreement with the findings of pH data

as the calcium ions present in the curing medium are mainly related to the liberated $\text{Ca}(\text{OH})_2$ resulted from the hydration of both C_3S and TTCP phases. The data given in Fig. 5 emphasized an increased Ca^{2+} ion value for samples immersed in DW than those cured in the bacterial solution. On the other hand the data of phosphorus ion concentrations given in Fig. 6 showed an increased values of phosphorus ions for bacterial solution than distilled water at all curing ages that may be due the high dissolution of tetra-calcium phosphate phase (TTCP) in the bacterial medium by the action of *Streptococcus thermophilus* bacteria as well as the free Na^+ and Cl^- ions. The XRD patterns of 3-day samples given in Fig. 7 showed an increase in peak intensities characteristic for C–S–H and HA of the samples cured in bacterial solution together with a clear decrease in the peak heights of the anhydrous composite powder comparing with the samples cured in distilled water that is due to the acceleration and activation effect at early curing ages in the bacterial solution. On the other hand, the XRD patterns of 14-day samples immersed in DW illustrated in Fig. 8 showed more diminishing of the XRD peak height of the anhydrous phases than those cured in the bacterial medium and an increase in the peak heights of the hydrated phases which is attributed to the encapsulation of the anhydrous particles by the fast precipitation of the hydrated phases on the particle surface keeping the powder inside dry as was discussed above. The changes in FTIR bands of the 7-day samples given in Fig. 9 agreed with the XRD findings as there was an increase in the intensities of IR bands corresponding to C–S–H phase at $\sim 445, 815\text{--}950\text{ cm}^{-1}$, those for $\nu_4\text{PO}_4^{-3}$ at $\approx 560\text{--}600$ (amorphous calcium phosphate), $\nu_3\text{PO}_4^{-3}$ at $\approx 1030\text{ cm}^{-1}$ (antisymmetric stretching) characteristic for hydroxyapatite (HA) and the bands corresponding to OH^- related to free lime for the samples cured in DW more than those cured in bacterial medium due to the encapsulation effect of the anhydrous phase particles rather than the phase dissolution in the curing medium. The micrographs of the 14-day samples given in Fig. 10 illustrate the influence of the bacterial medium on the microstructure of the hardened samples in comparison with distilled water (DW). The samples cured in DW showed more closed structure with a huge precipitated C–S–H fibrous and platelets of hydroxyapatite (HA) in a homogenous microstructure. The micrograph of the sample cured in the bacterial solution showed a more open structure with agglomerations of anhydrous particles coated with crystals of the hydrated phases in a highly pores structure. The elemental analyses (EDX) illustrated in Figs. 11 and 12 emphasized the presence of phosphorous in both samples and the increase in its concentration in the sample cured in the bacterial medium due to the enhanced dissolution of the TTCP

phase in the curing medium that leads to the precipitation of more HA crystals in this pores microstructure.

Sealing ability at both evaluation periods was better for distilled water than bacterial media, and this could be attributed to the adverse effect of bacterial media on the structure of the material which was also shown in decreased hardness in the bacterial media.

Conclusions

The added amount of the experimental nano-size tetracalcium phosphate (TTCP) in a 1:1 ratio to the synthesized nanoparticles of the ultra-pure single MTA phase (C_3S) improved the biocompatibility and setting properties of the tricalcium silicate phase for dental applications. Tetracalcium phosphate (TTCP) reacts with distilled water in a process called hydration process similar to that of tricalcium silicate (C_3S) but in a slower rate of hydration reactions. The main hydration product of C_3S phase that is C–S–H is mainly responsible for the mechanical properties of the hardened paste, while hydroxyapatite (HA) for TTCP phase has its main role in setting process. The use of *Streptococcus thermophilus* bacteria in nutrient broth media (NB media) as curing solution markedly affects the hydration process of the experimental material due to the adverse bacterial softening influence of the hardened particle surface and great acceleration and activation of the hydration reactions of both phases which results in an encapsulation of the anhydrous particles forming a much more open pores microstructure. This highly ionized curing medium enhanced the dissolution of TTCP phase in the pore system which results in a precipitation of more HA hydrated phase in the porous system. However, it affected the sealing ability of the material when used as a furcation repair material.

Abbreviations

TTCP: Tetracalcium phosphate; TCP: Tricalcium phosphate; BCP: Biphasic calcium phosphates; HA: Hydroxyapatite; C_3S : Tricalcium silicate; MTA: Mineral trioxide aggregate; XRD: X-ray diffraction; TEM: Transmission electron microscope; SEM: Scanning electron microscopy; NB: Nutrient broth.

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Author contributions

MR synthesized and prepared the experimental material and performed the characterization tests for the prepared material, and MK performed the physical properties tests and the ex vivo study. Both authors have read and approved the manuscript.

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Availability of data and materials

The authors declare that the data supporting the findings of this study are available within the article.

Declarations

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Competing interests

The authors declare no competing interests.

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