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Biosynthesis, Characterization and Antibacterial Activity of Hydroxyapatite

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Abstract: In the present study, focuses biosynthesis of hydroxyapatite from *klebsiella* sp PSM7 which was isolated from bone decomposing area. The synthesized hydroxyapatite have been characterized by FTIR, XRD, FESEM and EDX. The characterization analysis reveals that the biosynthesized hydroxyapatite shows irregular spherical structure and irregular pore ranges in these crystals from 150 to 270 nm. The microbial synthesized hydroxyapatite composite with natural biopolymer of chitosan, gelatin and bone ash (HApGCB) possessed the important feature that needed for bone tissue engineering. Chitosan is binds to the bacterial cell membranes and increases their permeability resulting in the rupture and leakage of intracellular components. Chitosan complexes with biopolymer of gelatin have a good blending capability hydroxyapatite. In this study, bone composite of nature biopolymers possess good antagonistic against to the bone pathogenic microorganisms of Gram positive (*S. aureus*) and Gram negative bacteria (*E. coli*). The HApGCB composite could be applicable as bone substitute for bone tissue engineering and bone regenerative medicine.

Keywords: Hydroxyapatite, chitosan, gelatin, bone ash, HApGCB

1. INTRODUCTION

Stress fractures are well recognized in military training and athletes, with the first reported case being identified in 1855 by Breithaupt and the first imaging of a stress fracture recorded by Stechowin1897. The incidence of sustained stress fractures in military recruits can be as high as 12%, as compared with a rate of 21.1% of elite athletes and 1% of the general population (Friedl et al., 1992). The injuries can be responsible for a significant of attrition in military training with consequent financial implication for military budgets (Almeida et al., 1999, Almeida et al., 1999). Soldiers and athletes are the two main groups that have motivated research in bone tissue engineering due the risk of training injuries. Over the past 20 years, Various biomaterials has expanded the use of nature polymers including inorganic composite such as collagen, hydroxyapatite, β-tricalcium phosphate, hyaluronic acid and saturated aliphatic polyesters like poly (lactic acid) (PLA), Polyphosphazenes, copolymers polylactic-coglycolic acid (PLGA) and polycaprolactone (PCL) as well as poly (glycolic acid) (Jagur-Grodzinski et al., 1999, Pitt et al., 1981) (PGA) in bone research. Bone tissue repair accounts for approximately 500,000 surgical procedures per year in the United States alone. Angiogenesis, osteogenesis and chronic wound healing are all natural repair mechanisms that occur in the human body. The bone grafting materials worldwide commonly used for bone transplantation such as auto grafts, allografting and xenografting have the disadvantages of causing immune reaction and transferring pathogens.

Hydroxyl apatite or also called hydroxyapatite (HAp) is a form of calcium apatite with the formula HAp $Ca_{10}(PO_4)_6(OH)_2$ naturally occurred in mineral form. Pure HA powder is white,

while naturally occurred appetites can also brown, yellow or green coloration. The HAp implant also beneficial as it is acceptance by the body's immune system. Furth ermore, the function of HAp in this biomedical application is largely determined by its similarity in chemical structure with biological apatite, which comprises the mineral phases of calcified tissues in the enamel, dentine and bone (Li, 1996).

Commercially available needle shaped nanocrystalline HAp particles damage the cells around implantation and proliferation of several types of cancer cells, including liver and throat cells, and also have little side effects on the normal cells (Zhang and Ma, 1996). Some reports have suggested that Hap coatings can produce nanoparticulate debris causing inflammation in the body. Several works have reported that phosphate released to solubilisation during minieral-phosphate solubilisation often shows an oscillatory kinetics. This feature has been observed in cultures of several phosphate–solubilizing microorganisms including *Pseudomonas* sp, *Bacillus* sp, *Azospirillum* halopraeforans, *Pencillium* sp, *P. radicum* and *A. niger* suggested as a possible explanation, a process of re-reprecipitation and secondary solubilisation of intermediate phosphate species (Kpomblekou and Tabatabai, 1994). Isolated bacterial strains are transferred into mineralisation medium, which consist of a mineral nutrient for bacterial growth and along with tricalcium phosphate, under these conditions hydroxyapatite crystal are produced within the bacterial extracellular polymeric substance (EPS). The crystals are then sintered at 1200°C (Mounira et al., 2009 and Schratt et al., 2001).

Gelatin can be made in to composites by blending with other materials like hydroxyl-apatite (HAp) and bone ash. Gelatin, a product from partial hydrolysis of collagen, has gained interest in biomedical engineering, mainly because of its biocompatibility and biodegradability. Since it contains Arg-Gly-Asp (RGD)-like sequences that promote bone cell adhesion and migration, it has been blended with hydroxyapatite to improve the biological activity of composite scaffold. The chitosan is biopolymer of poly-1,4, D-Glucosamine and derived from chitin sources. A biopolymer has the broad application of antimicrobial activity, biodegradability and enhances neovascularization. The gelatin/chitosan composite is safe, haemostatic, with osteoinductive and wound healing properties (Schratt et al., 2001). In this work, we isolate phosphate solubilising bacterial strains in shake flasks methods, then synthesized HAp components is characterized by several techniques including Fourier transform infrared spectroscopy (FTIR), Thermal gravimetric analysis (TGA), Scanning electron microscope (SEM), X-ray crystallography (XRD).

2. MATERIALS AND METHODS

2.1 Screening of hydroxyapatite solubilizing bacterial strains: Clinical isolates were collected from clinical laboratories of Kovi Medical Center (KMC), Coimbatore, India and soil samples were collected from municipal waste dumping area around Coimbatore, Tamilnadu. Soil sample were taken approximately 50g were homogenized in sterile solution. Then, serially diluted soil samples spread on the National Botanical Research Institute's phosphate growth medium (NBRIP) containing the following ingredients per liter: glucose, 10g: (NH4)2SO4, 0.1g: KCl, 0.2g: MgSO4.7H2O, 0.25g: hydroxyapatite, 2.5g and agar 20g (pH 7.0) the plate were incubated at 37 ºC for a week. The phosphate solubilization strains zone of clears around the bacterial colonies to be identified solubilization of hydroxyapatite.

2.2 Quantitative analysis of phosphatase activity: The overnight cultures 3.0 mL were harvested and suspensions were washed with same volume of Tris (0.05M). Toluene 0.1mL was added and kept in shaker at room temperature for 30 min, the suspensions were centrifuged. Further, was washed with 3 mL of Tris (0.05M), then to the cell suspension 1mg of *p*-nitro phenyl phosphate was added and incubated at 37 ºC for 1hr. After incubation 1 mL of 1N NaOH was added to

terminate the reaction. The amount of *p*-NPP released per mL of culture was determined at 420 nm. The organism which showed higher enzyme activity was selected for further work.

2.3 Selected phosphate solubilizing strain identified by biochemical test: The isolates were identified by gram staining, biochemical and sugar fermentation patterns with the scheme described in Bergey's Manual of Systematic Bacteriology.

2.4 Synthesis of hydroxyapatite: HAp was done by using the one strain that showed maximum phosphatase activity. The selected strain was grown in 250 mL conical flask containing 150 mL NBRIP broth (pH 7.0). The flasks were incubated at 37ºC on orbital shaker at 180 rpm for a week. The culture sample were centrifuged at 10,000 g for 15 min, the supernatant was decanted and remaining bacterial residues were dried at 80 ºC for 8hrs and the powder was sintered at 600 ºC in muffle furnace for 4hrs to obtain the increased in porosity.

2.5 Characterization of hydroxyapatite: The presence of the various vibrational frequencies of functional molecules (groups) of hydroxyapatite were analyzed by Fourier Transform Infrared spectroscopy (JASCO FT/IR-680). The X-ray diffraction (XRD) data recorded on Philips X-pert-XPER diffractometer with using monochromatized CuK_a radiation (1.5406 Å) at 40KV and 30mA. The scanning electron microscopy (SEM) was attached with an EDX microanalysis [JEOL-JSM-6490LA FESEM (JEOL, Tokyo, Japan)]. The samples were prepared for thermo gravimetric analysis using a Seiko SSC 5200 H in nitrogen atmosphere (80 mL min^{-1}) at a heating range of 10ºC min^{-1}.

2.6 Preparation of bone composite components

2.6.1 Preparation of gelatin and chitosan: About 5 g of gelatin powder was suspended in 20 mL of deionized water. After the suspension were liquefied by heated on a heating metal at 55 ºC with constant stirring. 3 g of chitosan was dissolved in 20 mL of deionized water at 60 ºC with the addition of 2 to 3 drops of 2 % acetic acid.

2.6.2 Preparation of bone ash: The cattle bone was hygienically collected from a nearby slaughter house. Physiologically bones were cut into small pieces using a Prebreaker. After that the bone pieces were incinerated at 300 ºC until reducing fume. Then sintered at 750 ºC for 4hrs by this process all organic maters are removed from the bone and results in the formation of bone ash.

2.6.3 Preparation of bone composite: Hydroxyapatite obtained from bacterial residues at maximum temperature (sintered at 750 ºC for 5hrs). About 5 g of microbial synthesized HAp powder and bone ash were added to 3 mL of gelatin (G) solution and 3 mL of chitosan (C) and bone ash (B). Contents mixed well and make into paste, and cylindrically shaped implant (6 mm in diameter 3 mm in thickness) were prepared with the help of glass tube. The implants were air dried for 48 hrs. This composite was denoted as HApGCB.

2.7 Antimicrobial activity: Antimicrobial activity was determined by the zone of incubation test using *Staphyolococcus aureus* (gram positive bacteria) and *E. coli* (Gram negative bacteria). Overnight cultures were prepared. A 100µL aliquot of the overnight culture of bacteria (1×10^8 CFU/mL) was spread onto MH agar medium, and implant bone material prepared into fine powder and diluted different concentration in distilled water. The plates were incubated overnight at 37ºC (aerobically). The zone of inhibition was measured.

3. RESULTS AND DISCUSSION

3.1 Isolation of phosphate solubilizing bacteria: The isolation of phosphate solubilization bacteria used by NBRIP agar medium containing inorganic phosphate source of hydroxyapatite. A total of 2 bacterial isolates with clear zone of phosphate solubilization around their colonies were selected among the 8 isolates tested in this primary screening studies. The preliminary observation suggested the ability

of phosphate efficiencies in the NBRIP medium. The selected only one bacterial strain based on the maximum sized (>8 mm) of clear zone (Fig. 1).

Fig.1: Hydroxyapatite solubilizing bacteria of PSM7 on NBRIP medium

The selected bacterial strains denoted as PSM7, it's compared to *Serratia marcescens* MTCC 97 strain. The PSM7 strain was higher activity then compared *Serratia marcescens* MTCC 97.

3.2 Quantitative determination of phosphatase activity in bacterial cells: The phosphatase activity of all the isolates was determined by using *p*-nitro phenyl phosphate as substrate. After incubation, the colour change was noted as the phosphatase acts onto the *p*-nitro phenyl phosphate which forms *p*-nitrophenyl and phosphate. The *p*-nitro phenyl an alkaline indictor causes colour change in clear medium to fluorescent yellow and the colour intensity was measured by spectrometric method. All the isolates showed the positive enzyme reaction in the NBRIP broth. Since the higher enzyme activity was noticed in the isolates of PSM7, it was selected for further analysis (Table 1).

Table. 1: Quantitative determination of phosphate production

Sample code	420nm
PSM1	0.095
PSM2	0.102
PSM3	0.132
PSM4	0.207
PSM5	0.187
PSM6	0.192
PSM7	0.213
PSM8	0.153
Serratia marcescens MTCC 97	0.090

3.3 Biochemical characterization of selected phosphate solubilizing strain: The bacterial strains were isolation from municipal waste dumping area around Coimbatore, Tamilnadu, and the selected strain was identified based on its physiological and biochemical characteristics (Table 2). The selected PSM7 bacteria strain was conformed *klebsiella* sp based on Bergey's Manual of Systematic Bacteriology.

Table.2: Biochemical test of hydroxyapatite solubilizing strains PSM7.

Characteristics	Results
Gram staining	- ve, Rod shape
Capsule	+ ve
Catalase	+ ve
Citrate	+ ve
MR (Methyl Red)	- ve
VP (Voges Proskauer)	+ ve
Oxidase	- ve
Spore	- ve

3.4 HAp solubilization experiment in NBRIP broth cultures: The isolated strains of *klebsiella* sp PSM7 broth cell culture was inoculated into NBRIP broth containing inorganic phosphate. The biological solubilization process of HAp in flasks methods, pH act as indirect parameter in the solution. During incubation period biomass production showed two stages, first one fast growth up to 4^{th} day and second one steady growth afterwards. The broth culture up to 4^{th} day as biomass production increased, pH decreased and to toward the end biomass value slightly decreased in the system. Evolution of total phosphate concentration availability in broth of uninoculated controls stayed almost constant during experiments. These biomass production inoculated experimental exceed to higher then uninoculated culture flask. The maximum HAp solubilization of *klebsiella* sp PSM7 strain at the end yield showed 657 mg L^{-1} at 37 ºC for a week, observed an oscillation trend as in shown (Fig. 2).

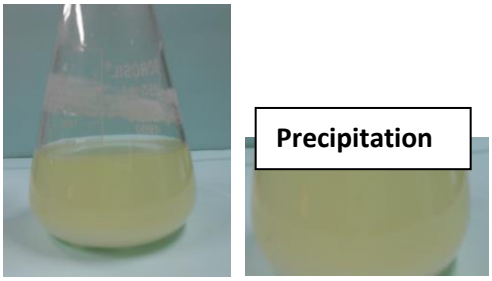

Fig. 2: Hydroxyapatite precipitation of *Klebsiella* sp PSM7 on NBRIP broth.

Once the oscillation occurred biomass were collected and dried at room temperature and then sintered at 600 ºC for 4 hrs for further analysis.

3.5 Physico-chemical properties of HAp synthesized by *klebsiella* sp PSM7.

3.5.1 FTIR analysis: The FTIR spectrum of HAp synthesized through *klebsiella* sp PSM7 bacterial strain was performed. In this study, spectrums (Fig. 3) were observed in broad peaks at 1081.87 and 1414.53 cm^{-1} indicating P-O asymmetric stretching in orthophosphate and at 605.539 and 561.112 cm^{-1} indicating P-O-P deformation of the PO_4^{3-} ion.

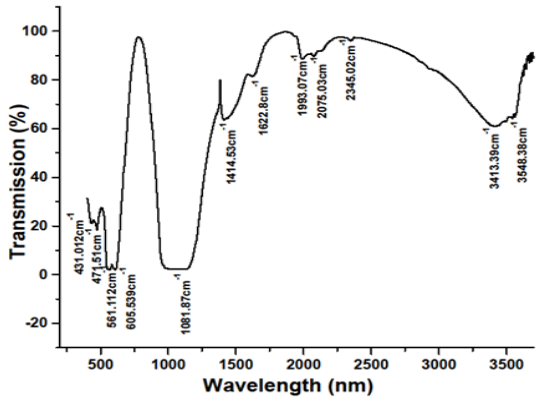

Fig. 3: FTIR analysis of microbial synthesized hydroxyapatite by *Klebsiella* sp PSM7.

The peak at 3413-3548.38 cm^{-1} represents O-H bonding in water. It shows a band in the 561.112 and 605.539 cm^{-1} region due to the simultaneous presence of crystallized apatite phase and C-O vibrations in CO_3^{2-} groups.

3.5.2 XRD analysis: The x-ray diffraction analysis (XRD) profile of the Hydroxyapatite shown in (Fig. 4) The newly synthesized HAp powder an excellent agreement between the experimental data and JCPDS standard for calcium hydroxyapatite (Reg. code 89-6439) in the terms of intensity was obtained. The strong diffraction peaks at 2θ position 31.35 (211), it was corresponding to hydroxyapatite and plane together with other similar peaks 32.33, 33.27and 34.70 indicate reflection from 112, 310, 202 Crystal plane, these reflection was confirmed to structure form of the hydroxyapatite crystal. In the case other peaks at 28.07 (210) referred to as calcium carbonate was observed. This data indicate that bacterial residues contains of the crystallographic structure of hydroxyl-apatite.

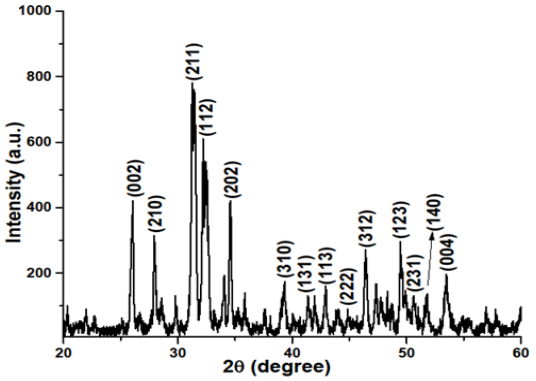

Fig. 4: XRD pattern of the *Klebsiella* spPSM7 synthesized hydroxyapatite

3.5.3 FESEM-EDX analysis: The surface morphology of the bacterial residues analysis by field emission scanning electron microscopic (FESEM) is show in (Fig. 5).

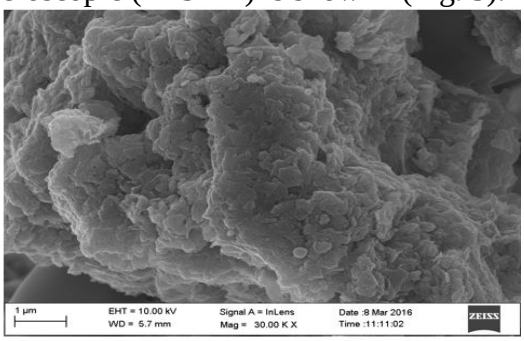

Fig. 5: FESEM image showing irregular spherical structure of hydroxyapatite synthesized by *Klebsiella* sp PSM7

The sample surface with irregular spherical structure and irregular pore ranges in these crystals from 150 to 270 nm. This is porous in nature and supports the bone cells attachment along with induced nucleation of the HAp for the bone in growth. The energy dispersive spectroscopy (EDX) spectrum determines the chemical composition of the sample surface (Fig. 6).

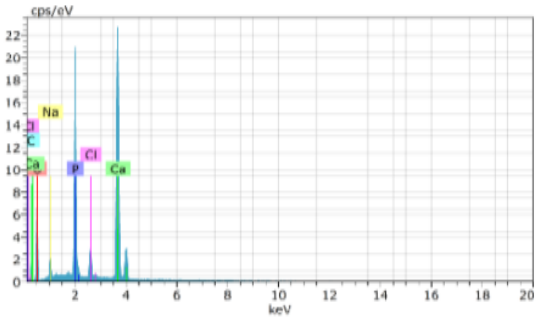

Fig. 6: EDX profile showing the Ca and P signal of hydroxyapatite powder.

The elemental analysis shows the presence of calcium and phosphate group in the sample, the Ca and P was clearly indicated in the formation of hydroxyapatite. The calcium and phosphate found were similar to that commercially available hydroxyapatite. The theoretical stoichiometric of the hydroxyapatite in nature bone ratio (Ca/P= 1.67) as compared to the synthesized hydroxyapatite value (Ca/P= 1.8) was obtained.

3.5.4 TGA analysis: The thermo gravimetric analysis of newly synthesized HAp is shown in (Fig.7).

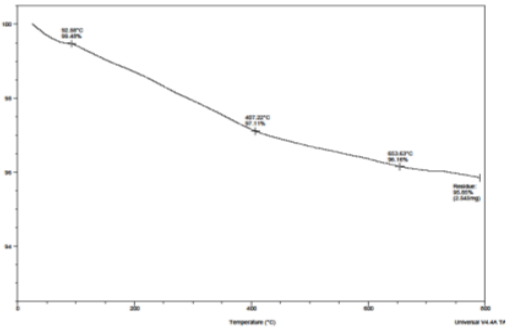

Fig. 7: Thermogram analysis of the *Klebsiella* sp PSM24 synthesized hydroxyapatite

The component weight loss was observed within two steps. About 12 % of weight loss due to dehydration of the precipitating complex and the loss of physically adsorbed water bound of the HAp powder was observed from 92.58 to 402.22 ºC. The second weight loss of 25 % was observed at 653.63 ºC, which is due to the denaturation of unwanted mineral components. About 63 % of inorganic material is accepted in the form of HAp.

3.6 Antimicrobial activity of bone composite HApGCB: The antimicrobial nature of HApGCB (Fig.8) bone implant material aids in the persistence of the implant by preventing microbial attacks associated with surgeries.

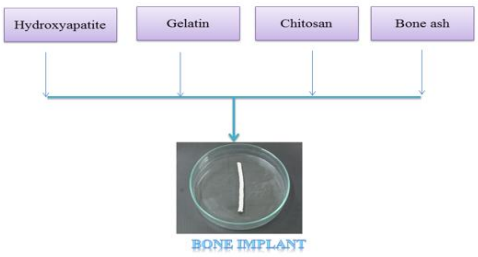

Fig. 8: Nature biopolymer of Bone composite

The bone composite exhibited a bactericidal effect against Gram positive and Gram negative bacteria such as *E. coli* and *Staphylococcus aureus*, respectively. The zone of inhibition *Staphylococcus aureus* 5±0.1mm and 4±0.1mm (Fig. 9).

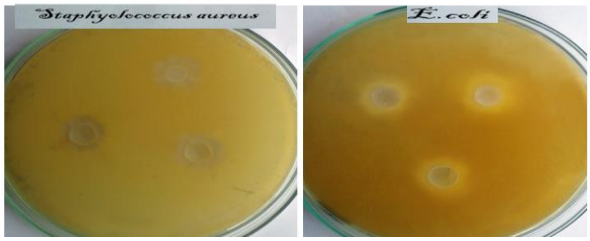

Fig. 9: Zone of inhibition against Gram-positive and Gram-negative organisms

The antimicrobial activity of the bone composite such as microbially synthesis hydroxyapatite, gelatin, chitosan and bone ash. The chitosan and gelatin has been possessing good antagonistic activity against bone pathogenic microorganisms.

4. Conclusion

In the present study, synthesis of hydroxyapatite by *klebsiella* sp PSM7 isolated from bone decomposing area. The microbially synthesized nanocrystalline HAp composite with natural biopolymer of chitosan, gelatin and bone ash (HApGCB) possessed the important feature that needed for bone tissue engineering. The bone composite of nature biopolymers possess good antagonistic against *S. aureus* and *E. coli*. The HApGCB composite could be applicable as bone substitute for bone tissue engineering and bone regenerative medicine.

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