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Preparation of microspheres using poly-3-hydroxybutyrate biopolymer and its characterization

Authors Info

C. Swornakumari,
S. Meignanalakshmi*,
R. Legadevi and A. Palanisammi

Department of Animal Biotechnology,
Madras Veterinary College,
Tamil Nadu Veterinary and Animal Sciences University,
Chennai-600 007, India

*Corresponding Author Email : smeignanalakshmi@gmail.com

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Abstract

Aim : Biodegradable poly-3-hydroxybutyrate is one of the most common biopolymer which is used in different fields such as medicine, agriculture, textile, industrial and food packaging. PHB microspheres are useful for targeting drugs to specific infection sites and for prolonged drug release. The present study focus on microsphere preparation for effective controlled drug release using poly-3-hydroxy butyrate biopolymer.

Methodology : In the present study, poly-3-hydroxy butyrate microspheres were prepared using solvent evaporation technique and characterized by SEM and FTIR. Poly-3-hydroxy butyrate microspheres were encapsulated with BSA and gentamicin. Drug encapsulation efficiency was determined. *In vitro* drug release profile and *in vitro* cytotoxicity was also studied.

Results : The prepared microspheres were of 2µm in size. Microspheres fabricated with 1% polyvinylalcohol showed encapsulation efficiency of 94.3% and 90.27% with BSA and gentamicin, respectively. The *in vitro* release studies in simulated body fluid, phosphate buffered saline and contact lens solution showed initial burst release followed by controlled release. *In vitro* cytotoxicity analysis showed 98% and 95% viability of cells in 3T3L1 cell line for microsphere encapsulated with BSA and gentamicin, respectively.

Interpretation : Poly-3-hydroxy butyrate microspheres were found to release BSA and gentamicin in a controlled manner and were found to be non-toxic by *in vitro* cytotoxicity studies.

Preparation of microsphere using poly-3-hydroxy butyrate by solvent evaporation technique (PHB)

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Characterization of PHB microsphere by SEM and FTIR
Size of microsphere was found to be 2µm

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Encapsulation of PHB microsphere with BSA and Gentamicin and the encapsulation efficiency was 94.3% and 90.27% respectively

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Studies on *in vitro* drug release from PHB microspheres revealed initial burst release followed by controlled drug release

↓

PHB microspheres were found to be non-toxic when tested for *in vitro* cytotoxicity using 3T3L1 cell line

Introduction

Biopolymers are a type of polymers that are degradable and environmentally safe. Polymers have an undesirable influence on the environment and cause problems with waste deposition and utilization (Emily and Rolf, 2013). There is a need to substitute for polymer having the ability to undergo biodegradation processes. Biodegradable polymers are gaining increasing attention due to their properties such as biocompatibility, nontoxic and high degradability in different environments and processing versatility. Biodegradable poly-3-hydroxybutyrate (PHB) is considered as one of the most common intracellular biopolymer first discovered in bacteria (Muralidharan and Radha, 2015) and is used in different fields such as medicine, agriculture, textile, industrial and food packaging (Siracusa *et al*., 2008).

This biopolymer is used in drug delivery systems in the form of microsphere (Atul, 2014; Aggarwal *et al*., 2012). PHB microspheres are useful for targeting drugs to specific infection sites and for prolonged drug release (Mao *et al*., 2012; Joshi and Patel, 2012). The product of degradation of PHB is 3-hydroxy butyric acid which is normally present in human blood and this is advantageous for PHB to be used in medical field (Siraj *et al*., 2014). Controlled drug release which increase the therapeutic activity, decrease the side effects and reduce the number of dosages required during the treatment. Factors such as morphology, size of particles, their chemical composition, rate of degradation and the type of drug affects the drug delivery, by controlling these factors drug delivery can be controlled (Grillo *et al*., 2011). Several different kinds of drugs can benefit from controlled delivery, such as anti-inflammatory agents, antibiotics, chemotherapeutic drugs, immune suppressants, anesthetics and vaccines. PHBs have been used for a number of biomedical applications such as wound management, orthopaedics, drug delivery and vascular system applications (Giovana *et al*., 2012). In view of the above, in the present study, microspheres were prepared by using PHB biopolymer and characterized for controlled drug release by encapsulating with BSA and gentamicin.

Materials and Methods

PHB biopolymer produced by *Bacillus* sp. available in laboratory was used for the preparation of microspheres.

Microsphere preparation : PHB microspheres were prepared by triple emulsion solvent evaporation technique. 600 mg PHB and 200µl of polyethylene glycol (PEG) were dissolved in 10 ml of dichloromethane. To the mixture, 1ml of 6% gelatin solution at 40ºC was added and the mixture was shaken. Then, 150 ml of 0.5% or 1% PVA was added (poly vinyl alcohol) and stirred mechanically with different stirring rate 600, 800 and 1000 rpm until the solvent was completely evaporated. After 24 hrs of stirring, microspheres were collected by centrifuging at 5,000 rpm for 15 min and rinsed in distilled water for 7-8 times (Shishatskaya *et al*., 2008).

Characterization of PHB microspheres

Scanning Electron Microscope analysis : Surface morphology of PHB microspheres was analyzed using scanning electron microscope (HITACHI model S-3000H).

Fourier transform infrared spectroscopy analysis for PHB and PHB microspheres : PHB polymer and PHB microspheres were subjected to Fourier Transform Infrared Spectroscopy analysis by FTIR spectrophotometer. Spectra were documented in 4000 cm^{-1} to 400 cm^{-1} range

Determination of residual PVA content for drug loading efficiency : Two mg of microspheres were treated with 2 ml of 0.5 M sodium hydroxide for 15 min at 60ºC. To the mixture, 900µl of 1 N hydrochloric acid was added to neutralize the samples and the volume was adjusted to 5 ml with distilled water. To the mixture, 3 ml of 0.65M boric acid, 0.5 ml iodine solution and 1.5 ml of distilled water were added. After 15 min incubation, absorbance was measured at 690 nm.

Encapsulation of PHB microsphere with BSA and gentamicin : Microsphere preparation (1% PVA at 800 rpm) procedure was followed with addition of BSA and gentamicin along with PHB (Shishatskaya *et al*., 2008)

***In vitro* drug release studies for PHB microsphere encapsulated with BSA and gentamicin :** Five mg of BSA and gentamicin loaded microspheres were immersed separately in 3 ml of contact lens solution, phosphate buffer (PBS) (pH, 7.2) and simulated body fluid (SBF) (Kokubo *et.al*., 1990). The Composition of simulated body fluid consisted pure water, sodium chloride, sodium bicarbonate, potassium chloride, potassium phosphate dibasic trihydrate, magnesium chloride hexahydrate, calcium chloride, Sodium sulphate and Tris base. They were kept in a shaker at 250 rpm at 37ºC. About 1ml of the sample was collected at regular intervals, till 24hrs. Each sample was replaced with fresh medium and the tubes were returned to the shaker. At each time point, samples were taken out in triplicates and the results were averaged. The BSA and gentamicin content in the aliquot was determined by Coomassie brilliant blue and measuring the absorbance at 450-595 nm and quantified using BSA and gentamicin standard curves.

Encapsulation efficiency of BSA and gentamicin : Ten mg of BSA and gentamicin loaded microspheres were dispersed in chloroform with vigorous shaking at room temperature for 24hrs.The samples were collected at 0hr and 24hr, BSA and gentamicin content were determined by Coomassie brilliant blue by measuring the absorbance at 450-595 nm. Encapsulation efficiency was determined by the following formula :

$$\text{Encapsulation efficiency \%} = \frac{\text{Retained BSA/ Gentamicin amount}}{\text{Initially loaded BSA/ Gentamicin amount}}$$

***In vitro* cytotoxicity study :** *In vitro* cytotoxicity of PHB microsphere (prepared with 1% PVA at 800 rpm), PHB microspheres encapsulated with BSA and gentamicin, was carried out using 3T3L1 cell lines by MTT assay at different concentrations (1mg, 10 mg and 100 mg). 3T3L1 cell lines were commonly used for *in vitro* cytotoxicity studies. Briefly, 3T3L1 cell line was subjected to serum starvation for 3-4 hrs followed by incubation with various concentrations (1mg, 10 mg and 100 mg) of samples for 24 hrs. After 24hrs of incubation, 100 µl of 0.01% triton X-100 was added to positive control well and incubated for 30 min. The medium from the wells was completely aspirated and 100 µl of 1X PBS was added to the wells and incubated for 10 min. A 20 µl of MTT (3-[(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide]) was added to the wells and incubated for 4 hrs in dark at 37°C. A 100 µl of solubilization buffer (DMSO) was added to the wells and incubated in dark for 1 hr. Absorbance was measured at 570 nm and percentage cell viability was determined.

Results and Discussion

The SEM analysis of microspheres prepared by using PHB revealed the presence of uniform spherical shaped microspheres of 2 µm size (Fig.1). In the present study, dichloromethane was selected instead of chloroform due to lesser miscibility of chloroform in water as compared to dichloromethane, as the type of solvent influences the surface morphology of the microsphere. Siraj *et al*. (2014) used chloroform as a solvent for PHB microsphere production and obtained a spherical shaped porous microspheres of 10-50 µm in diameter with rough surface. In the present study, use of dichloromethane resulted in uniform spherical shaped microspheres of smaller size when compared to chloroform Shishatskaya *et al*. (2008) also reported uniform spherical shaped microspheres when dichloromethane was used.

The FTIR analysis showed that, the peak which is assigned for PHB material such as 1647.5 cm^{-1} and 1238.3 cm^{-1}, 1402.2 cm^{-1}, 1238.3 cm^{-1}, 1076.7 cm^{-1} and 3447.50 cm^{-1} were present in the PHB microsphere (Fig.2), Functional groups C=0, CH_3, CH_2 present in the PHB microsphere indicates the presence of PHB and without any disturbances or merging of solvent functional groups in the PHB microsphere.

FTIR spectrum of PHB microsphere encapsulated with gentamicin (Fig.3) showed characteristic peaks of both PHB microsphere and gentamicin, which confirms the presence of gentamicin within the microsphere. FTIR spectrum of PHB microsphere encapsulated with BSA (Fig.4) showed characteristic peaks of both PHB microsphere and BSA, which confirmed the presence of BSA within the microsphere.

When PVA was used at 0.5% and 1% concentration with 800 rpm stirring rate, uniform spherical shaped microspheres were obtained. PVA at 0.5% and 1% concentration with stirring rate at 600 rpm and 1000 rpm resulted in microsphere with breakage and also the shape of the microspheres were not spherical and uniform. The residual PVA content of microspheres were higher, when microspheres were prepared at stirring rate 800 rpm when compared to stirring rate at 600 rpm and 1000 rpm (Fig.5). Quantity of PVA influences the microsphere formation because of their hydrophilicity and miscibility in water.

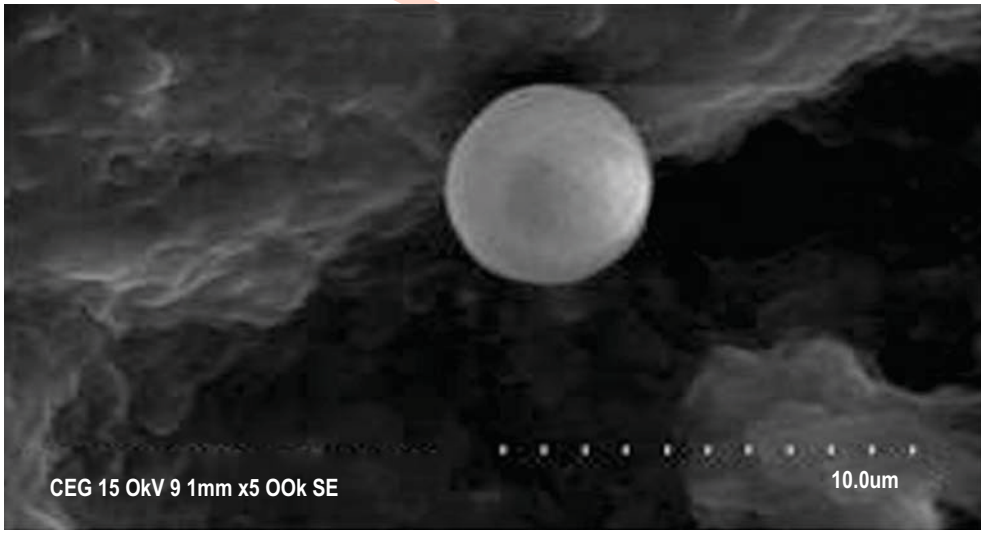

Fig. 1 : Scanning Electron Micrograph of PHB microspheres

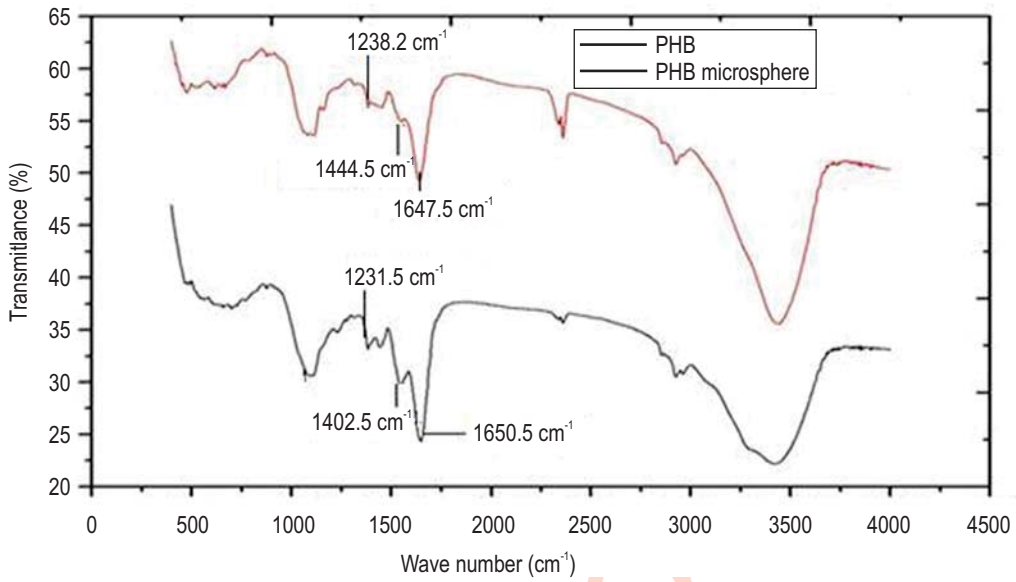

Fig. 2 : FTIR spectrum of PHB and PHB microspheres

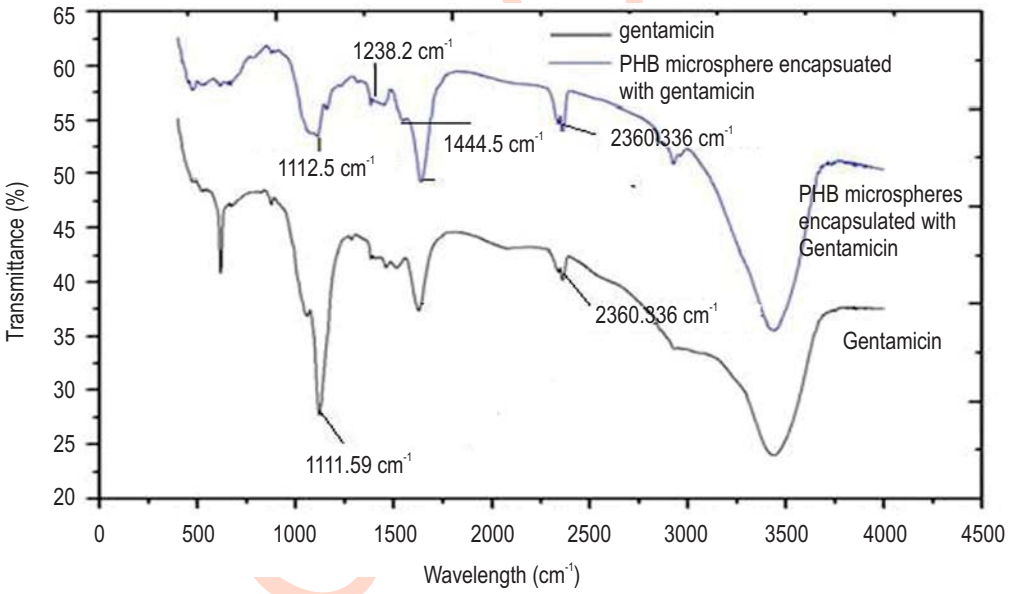

Fig. 3 : FTIR spectrum of gentamicin and PHB microsphere encapsulated with gentamicin

Encapsulation efficiency of BSA and gentamicin (Fig.6) was found to be 94.3% and 90.27% when PVA was used at 1% concentration with a stirring rate of 800rpm.When PVA was used at 1% concentration with stirring rate 600 rpm and 1000 rpm, the encapsulation efficiency of gentamicin was found to be 77.09% at 600 rpm and 88.64% at 1000 rpm. The encapsulation efficiency of BSA was found to be 89% at 600 rpm and 82% at 1000 rpm. Similarly, Francis *et al*. (2011) also observed maximum encapsulation efficiency of drugs of about 48% using 1% PVA at 800 rpm. Yang *et al.* (2001) also reported that 1% PVA concentration resulted in higher encapsulation of drugs when compared with lower encapsulation with 0.5% PVA concentration. Hence, the microsphere preparation was proceeded with 1% PVA concentration.

The *in vitro* release of PHB microsphere encapsulated with gentamicin and BSA is shown in Fig. 7 and 8. In the present study, the initial burst release followed by controlled release was observed with PHB microspheres encapsulated with gentamicin and BSA. Initial burst (0 to 1 hr) release was found to be 30.4%,

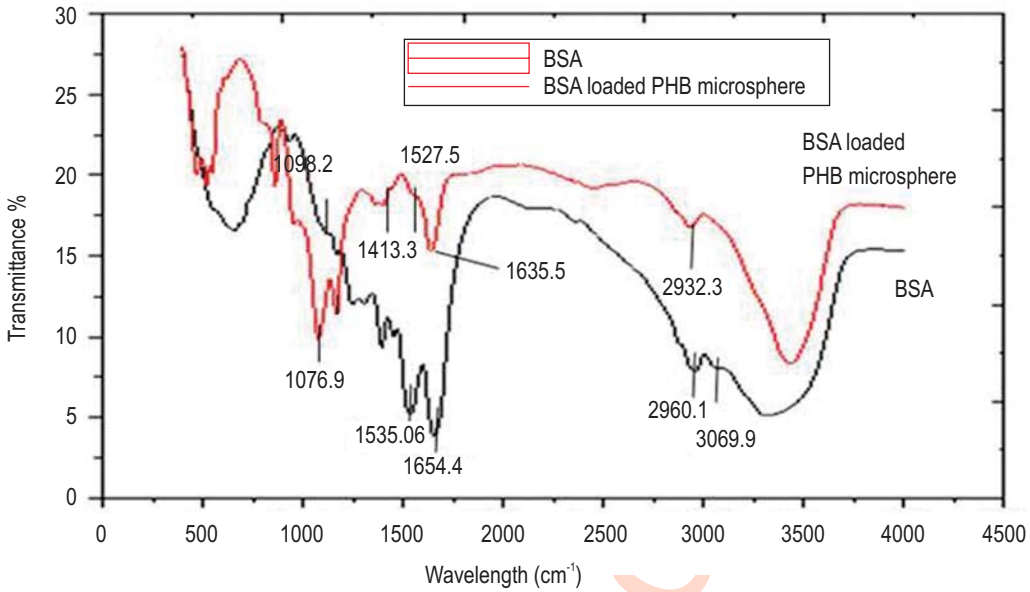

Fig. 4 : FTIR spectrum of BSA and BSA loaded PHB microsphere

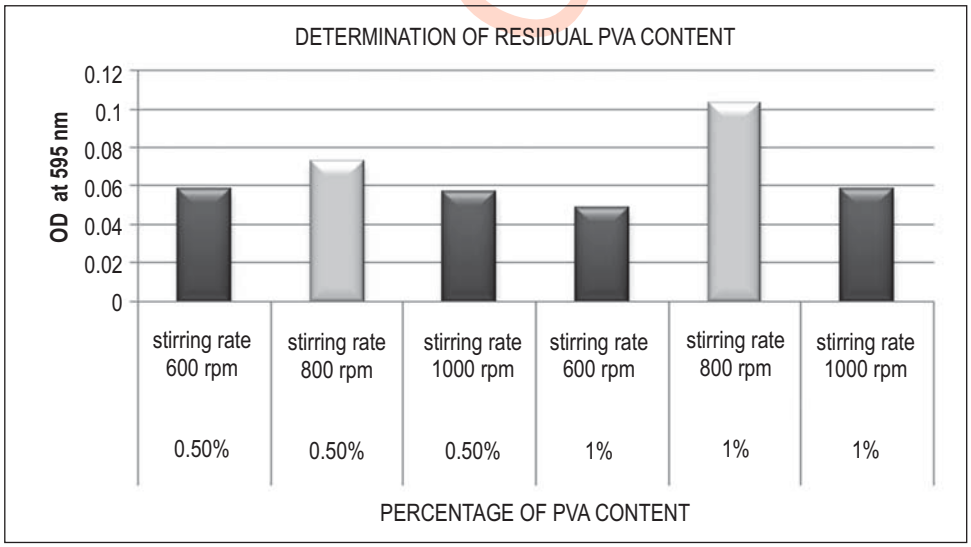

Fig. 5 : Residual PVA content at different concentration of PVA at different stirring rate during microsphere preparation

31.89%, 32.915% in SBF, PBS and CLS for BSA and 30%, 31%, 29.7% in SBF, PBS and CLS for gentamicin. This release continued up to 24 hr and the total release was found to be 78.86%, 75.24% and 72.91% in SBF, PBS and CLS for BSA. The total release for gentamicin was found to be 79%, 77.4% and 80.8% in SBF, PBS and CLS, respectively. Similarly, Francis *et al*. (2011) observed the *in vitro* drug release of gentamicin in simulated body fluid at 37 ºC for a period of 20 hr. An initial burst release of 90.6 μgml^{-1} at 0-1hr was reported, which was 60% of the total encapsulated drug followed by the controlled drug release upto 20 hrs. Controlled drug release was found to be 24.6 μgml^{-1} at 5 hr and 18 μgml^{-1} at 12 hrs. After 20 hrs, the total drug release of 95.33% was noted.

Ismail *et al*. (2012) observed the *in vitro* release of gentamicin in PBS at 37ºC for a period of 6 days at a regular interval of 1hr. Approximately, 50% of gentamicin was released within 10hrs and release continued to be minimal for next few hrs with total release of less than 60% for a period of 6 days. Rossi *et al*. (2004) observed the release of gentamicin in phosphate buffered saline at 37ºC, and reported a cumulative drug release of 33% and 37% of gentamicin from gentamicin

loaded PHBV. Daniela *et al*. (2015) observed the release of BSA from loaded PHBV and reported that 47% drug release followed by 60% and 63% in next 21days. Gursel *et al*. (2008) observed the controlled release of gentamicin at 22% for a period of 2 months. Rapid early phase followed by slower and prolonged phase was observed by them. Li and Chang (2005) also observed the controlled release of gentamicin at 70% during first three days in a period of 12 wks in SBF and PBS.

Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) microspheres were developed with a mean particle size 4±2 µm loaded with daidzein using oil-in-water single emulsion solvent

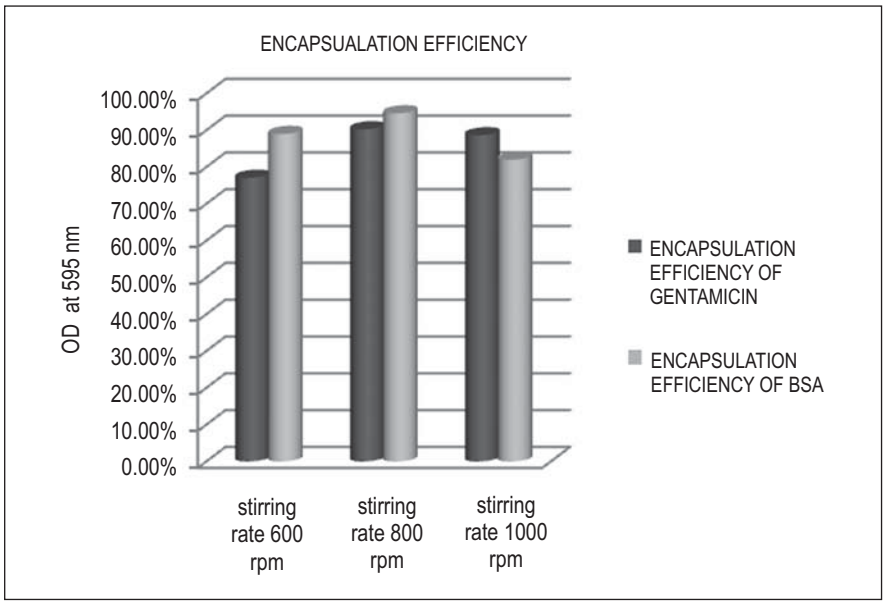

Fig. 6 : Encapsulation efficiency of PHB microsphere encapsulated with gentamicin and BSA at different stirring rate

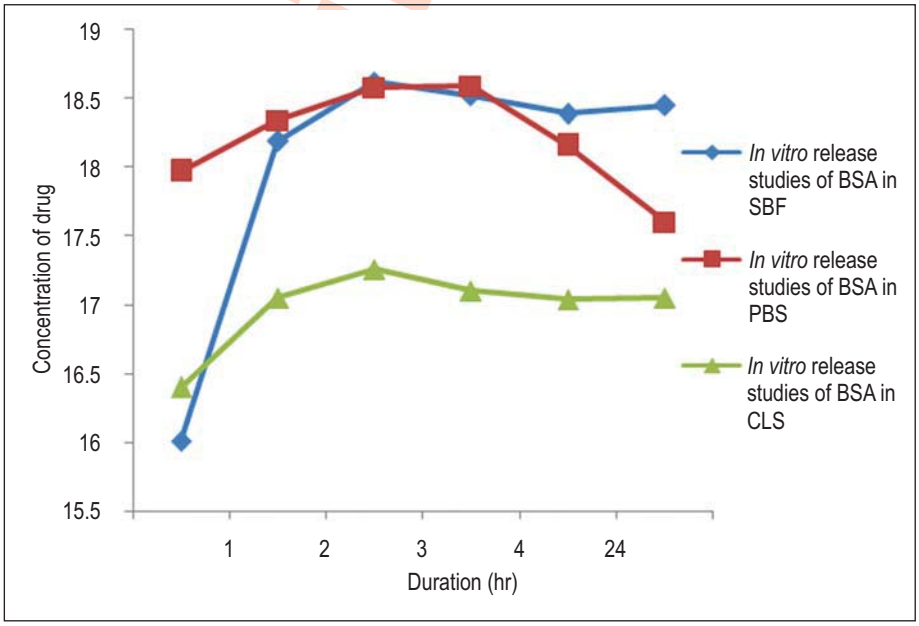

Fig. 7 : *In vitro* release of BSA from PHB microspheres

evaporation method and applied to the surface of bio active scaffolds by dip coating technique and also reported that the daidzein release from the microsphere loaded scaffolds lasted for almost one month. (Macías-Andrés *et al*., 2017)

In the present study, the percentage viability of cells in 3T3L1 cell line at 1mg, 10mg and 100mg of concentration of PHB microspheres was found to be 97%, 87% and 53%, respectively. At same concentration, the percentage viability of PHB microsphere encapsulated with BSA and gentamicin was found to be 98%, 91%, 32% and 95%, 53%, 47% respectively (Fig.9). On increasing the concentration of PHB microspheres, decrease in cell viability was observed. Similarly, Shishatskaya *et al*. (2008) used 3T3 mouse fibroblast cell lines to evaluate the biocompatibility (*in vitro* cytotoxicity) of PHB microsphere. PHB microspheres showed 99.8% cell viability and did not produce any toxic effects in 3T3 cell line. As the concentration increased, it became toxic to cell lines. In the present study, 98% cell viability was observed for PHB microspheres at lowest concentration.

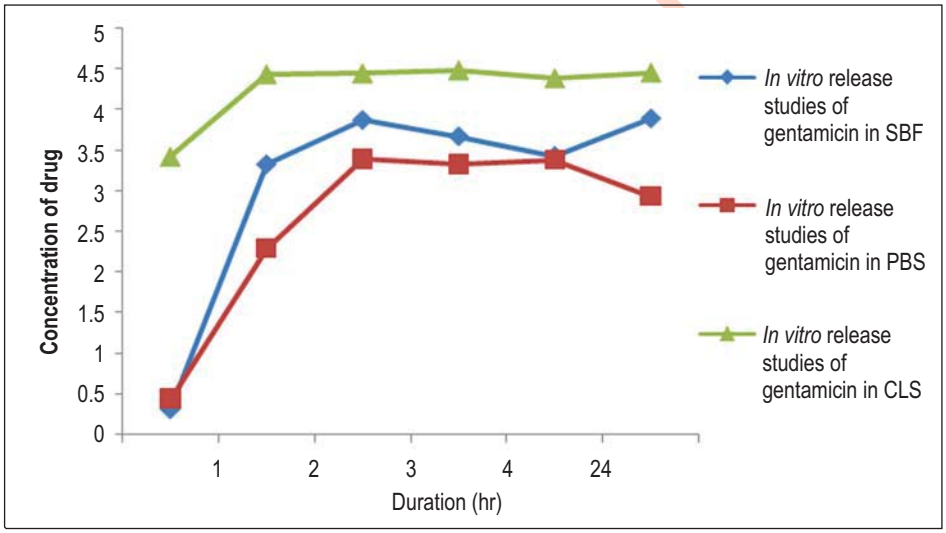

Fig. 8 : *In vitro* release of Gentamicin from PHB microspheres

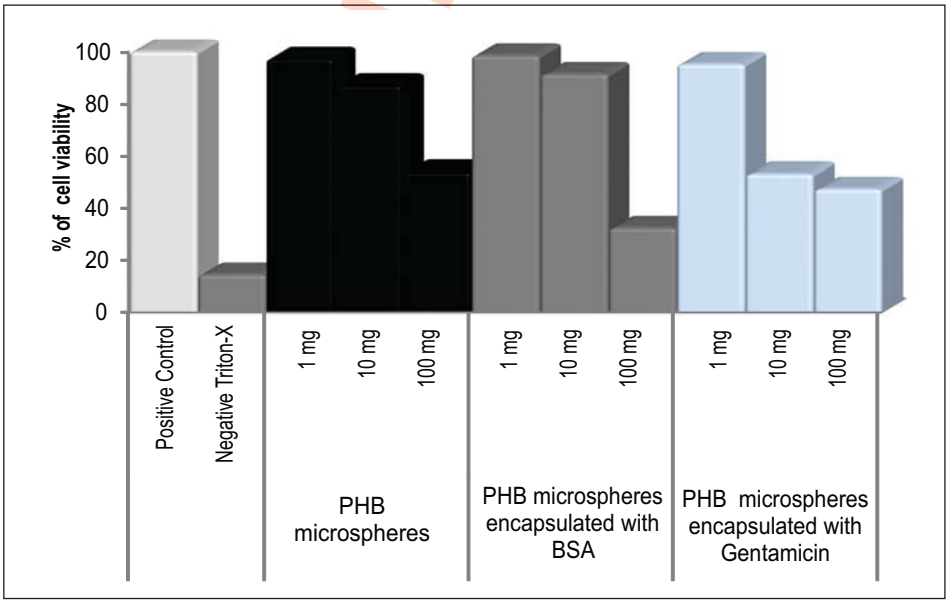

Fig. 9 : *In vitro* cytotoxicity assay of PHB microsphere encapsulated with BSA and gentamicin in 3T3L1 cell line

It can be concluded that the microspheres prepared by using PHB produced by *Bacillus* sp. by solvent evaporation method using dichloromethane and PVA at 1% concentration at 800rpm showed better encapsulation efficiency when compared to others. Also, the *in vitro* drug release studies with PHB microspheres encapsulated with BSA and gentamicin showed initial burst release, followed by controlled drug release. Microspheres prepared from PHB produced by *Bacillus* sp. can be used for encapsulation of antibiotic for controlled drug release.

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References

Aggarwal, S., A. Goel and S. Singla: A review on drug delivery: Special emphasis given on biodegradable polymers. *Int.J. Sci*., **2**, 1-15 (2012).

Atul, N.W.: Biosynthesis of polyhydroxybutyrate (PHB): An ecofriendly biopolymer with therapeutic application. *Int. J. Inform. Futur. Res*., **4**, 1068–1080 (2014).

Daniela, P.P., M. H. Amaral, R.L. Reis, A.P. Marques and V.M. Correlo: Development of injectable PHBV microparticles-CG hydrogen hybrid system for regenerative medicine. *Int. J. Pharm*., **478**, 398-408 (2015).

Emily, N. and U. Halden Rolf: Plastics and environmental health : The road ahead. Rev. *Environ. Hlth*., **28**, 1-8 (2013).

Francis, L., D. Meng, J. Knowles, T. Keshavarz, A.R. Boccaccini and I. Roy: Controlled delivery of gentamicin using poly (3-hydroxybutyrate) microspheres. *Int. J. Mol. Sci*., **12**, 4294–4314 (2011).

Giovana, B.C., A.T. Macedo, J. P. Crenca, V.E. Silva, E. M. Pereira, M. Zetola and B.R. Pezzini: Microspheres prepared with biodegradable PHBV and PLA polymers as prolonged-release system for ibuprofen : I*n vitro* drug release and *in vivo* evaluation. *Braz. J. Pharm. Sci*., **48**, 773–780 (2012).

Grillo, R., A.E. Pereira, N.F. de Melo, R. M. Porto, L.O. Feitosa, P.S. Tonello, N. L. Dias Filho, A.H. Rosa, R. Lima and L.F. Fraceto : Controlled release system for ametryn using polymer microspheres : Preparation, characterization and release kinetics in water. *J. Hazard. Mater*., **186**, 1645–1651 (2011).

Gursel, I., F. Yagmurulu, F. Korkusuz and V. Hasirci : The In vitro antibiotic release of PHBV rods. *J. Microencapsulation: Micro* and *Nanocarriers*, **19**, 153-164 (2008).

Ismail, A.F., A.M. Awang and M. Farahidah : High initial burst release of gentamicin formulated as PLGA microspheres implant for treating orthopaedic infection. *Int. J. Pharm. Pharm. Sci*., **4**, 685–691 (2012).

Joshi, J.R. and R. P. Patel: Role of biodegradable polymers in drug delivery. *Int. J. Curr. Pharmaceut. Res*., **4**, 74–81 (2012).

Kokubo,T., H. Kushitani, S. Sakka, T. Kitsugi and T. Yamamuro: Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic. *J. Biomed. Mater.Res*., **24**, 721-734 (1990).

Li, H. and J. Chang, : Preparation, characterization and *in vitro* release of gentamicin from PHBV/wollastonite composite microspheres. *J. Controll. Rele*., **107**, 463-473 (2005).

Macias-Andrés, V.I., W. Li, E.A. Aguilar-Reyes, Y. Ding, J.A. Roether, L. Harhaus, C.A. León-Patina and A.R. Boccaccini: Preparation and characterization of 45S5 bioactive glass-based scaffolds loaded with PHBV microspheres with daidzein release function. *J. Biomed. Mater. Res. Part A*., **105 (A)**, 1765–1774 (2017).

Mao, S., C. Guo, Y. Shi and LC. Li: Recent advances in polymeric microspheres for parenteral drug delivery—part 2. *Expert Opinion on Drug Delivery*., **9**, 1209–1223 (2012).

Muralidharan, R. and K.V. Radha: A kinetic study of polyhydroxybutyrate production on nitrogen limited medium using *Bacillus subtilis* MTCC 9763 through a two stage cultivation strategy. *J. Environ. Biol*., **36**, 537-542 (2015).

Rossi, S., A.O. Azghani and A. Omri: Antimicrobial efficacy of a new antibiotic-loaded poly (hydroxybutyric-co-hydroxyvaleric acid) controlled release system. *J. Antimicro. Chemother*., **54**, 1013–1018 (2004).

Shishatskaya, E. I., O.N. Voinova, A.V. Goreva, O. A. Mogilnaya and T.G. Volova: Biocompatibility of polyhydroxybutyrate microspheres: *In vitro* and *in vivo* evaluation. *J. Mater. Sci.: Mater. Med*., **19**, 2493–2502 (2008).

Siracusa, V., P. Rocculi, S. Roamin and M.D. Rosa: Biodegradable polymers for food packaging : A review. *Trends in Food Sci. Techno*., **19**, 634-643 (2008).

Siraj, S., P. Sudhakar, B. Mallikarjuna, K. Chowdoji Rao and M.C.S. Subha : Biodegradable PHBV/PEO blend microspheres for controlled release of Bosentan monohydrate: Preparation, characterization and *in vitro* release studies. *Int. J. Pharm. Sci. Rev. Res*., **25**, 103–109 (2014).

Yang, Y.Y., T.S. Chung and N.P. Ng: Morphology, drug distribution and *in vitro* release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/ evaporation method. *Biomaterials*, **22**, 231-241 (2001).