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In vitro Inhibition Study against *A. flavus* with *Stenotrophomonas maltophilia* Immobilized in Calcium Alginate Gel Beads

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Enhanced chitinase secretion was demonstrated in batch fermentation process by immobilizing a novel bacterial isolate SJ602 in alginate beads. The phylogenetic data affirmed the identity of the SJ602 as *Stenotrophomonas maltophilia* strain. Optimization of the conditions suggested the alginate concentration of 4% at a pH range of 6.0-7.0 and temperature of 37 °C, are the more convenient conditions for achieving maximal enzyme activity of immobilized cells. Amendment of alginate with 5% skimmed milk powder added greater stability, and also maintained the viability of bacterial cells inside the entrapped alginate beads. Thus, the alginate immobilization, described above, improve the bacterial life and the chitinase activity as compared to non-immobilized bacterial cells. The results described the effect of the biodegradable polymers as the nutrient source in maintaining the critical biomass and enhanced stability during long term storage at ambient temperature. The study showed *S. maltophilia* SJ602 as a potential candidate for agricultural and biotechnological applications.

Key Words: *Stenotrophomonas maltophilia*, Chitinase, Immobilization, Alginate beads, Biocontrol.

Chitinases (E.C 3.2.1.14) exist in a wide spectrum of organisms including the bacteria, fungi, yeasts, plants, actinomycetes, arthropods and human beings. They are essentially glycosyl hydrolases with molecular mass ranging from 20-90 kDa[1]. The chitinases have the ability to decompose chitin directly to low molecular weight chitooligomers, which serve as a functional entity in a wide range of industrial, agricultural and medical (elicitor action and antitumor) activities[2]. Chitinases have been divided into two main groups i.e. endo-chitinases and exo-chitinases. The enzymes, which randomly split chitin at its internal sites are endochitinases, and yield the di-acetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and chitotetraose[3]. Chitinase producing bacteria have been isolated from varying habitats that includes shellfish waste[4], garden and park waste compost[5]. A number of bacteria viz. *Aeromonas*[6], *Bacillus*[7], *Streptomyces*[8], *Alteromonas*[9], *Serratia*[10], and *Enterobacter*[11] have exhibited the ability to

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produce chitinases. Furthermore, the bacterial strain, *Stenotrophomonas maltophilia* C3, produces specific chitinase against *Bipolaris sorokiniana*[12], and *S. maltophilia* W81 produces extracellular proteolytic enzymes for the degradation of *Pythium ultimum*[13]. Chitinases have been shown to exhibit a broad range of antifungal activity[14] and, therefore, attracting increased attention due to their role in biocontrol of fungal phytopathogens[15] and insect pests[16]. They have also had biotechnological applications in producing sphaeroplasts and protoplasts *in vitro* from yeast and fungal cells[17, 18]. Owing to their environmental and industrial significance, it is imperative to develop stable microbial bioresource for efficient, economical and large scale production of chitinases. In this regard, several technologies have been developed for the safer exploitation of microorganisms by adsorbing them with different carriers such as peat, charcoal, vermiculite, and other organic materials[19, 20, 21, 22]. Alternatively, microbial cells can also be used[23] by attaching and/ or entrapping cells to inert supports for efficacy, stability, safety, and flexibility of applications[24]. Several studies have demonstrated the use of alginate polymer for immobilization of beneficial microorganisms for developing agricultural inoculants as biocontrol agents[24]. Entrapment of cells in alginate beads is regarded as a modest immobilization method, which maintains cell viability of cells for advantages of heterogeneous catalysis[25]. The encapsulation provides enhanced metabolic activity even when the cells are used again and extends protection against inhibitory compounds or metabolites[26]. Akimoto *et al*.[27] have demonstrated that the pH and temperature optima of chitinase producing alkalophilic *Bacillus* sp. BG-11 cells immobilized on chitosan and alginate remain same as that of free enzyme.

According to the USDA, India is the second leading producer of groundnut[28], and as per the reports of Indian Council of Medical Research (ICMR), 21% of the groundnut samples are contaminated with aflatoxins and thus, not safe for human consumption[29]. Since India lies in sub-tropical region where molds thrive best, they damage about 80% of the crops[30]. Therefore, the aim of the present study was to isolate an indigenous biocontrol microbe against *A. flavus* and investigate the effect of various alginate concentrations on bead formation and antifungal producing ability of immobilized bacterial cells in alginate beads as compared to those of free bacterial cells. In addition to this, the effects of pH, temperature, and drying on the bacterial survival, and efficiency of chitinase production on long term storage were also studied.

MATERIALS AND METHODS

Bacterial Culture and Growth Conditions

The bacterial isolate SJ602 was obtained through the screening of rhizospheric bacterial population of *Arachis hypogea* L. plants from the experimental plots of Jamia Hamdard, New Delhi, India. The isolate SJ602 was sub-cultured in sterilized nutrient broth (NB) and incubated in a rotary shaker (180 rpm) at 37°C for overnight. All operations were carried out in aseptic conditions under laminar flow unit.

Antagonistic effect of isolate SJ602 against *A. flavus*

Antagonistic activity of the isolate SJ602 was determined by mycelial inhibition assay. On potato dextrose agar (PDA) plate, freshly grown SJ602 isolate was grown alongwith fungus *A. flavus* MTCC 277 (10^6 spores/ml) and incubated for 3 days at 37°C. The plates were then observed for antagonistic activity against the fungus.

Elucidation of chitinase production by isolate SJ602

On a chitin agar plate where chitin was taken as sole carbon source with minimal salts, freshly grown isolate SJ602 was inoculated at five equidistant points followed by incubation of plates for 3 days at 37°C and were subsequently evaluated for the zone of hydrolysis around the inoculated area.

Identification of Bacterial Isolate SJ602 by 16S rRNA Sequence

DNA from the bacterial strain SJ602 was isolated by standard procedure[31]. Using the purified genomic DNA as target, the gene coding for 16S rRNA was amplified employing primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'), complementary to the 5' and 3' regions of eubacterial 16S rRNA gene, respectively. The PCR amplification was carried out in a final volume of 25 µl. Briefly, the amplification reaction containing

50 ng template DNA, 30 pmole each of universal primers, 0.2 mmol/l dNTPs and 2.5 U *Taq* polymerase in 5 µl of 10X PCR buffer (Sigma, USA) was run on thermocycler (T-Cy, Creacon Technology). The amplification reaction with initial denaturation at 95 ºC for 1 min was cycled as follows: 94 ºC for 1 min; 55 ºC for 1 min; 72 ºC for 1.5 min for 35 cycles; and incubation at 72 ºC for 3 min". The amplicons were sequenced on ABI prism 3130 XL sequencer. The nucleotide sequence was finally submitted to NCBI database. The 16S rRNA gene sequence was subjected to ClustalW multiple alignment using BioEdit (ver. 5.0.9). Phylogenetic tree was constructed by the neighbor-joining (NJ) method with nucleotide pairwise genetic distances corrected by the Kimura two-parameter method using TreeCon tool.

Formulation of Cell Immobilized Alginate Beads

Sodium alginate solution was prepared by dissolving it in 100 ml boiling water, and then sterilized by autoclaving[32]. Both the alginate slurry and bacterial cell suspension, equivalent to 0.03 g dry cell weight (DCW) were mixed and stirred for 2 h to obtain a uniform mixture. The slurry was collected in a sterile syringe and added drop wise into 0.1 M $CaCl_2$ solution from 5 cm height, and left for curing in 0.05 M $CaCl_2$ solution at 4°C for 24 h with gentle stirring. The cured beads were then washed several times with sterilized MilliQ water to remove $CaCl_2$ completely from the bead surface, and transferred to freshly autoclaved nutrient broth and incubated at 37^0C for overnight with gentle agitation[33]. After incubation, the beads were again washed thoroughly with sterile MilliQ water and stored at 4^0C until used.

Optimization of Alginate Concentration

Alginate entrapment was carried out by following the methods of Johnsen and Flink[34] and Begin *et al*.[35]. Sodium alginate at variable concentrations (1-6% w/v) with a constant amount of inocula (equivalent to 0.03 g DCW) was used for the immobilization purpose[32]. One gram of alginate beads with entrapped bacterial cells were added to the 100 ml of nutrient broth and incubated at 37^0C for overnight. After incubation, the optical density (OD) was read at 600 nm to assess the extent of release of entrapped bacteria from loaded beads.

Amendment of Alginate Beads with Different Polymers

Alginate preparation was supplemented with starch and skimmed milk at 5% w/v respectively, to assess the role of different polymers for providing additional stability to alginate beads. Sterilized polymer was added prior to alginate solution at dissolving stage. Further procedure was followed as mentioned above.

Bacterial counts and viability of cells within alginate beads amended with different polymers were assessed by dissolving the beads at different time intervals, and then incubating for 24 h. The culture media (0.1 ml) was then spread on nutrient agar plate and the viability determined in terms of CFU/ml.

Effect of Temperature and pH on Release of Bacteria from Immobilized Alginate Beads

The conditions for the controlled release of bacteria were optimized by varying the temperature and pH of the slurry containing 1 g of immobilized beads[35]. The trials were performed in batch processes in 250 mL Erlenmeyer flasks. The beads were inoculated in NB media at a temperature range of 25-42°C. After optimizing the temperature, another separate set of experiment was run at a pH range from 2-9. The bacterial release was determined spectrophotometrically (Spectronic 20 Genesys) OD_{600}.

Scanning Electron Microscopy

A thin layer of wet, and freeze-dried beads was prepared completely at room temperature under aseptic condition in laminar flow and kept at 4^0C until further used. For each observation of scanning electron microscopy, 10 beads were fixed for 3 h in 5% glutraldehyde solution, washed three times in 0.2 M phosphate buffer (pH 7.2) and finally dehydrated with ethanol. The whole procedure was carried out at 4±1°C and all samples were dried in desiccators. The wet-dried, freeze-dried samples were stuck, followed by gold coating was done on them. The coated samples were finally viewed under scan electron microscope (Zeiss EVO40) at 20 kV.

Evaluation of chitinase production by alginate immobilized bacterial cells

On potato dextrose agar (PDA) plate, *A. flavus* fungus was grown alongwith freeze-dried immobilized bacterial cells at five equidistant points. The plates were incubated for 3 days at 37°C and were subsequently evaluated for chitinase production.

Determination of Chitinase Activity

Chitinase activity of the wet and freeze-dried alginate immobilized bacteria was measured at different time-intervals of 1-6 months, in a batch fermentation process following the method of Miller *et al*.[36]. Briefly, 100 mL of NB media supplemented with chitin as a carbon source was inoculated with one gram of wet and freeze-dried immobilized beads and incubated for 24 h at 37°C in an orbital shaking incubator (Remi Instruments Ltd.) with gentle agitation[37].

The chitinase activity in the culture supernatant was estimated, periodically after every two month, using hydrolyzed chitin as the substrate[38]. The reaction mixture for chitinase assay contained 1 ml 5% acid swollen chitin, 1 ml 50 mM acetate buffer, pH 5.0 and 1 ml enzyme solution that was incubated at 50°C for 1 h and the reaction was stopped after boiling it for 15 min. The mixture was centrifuged at 5000 rpm for 20 min and the concentration of GlcNac produced was assayed at 530 nm spectrophotometrically with colloidal chitin as substrate from the aliquots following DNS sugar estimation test using GlcNac as standard.

RESULTS

Growth conditions and production of antifungal agent by isolate SJ602

The bacterial isolate SJ602 exhibited optimum growth after 24 h of incubation at 37°C at pH 7.0 under shaking (180 rpm). Mycelial inhibition assay shows the inhibition of *Aspergillus* fungus (Fig. 1) due to the production of chitinase enzyme (Fig. 2).

Phylogenetic Analysis of Bacterial Strain SJ602

For molecular characterization, the 16S rRNA gene was amplified, and the purified amplicon (1.2 kb) was sequenced. Multiple sequence

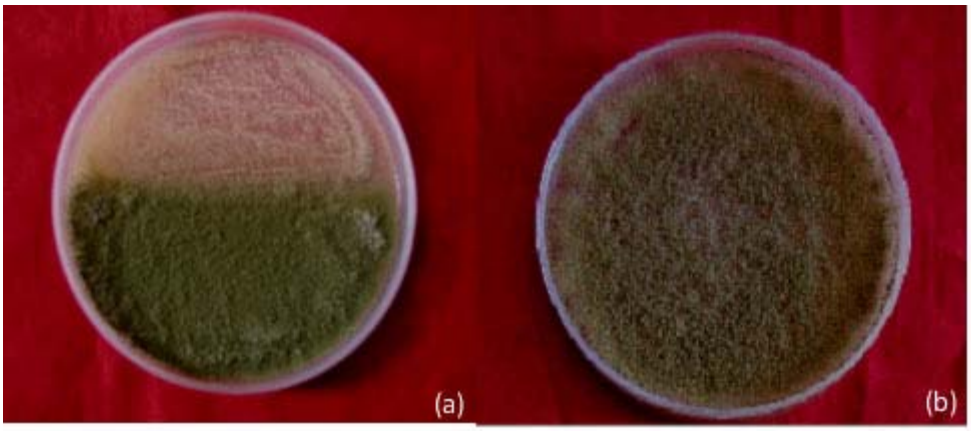

Fig. 1. Screening for antifungal activity on PDA plate (a) isolate SJ602 grown with *A. flavus* in dual culture assay, (b) control plate of *Aspergillus* fungus.

Fig. 2. Formation of halo around isolate SJ602 on colloidal chitin agar plate showing secretion of chitinase.

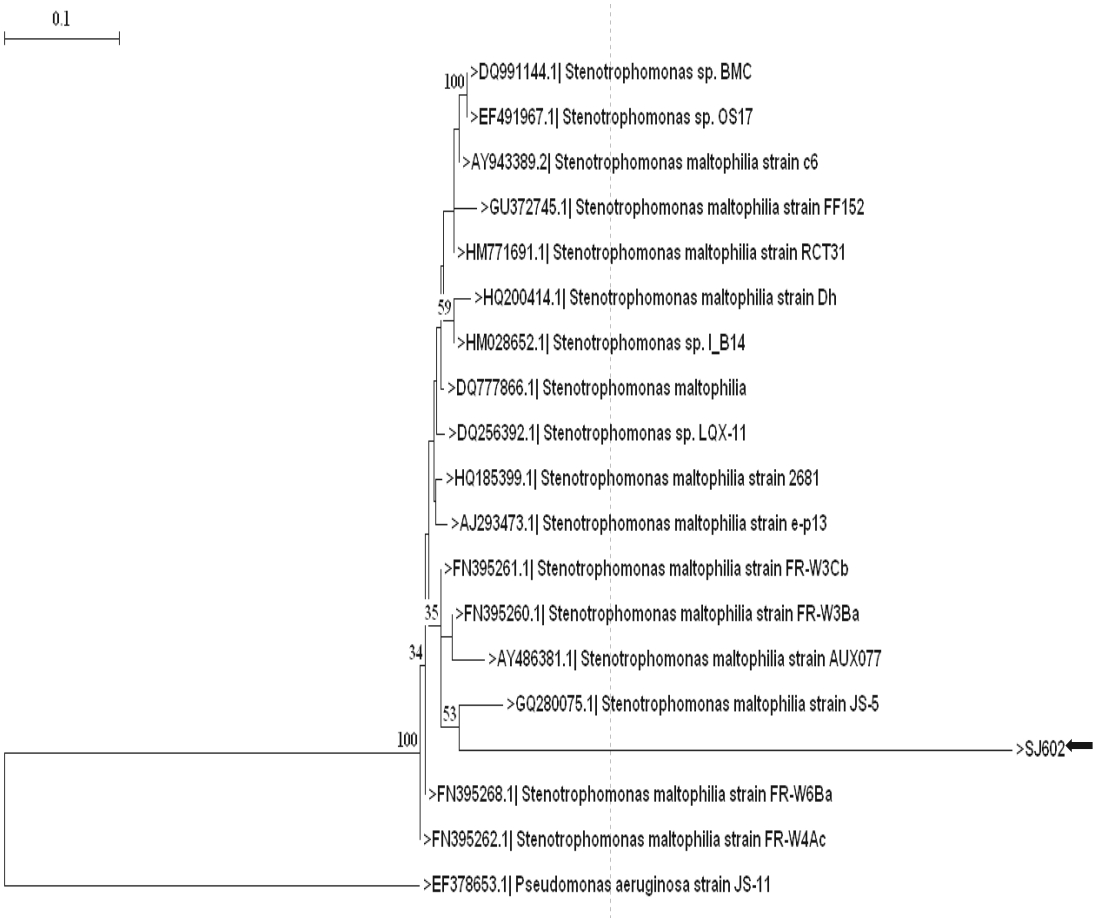

Fig. 3. Phylogenetic relationship between the *Stenotrophomonas maltophila* strain SJ602 and reference sequences retrieved from NCBI GenBank, based on 16S rRNA gene nucleotide sequences.
All bootstrap values of 34% or greater are indicated on the tree.
The scale bar indicates the numbers of nucleotide substitutions per site.

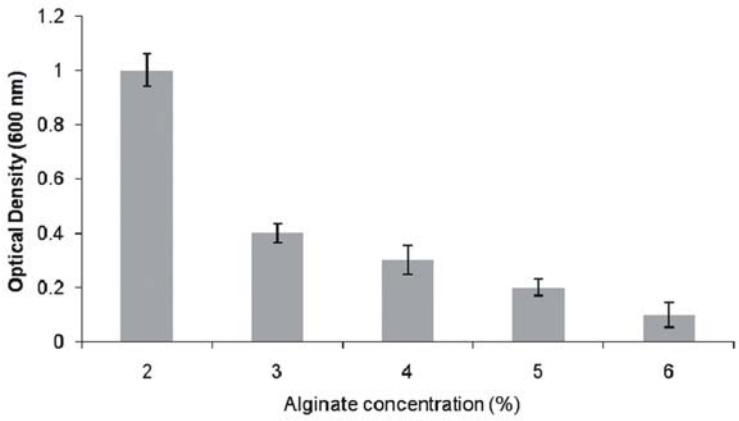

Fig. 4. Effect of different concentrations of alginate on the release of entrapped *Stenotrophomonas maltophila*.

alignments of the submitted 16S rDNA (Accession no. EU492391) with the sequences in NCBI GenBank revealed homology with the members of the genus *Stenotrophomonas*[39]. The phylogenetic tree (Fig. 3) showed closest relationship with *Stenotrophomonas maltophilia* JS5.

Effect of Alginate Concentrations

The absorbance data shown in Fig. 4 clearly demonstrated the maximum release of cells from 2% alginate vis-à-vis the moderate and slow release at 4% and 6% alginate, respectively. The average size of the wet beads obtained was determined to be 2.5-3.0 mm. Curing further improved the size uniformity and texture of the beads.

Effect of Starch and Skimmed Milk

The starch and skimmed milk at 5% concentration provided better stability as compared to unamended beads. Comparative analysis revealed that the skimmed milk powder adds greater stability than starch, and also maintains the viability of bacterial cells inside the entrapped alginate beads. At an initial stage (1 month) of storage starch provided greater firmness to the beads, where as in subsequent period the skimmed milk offered greater consistency.

The bacterial release was evaluated for all three types of beads which showed that alginate with skim milk powder discharged maximum bacteria when plated on petri plates for 6 months continuously (Fig. 5).

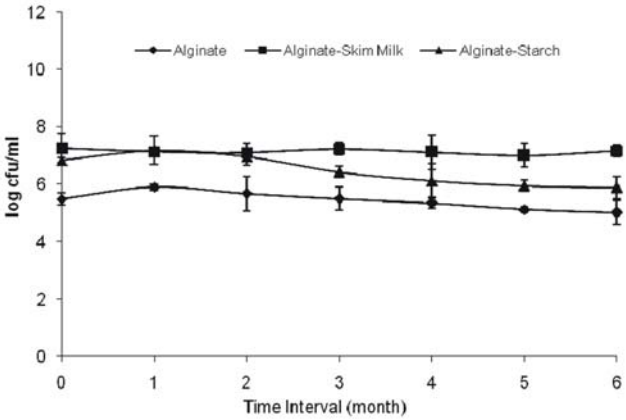

Fig. 5. Effect of different polymers on shelf life of bacteria entrapped in alginate beads. *Symbols*: diamond (♦) bacterial count in log cfu released through alginate beads; triangle (▲) indicate bacterial count in log cfu released from alginate-starch mix beads; square (■) indicate the bacterial release in log cfu from alginate-skim milk mixed beads.

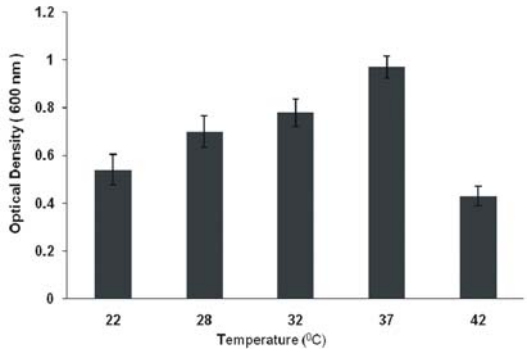

Fig. 6. Effect of different temperatures on the release of entrapped bacterial cells from alginate beads.

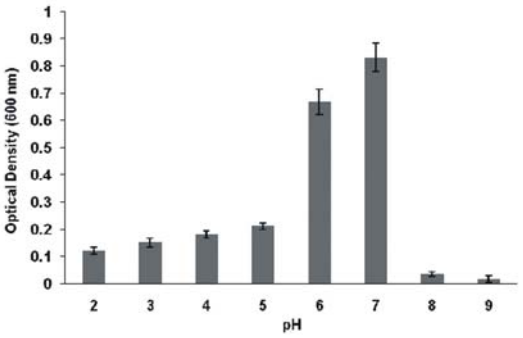

Fig. 7. Effect of different pH range on the alginate bead for release of immobilized bacterial cells.

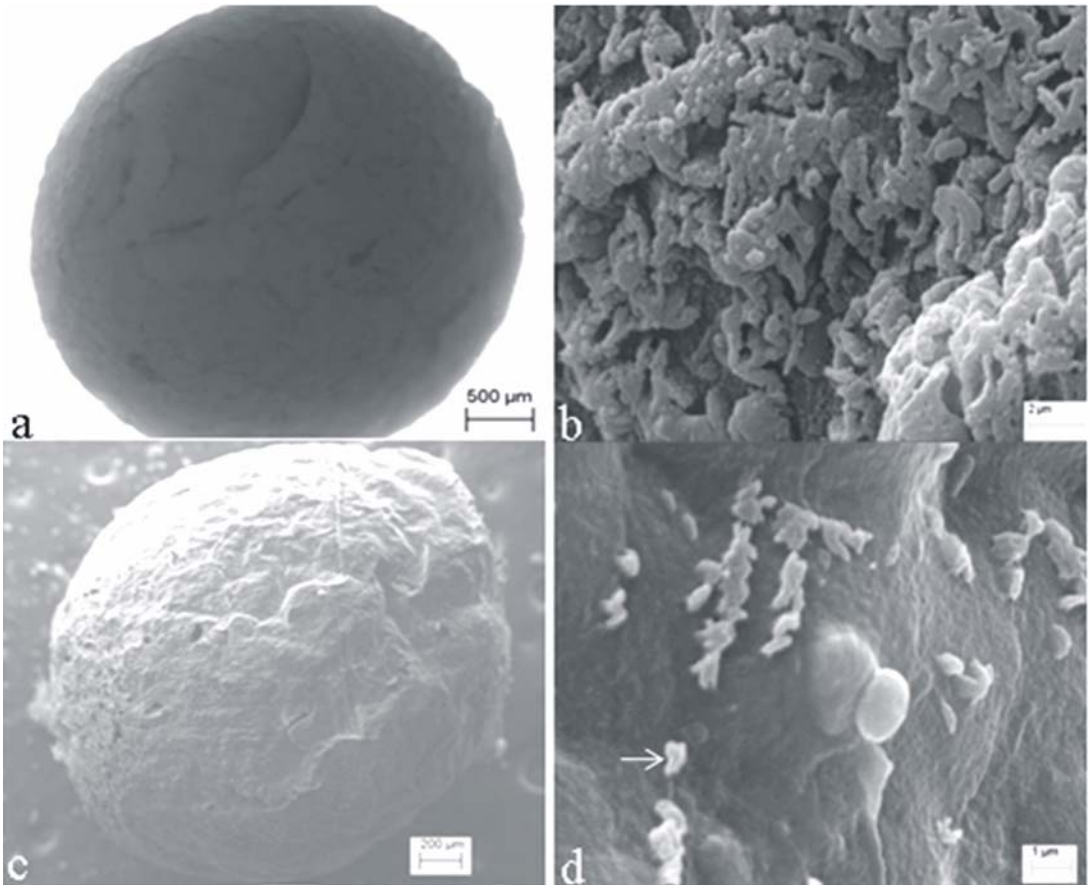

Fig. 8. Scanning electron microscope of wet and freeze-dried alginate beads. (a) Wet alginate beads with bacteria, (magnification, 40 X) (b) Micro colonies of bacteria on the surface of a wet alginate bead (magnification, 15.88 KX) (c) Freeze-dried alginate beads (magnification, 193X) (d) Surface of freeze-dried alginate beads (magnification, 17.50 KX) arrow indicating bacteria on bead surface.

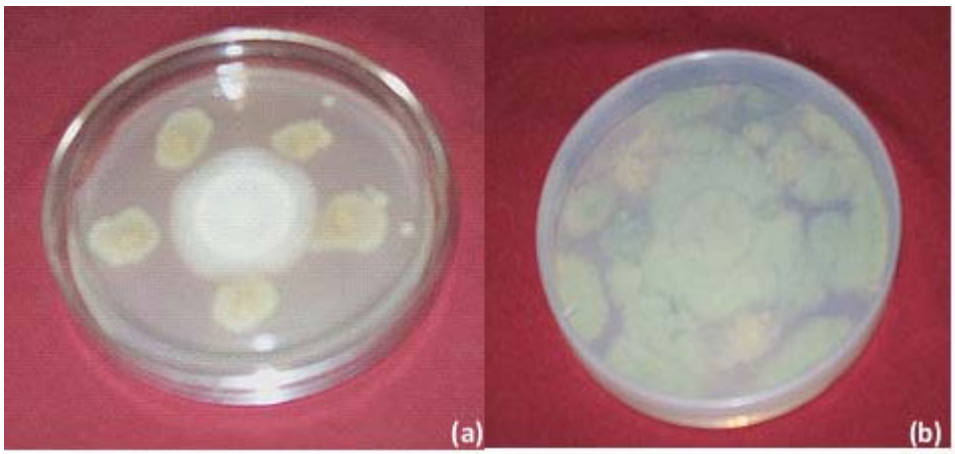

Fig. 9. *In vitro* inhibition of *A. flavus* by freeze-dried bacterial cells (a) Immobilized *Stenotrophomonas maltophilia* SJ602 at five equidistant points (b) alginate bead without bacterial cells with *A. flavus* as control.

Effect of Temperature and pH on Release of Entrapped Bacteria

Maximum release of entrapped bacteria from the beads occurs at temperature of 37°C (Fig. 6) and pH range of 7.0 (Fig. 7).

Scanning Electron Microscope Study

Assessment of beads revealed that the alginate bead has a circular structure with superficial groves and a few cavities on its coarse surface. Multiplication of bacteria inside the bead consequently resulted in the development of abundant bulges all over the bead surface area inside which the bacteria population multiplies. An additional examination of a bead showed a growth of bacterial micro colony on inner side covered with a solidified matrix, while the peripheral surface was covered by bacteria. This pattern of multiplication was found in alginate beads with both wet and freeze-dried immobilized bacteria (Fig. 8).

In vitro* inhibition study against *A. flavus

The inhibition of fungal hyphal growth was recorded as the release of freeze-dried immobilized bacterial cells from alginate hindered the growth of *A. flavus* while the control (alginate beads without bacteria cells) showed the growth of fungi (Fig. 9).

Chitinase Activity of Immobilized Bacterial Strain *Stenotrophomonas maltophilia* SJ602

Chitinase activity (U/ml) was assessed for *Stenotrophomonas maltophilia* SJ602 entrapped in alginate beads after every 2 months. Both wet and freeze-dried immobilized bacterial cells after its release showed the significant increase in the chitinase activity as compared to the non-mobilized bacterial cells confirming the fact that entrapped bacterial cells have not lost their chitinase activity upon long term storage. Interestingly, the freeze-dried entrapped cells showed a relatively higher chitinase production than wet immobilized cells (Fig. 10).

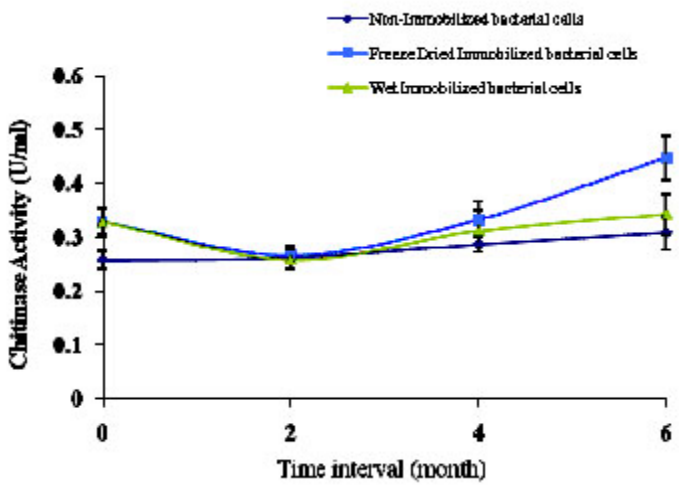

Fig. 10. Chitinase activity of both immobilized cells (wet and freeze-dried) and non-immobilized bacterial cells.

DISCUSSION

Cell immobilization is one of the common techniques for increasing the overall cell concentration, efficiency and productivity[33]. It also protects the cells from external shear forces and therefore, has been exploited for immobilizing the microbes present in nature with promising antagonistic potential for the development of biocontrol agents with improved efficacy. The main purpose of immobilizing the *S. maltophilia* SJ602 was that it was an indigenous bacterium that had been isolated from the indigenous soil source rather than using some other microbe from different geographical location. Furthermore, the isolated bacterium is a gram negative, the encapsulation increases its shelf life.

The data revealed that the alginate

concentration at lesser percentage (2%) does not entrap bacterial cells for longer duration as lower concentration of alginate make the beads porous due to larger pore size. Moreover, at a higher concentration of 6%, a less number of bacterial cells were released because of the smaller pore size and also reduced microbial growth and enzyme production was observed owing to a limited diffusion of nutrients and oxygen[40]. Although, it has been reported that 2% alginate concentration was best for the survival of *Trichoderma* spp.[26], *A. flavus*[41], and 3% for *Kitasatospora* sp. of actinomycetes[42], our studies suggested that 4% of alginate concentration is optimal for the survival and release of *Stenotrophomonas maltophila* SJ602 cells in comparison to either lower or higher concentrations.

Skimmed milk powder and starch were used for providing nutrients to bacterial cells and thereby increasing the bacterial activity, growth and/or cell survival[23]. Additionally, these adjuvants (starch and skimmed milk) provide mechanical support by acting as filler material between the alginate matrixes and help in preserving the porous cellular structure as their particles resist the collapse. The porous structure of alginate-skimmed milk combination possesses higher porosity and maximum void spaces thereby, releasing higher amount of bacteria. Also, the skimmed milk containing alginate beads have more void spaces, causing lower stiffness, thus reducing the mechanical compression which is one of the goals of the immobilization system. The encapsulated *Pseudomonas fluorescens* R2f cells grown in skimmed milk have reportedly exhibited higher cell survival rate as compared to non-immobilized or alginate immobilized only. However, cell survival and growth are more important parameters than the mechanical support for biocontrol potentials and our results re-validated the role of the polymer and corroborate well with the observations of van Elsas *et al*.[20].

Optimization of skimmed milk-alginate beads with entrapped bacteria for growth conditions at various pH and temperature ranges revealed that the acidic pH does not seem to be supportive for the release of bacterial cells, while at the pH 7.0 and temperature 37^{0}C, maximum growth or release of bacteria occurred. Also, the growth and release of entrapped bacterial cells got reduced significantly above pH 7.0 and 37^{0}C. Reduction in the dissolved oxygen content and oxygen transfer rate through outer gel matrix to the immobilized cell density are attributed for the reduced growth of cells[43] at temperatures higher than the optimum temperature. The oxygen transfer limitation through outer alginate matrix at higher temperatures results in growth of lower cell density and thereby reduced chitinase enzyme production[44].

Since the *Stenotrophomomas maltophila* SJ602 is a gram negative and motile strain, the exposed teichoic acid molecules on the cell wall surface and extracellular polysaccharides hold more water than those in the cell membrane, thereby helping bacteria to survive in desiccant environments[45]. Soil bacteria such as *Pseudomonas* spp. and *B. subtilis* produce extracellular polysaccharides when proliferating in dry environments[46].

Many strains of *S. maltophilia* reported earlier have shown chitinase production[47]. However, in our case better chitinase production was shown by the *Stenotrophomonas maltophila* SJ602 cells in the sixth month of the storage, there is a gradual increase in the chitinase activity after the second month. This may be due to the fact that bacterial cells acclimatize themselves within two months for the chitinase production. After that, the production of enzyme does not get affected even in the entrapped condition. Also, in comparison to the non-dried beads, their counterparts showed a significantly greater amount of chitinase production (Fig. 10). The higher chitinolytic activity in freeze-dried beads can be explained on the basis of different structures of wet and freeze-dried bead matrix. The alginate and divalent cation (Ca^{2+}) results in beads consisting of a dense three-dimensional lattice with pore size ranging ~0.005-0.2 mm in diameter[48]. Freeze drying of the w*et al*ginate beads gives a low density bead having much smaller pore-size and void space (in microns)[49]. These differences in bead structures provide a way for the increased release of bacteria and their products from freeze-dried beads. Another possible explanation for the increased release of chitinase is that freeze-dried beads have porous structure, thus providing a proficient metabolism in the immobilized cells and higher diffusion rates of large molecules[50]. Freeze

drying enhances the shelf life of the beads and simplifies its storage[51]. These dried products are also known for their water-absorption potential when rehydrated. The dried alginate beads' capacity to absorb a high amount of water in a short time enables rapid revival of the dormant cells and their return to activity[49].

As chitinases are known to show biocontrol activity, *Stenotrophomonas maltophilia* strain C3 inhibited the growth of the phytopathogenic fungus *Pythium ultimum* and *Bipolaris sorokiniana*, respectively, by producing the extracellular enzymes chitinase and protease[12]. The antagonism mechanism shown by *Stenotrophomonas maltophilia* strains proved that these bacterial cells under alginate immobilized conditions can serve as better biocontrol agents. Following the freeze-drying activity, alginate beads enable more efficient transport of the cells and their products. Consequently, higher bacterial densities and chitinolytic activities were distinguished in dried beads than in w*et al*ginate beads. The performance of the culture has been improved by alginate immobilization process and production of the bioactive compound has also enhanced. This form of bacterial cell cultivation is simple and can widely be used in the laboratory and industry. The above results show that bacterial isolate *Stenotrophomonas maltophilia* SJ602 has promising potential as a biocontrol agent to be applied for field application to prevent the contamination of legume crops by fungus *Aspergillus*.

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