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Chitinase Production by Marine Bacterium *Pseudomonas putida* Mb12 in Free and Immobilised form: A Comparative Investigation

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Abstract

The biotechnology sector has taken notice of the better performance of immobilised microbial cells for enzyme production. This promising technology removes the majority of the constraints that free cells experience. In the present study, chitinase production by *Pseudomonas putida* Mb12 was investigated using both free and immobilised cells. The low-cost prawn shell substrate was used for the optimization of chitinase enzyme production by Ca-alginate immobilised *P. putida* Mb12. Beads made with 3 % Na-alginate, 0.5 M $CaCl_2$, and a curing time of 60 min displayed the highest bead stability and chitinase production. The best conditions for maximal enzyme synthesis by immobilised *P. putida* Mb12 were found to be 0.5 percent prawn shell powder, 35 °C, pH 6.0, and agitation at 50 rpm. Free bacterial cell, on the other hand, requires agitation at 150 rpm, 40 °C, pH 7.0, and 0.4 percent shrimp shell powder for maximum enzyme production. Immobilised *P. putida* Mb12 was more resilient to environmental variations such as temperature and pH, retaining 90 % of its peak activity at 30-65 °C and 100 % activity within pH 5–10. Fermentation and thermodynamic parameters suggested that immobilised cells produce 1.5 times more enzyme than free cells. After one month, immobilised *P. putida* Mb12 retained around 90 % of its initial activity.

Key Words: *Pseudomonas putida*, chitinase, immobilization of bacteria, sodium alginate, prawn shell waste, optimization

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Introduction

Chitin is the second most prevalent polymer and is made up of *N*-acetylglucosamine through a β-(1 → 4) glycosidic bond (Gooday, 1995). Chitinases (E.C. 3.2.1.14, Poly {1, 4- N- acetyl D- glucosaminide} glucanohydrolase) are glycosidase enzyme that particularly degrade chitin at the bond between C_1 and C_4 of two consecutive *N*-acetylglucosamine monomers (Flach et al., 1992). Chitinase have variety of applications such as fungal protoplast isolation (Balasubramanian et al., 2003; Prabavathy et al., 2006), mosquito control by degrading cuticle of insects, which contain chitin as an essential component (Mendonsa et al., 1996), production of single cell protein (Vyas & Deshpande, 1991) and oligosaccharides and N-acetyl glucosamine synthesis (Makino et al., 2006).

Crustacean (shrimp, prawn, crab) shells contain high amount of chitin among all biological compounds (Muzzarelli, 1977). India is a major player in shrimp rearing and trading industry and disposal of shrimp shell waste generated during shrimp processing is a major problem in the fisheries sector (Dhillon et al., 2012). Most chitinous wastes are disposed through ocean dumping, cremation, or land filling. This results in natural capital loss, economic loss, and ecological pollution. The chitin can be recovered from chitinous waste products *via* chemical methods, which include demineralization and deproteinization by either a strong acid or base. Chemical treatments also present problems with waste disposal because neutralisation and filtration of the released waste water are necessary. Chitinases are involved in the enzymatic breakdown of chitinous waste, and they have a dual role in the exploitation of chitinous materials and the mitigation of microbial chitinases' manufacturing costs (Gohel et al., 2006). Bacterial exploitation of shellfish

debris for the synthesis of chitinases has been described by Wang et al. (2001), Rattanakit et al. (2002), and Paul et al. (2012). Chitinolytic microorganisms are found in abundance in nature and are good sources of chitinases due to the low cost of production and convenient procurement of raw materials for cultivation. The ability of such a microbial population to breakdown chitin is also critical for nitrogen recycling (Chandran et al., 2007). Bacteria like *Serratia marcescens, Xanthomonas maltophilia, Stenotrophomonas maltophilia* and *Paenibacillus illinoisensis* (Kobayashi et al., 1995; Zhang & Yuen, 2000; Jung et al., 2003) have been found to be chitinase producers.

Immobilization of microbial cells can be utilised to boost the effectiveness and affordability of fermentation process for chitinase production. Compared to free cell systems, whole cell immobilisation has various advantages such as better yield of enzyme activity, productivity, operational stability, catalytic efficiency, environmental resilience, cheaper enzyme cost, and biocatalyst reuse (Mamo & Gessesse, 2000; Nampoothiri et al., 2005; Konsoula & Liakopoulou, 2006). Alginate is the most widely utilised immobilisation matrix worldwide because encapsulation in Ca-alginate gels happens under extremely gentle circumstances and has a low cost (Bladino et al., 2001; Dey et al., 2003). The current study was undertaken to immobilise *P. putida* Mb12 in Ca-alginate gels for chitinase production. The objective of this work was to optimise immobilisation parameters and to compare the culture conditions of free and immobilised *P. putida* Mb12 for increased chitinase biosynthesis.

Materials and Methods

For immobilisation studies, *P. putida* Mb12, a newly isolated marine bacterial strain from Cochin, Ernakulam, Kerala, India, was employed. *P. putida* Mb12 culture (2×10^7 CFU/mL) was added to the cooled (25 °C) sodium alginate solution (1 %) and gently mixed. Using a 5 mL automated pipette, the mixture was then dropped into a beaker containing calcium chloride solution (0.5 M). To improve the cross- linking and stability of the beads, the beaker was gently swirled at 70 rpm at 40 °C for 1 h. Finally, the beads were washed with deionized water to remove superfluous calcium ions and free cells. To estimate ideal conditions the efficient polymerization of calcium alginate spheres with immobilised bacterial cells, immobilisation conditions were optimised using 1-8 % sodium alginate and 0.1–0.8 M calcium chloride. Immobilisation efficiency was also enhanced by changing the curing time from 30 to 135 min. Ten beads were randomly chosen from the fermentation broth and transferred to a 50 mL flask containing 20 mL of 0.2 M sodium citrate buffer (pH 6). At room temperature, the beads were subsequently solubilized by agitating with a magnetic stirrer. The cell suspension (100 µL) was then seeded onto nutrient agar plates and cultured for 48 h at 37 ºC. The number of colony-forming units was counted. For production of chitinase, fifty immobilised beads (4×10^5 CFU/bead) and one mL of free *P. putida* Mb12 culture (2×10^7 CFU/mL) were inoculated into 100 mL of a 1 % prawn shell powder suspension (pH 6.5) and cultured at 37 °C for 48 h in a shaking incubator at 100 rpm. The supernatant was collected after centrifuging the culture for 20 min at 8000 rpm to assess chitinase activity. One mL of the supernatant was mixed with 1mL of 1 % colloidal chitin in 0.05 M phosphate buffer (pH 7) and incubated at 40°C for one hour. According to the procedure outlined by Rojas-Avelizapa et al. (1999), the chitinase activity was determined by the reduction of 3,5-dinitrosalicylic acid in the presence of N-acetyl-D-glucosamine (NAG) produced by the enzymatic hydrolysis of colloidal chitin. The absorbance was measured at 530 nm. Readings were compared to a standard curve made with a series of dilutions of NAG. Under experimental conditions, one unit of chitinase was defined as the quantity of enzyme that generated one micromole of N-acetyl glucosamine per millilitre per minute. Using bovine serum albumin (Sigma) as a reference, the protein concentration was measured as described by Lowry et al. (1951). At 37 °C and 100 rpm, 1 % prawn shell powder suspension (pH 6.5) was inoculated with entrapped cells as well as free cells (control) and incubated for 24 h. The cells that leaked out of the encapsulated gels or beads were evaluated spectrophometrically by measuring optical density at 600 nm every hour, and one OD was found equal to 0.23 g cell dry weight/L. The variables were investigated in order and at a time, one factor was optimised. In the following stage, the optimal level of this component was integrated. All of the experiments were done in triplicates, and the average enzyme activity with standard deviations was calculated using SPSS 11.5 software. The culture was inoculated into a fermentation medium containing prawn shell powder and cultured for various

durations of time before being evaluated for enzyme production (12-108 h). The fermentation medium contained different quantities of prawn shell powder (0.1-0.9 percent). At 37 °C, immobilised *P. putida* Mb12 was cultured for 24 h and free form for 36 h, and the enzyme was estimated. The synthesis of chitinase was evaluated by changing the agitation rate of the incubator shaker (50-250 rpm). The optimum temperature and pH for production of enzyme were selected by varying the pH of the medium (3-8) and temperature of incubation (20-60 °C). The immobilised and free form of *P. putida* Mb12 was cultured in the presence of 5mM of metal ions such as $MnCl_2$, $CaCl_2$, $HgCl_2$, $CuSO_4$, $ZnCl_2$, and $MgCl_2$ as well as surfactants (1 % v/v) such as Tween 20, Triton X –100, and Tween –80 to see how they affected the growth of bacterium. The following batch-by-batch reuse strategy was used for the experiment: at the end of each production cycle beads were collected by centrifugation and washed with 0.9 % saline under aseptic condition and were added to fresh production medium. The shelf life of the beads was tested weekly for a month by culturing the preserved beads in new production media and comparing cell viability (CFU/mL) along with chitinase activity as mentioned in the previous section.

Results and Discussion

The concentrations of sodium alginate and calcium chloride are linked to the strength and hardness of beads. Immobilized beads must have sufficient mechanical strength to prevent cell leakage. A hard bead can disrupt nutritional transmission, but a soft bead lacks mechanical strength. As a result, the impact of different sodium alginate and $CaCl_2$ concentrations on the texture of the beads was assessed. Preliminary tests revealed that a 3 percent aqueous sodium alginate solution (Fig. 1) and a 0.5 M aqueous $CaCl_2$ solution (Fig. 2) were the best concentrations for production of immobilised beads. Immobilisation was observed at various curing durations (30 to 135 minutes). The result showed that chitinase production was highest at 60 minutes (Fig. 3). However, after 60 minutes of curing time, there was no improvement in efficiency, possibly due to the sphere's sustained hardness. The immobilized beads are shown in Fig. 4.

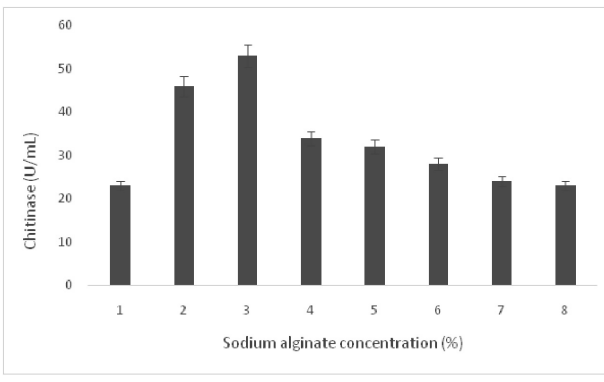

Fig. 1. Effect of sodium alginate concentration on chitinase production

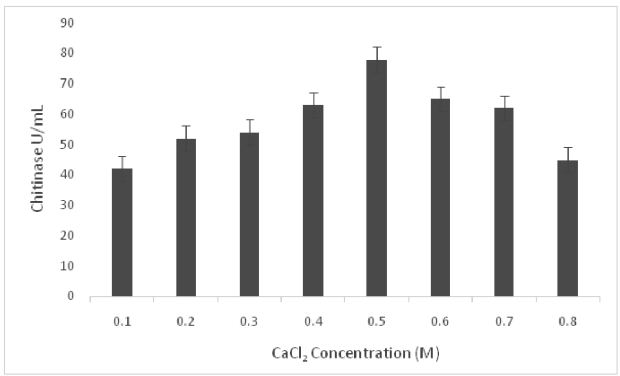

Fig. 2. Effect of $CaCl_2$concentration on chitinase production

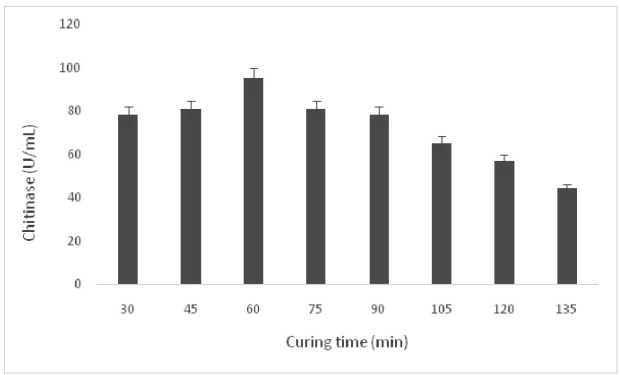

Fig. 3. Effect of curing time (min) on chitinase production

Chitinase production is regulated by a number of physical and chemical parameters in immobilised and free forms of this bacteria. Fig. 5a depicts chitinase synthesis by free and immobilised *P. putida* Mb12 during cultivation. After 24 h of incubation, immobilised *P. putida* Mb12 produced the highest enzyme, while the free form of bacteria produced the most after 36 h, and thereafter the enzyme production decreased.

Fig. 5b shows the impact of various percentages of prawn shell powder in media on chitinase release

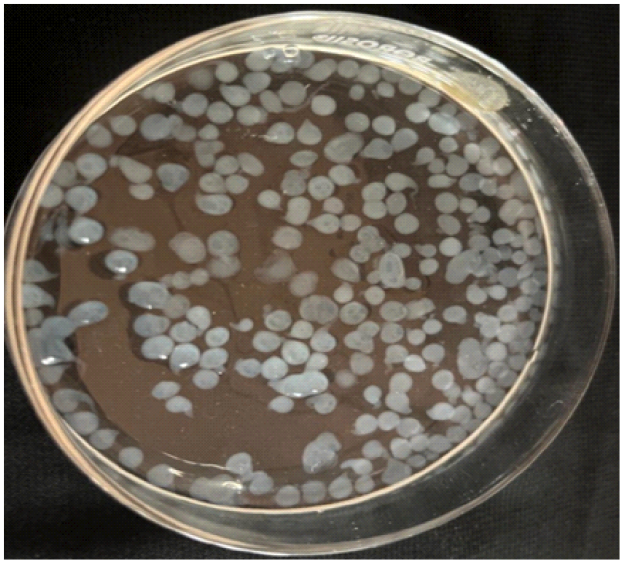

Fig. 4. Immobilised *P. putida* Mb12

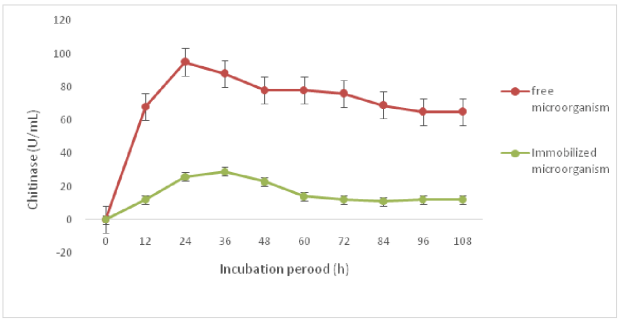

Fig. 5a. Effect of incubation period on chitinase production by free and immobilized *P. putida* Mb12

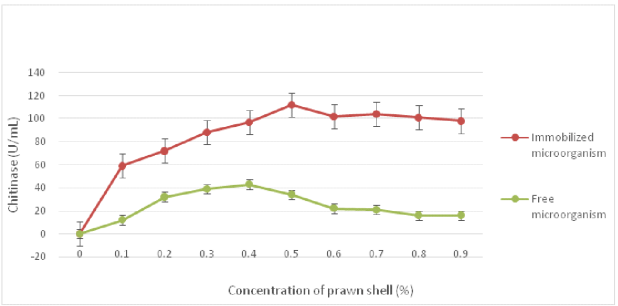

Fig. 5b. Effect of concentration of prawn shell on chitinase production by free and immobilized *P. putida* Mb12

by free and immobilised *P. putida* Mb12. The medium with 0.5 percent prawn shell powder and immobilised *P. putida* Mb12 produced the highest chitinase, whereas the free bacteria required 0.4 percent prawn shell powder.

Fig. 5c demonstrates the influence of agitation rate on chitinase synthesis by free and immobilised *P. putida* Mb12. Immobilized *P. putida* Mb12 had maximum enzyme production, when agitated at 50 rpm. But the free bacteria produced the most chitinase, when agitated at 150 rpm. When the agitation rate was increased to 250 rpm, the yield of immobilised microorganisms dropped.

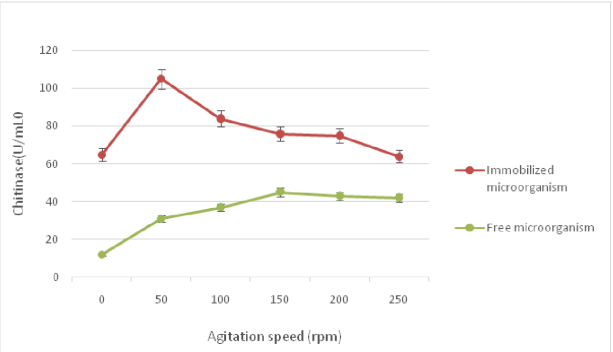

Fig. 5c. Effect of agitation speed on chitinase production by free and immobilized *P. putida* Mb12

The chitinase synthesis by free and immobilised *P. putida* Mb12 at different temperatures and pH are given in Fig. 5d and 5e, respectively. At 35 °C, immobilised *P. putida* Mb12 produced the highest chitinase, while the free cells had the highest production at 40 °C (Fig. 5d). Immobilized *P. putida* Mb12 produced the highest chitinase at pH 5, and chitinase production was steady between pH 5 and 10 (Fig. 5e), whereas the free cells produced the most at pH 7, and above pH 7, chitinase levels declined drastically.

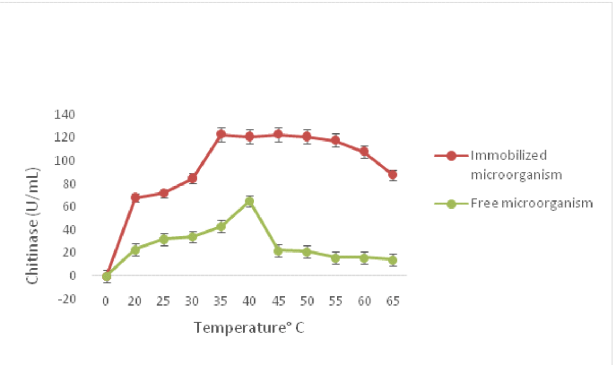

Fig. 5d. Effect of temperature on chitinase production by free and immobilized *P. putida* Mb12

The effect of metals and surfactants on the chitinase activity of free and immobilised *P. putida* Mb12 is shown in Table 1. Mg^{2+} and Ca^{2+} marginally boosted

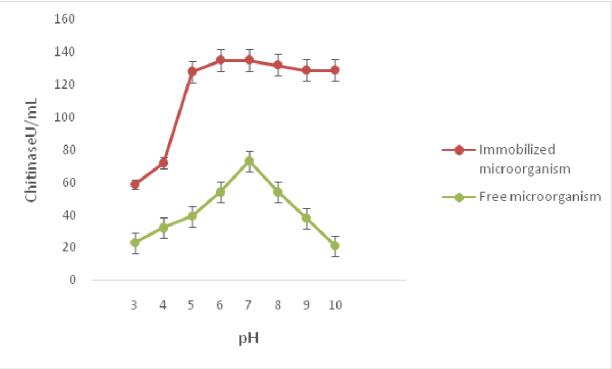

Fig. 5e. Effect of pH on chitinase production by free and immobilized *P. putida* Mb12

Chitinase production, whereas Mn2+, Zn2+, and Cu2+ inhibited the enzyme production (Table 1). The incorporation of surfactants in the media increased the enzyme production in both the cases.

Table 1. Effect of metal and surfactants on chitinase production in free and immobilized *P. putida* Mb12

Metal/Surfactants	Chitinase produced by immobilized *P. putida* Mb12 (U/mL)	Chitinase produced by free *P. putida* Mb12 (U/mL)
Control	136±1.23	76±0.34
$MnCl_2$	108±1.32	67±0.23
$CaCl_2$	145±1.79	78±1.48
$MgCl_2$	149±1.41	86±1.46
$CuSO_4$	98±1.58	54±1.38
$ZnCl_2$	106±1.6	46±1.21
Tween 20	148±1.73	86±1.39
Triton X –100	163±1.42	79±1.60
Tween -80	149±1.65	83±1.23

The immobilised *P. putida* Mb12 were able to make more than 60 % chitinase even after 10 cycles (Fig. 6). The results revealed that the quantity of enzyme production with immobilised cells gradually reduced from the first cycle onwards. Simultaneously, leakage of cell from the gel into the media was noted from the first batch itself. During the production process of the 12th batch, the beads disintegrated.

About 90 % of the initial chitinase activity of immobilised *P. putida* Mb 12 was retained even after one month while the cell growth reduced from 4.0 log to 3.6 log (Fig. 7).

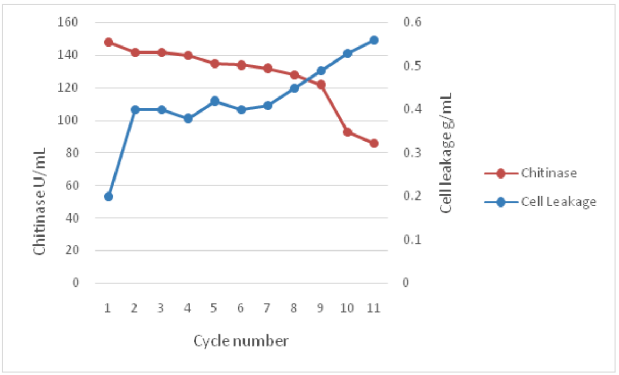

Fig. 6. Reuse of immobilized *P. putida* Mb12

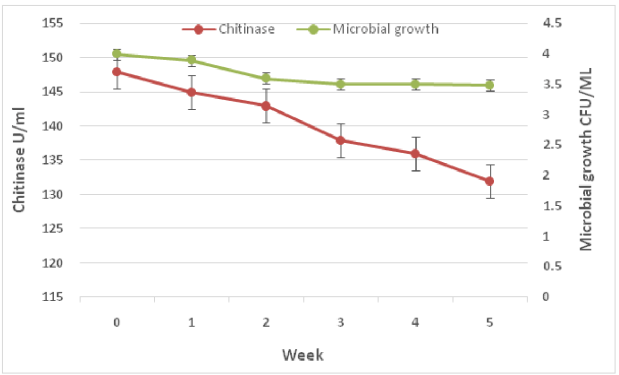

Fig. 7. Effect of beads storage time on cell viability and chitinase production

Cell immobilisation techniques have been used by many researchers to produce enzymes on a continual basis (Ramakrishna & Prakasham, 1999). Cell immobilisation provides a number of advantages over traditional techniques, including quick cell separation and reuse; protection against severe shear damage, high cell densities, easy reactor design with operation flexibility, greater substrate usage, a suitable milieu for the cell, and the normalisation of multiple cell functions (Dervakos & Webb, 1991). Despite these benefits, the immobilisation of microbial cells is limited by gel disintegration, significant mass transfer, low mechanical strength, and large pore size. For immobilisation of microbial cells for large-scale production of the extracellular chitinase enzyme, suitable material and methodology design are critical. They have numerous benefits, including user-friendliness, low cost, easy availability, and non-toxicity. The extracellular chitinase production by immobilised cells of the *P. putida* Mb12 strain was

optimised utilising Ca-alginate beads, as well as the validation of several parameters for enhanced chitinase output. Because of the entrapped cell's reusability and long-term storage ability, the findings of this study will be extremely beneficial for production of chitinase enzyme.

In this study, beads were prepared by altering the percentage of alginate in order to establish the appropriate strength for entrapping *P. putida* Mb12. The concentration of the gel has a big impact on enzyme synthesis. The optimal alginate concentration varies depending on the microorganism and the commodity of interest. Here, when beads were made with 3 % alginate and 0.5 M $CaCl_2$, chitinase production was at its peak and beads were exactly spherical. Beads with less than 3 % alginate were brittle and had a lot of cell leakage. However, over 3 % of Ca-alginate, enzymatic production decreased due to reduced porosity and cell proliferation inside the beads, creating an additional barrier to nutrient diffusion in the beads. The immobilised balls were opaque, rigid, and trailing at calcium chloride concentrations greater than 0.5 M, but had better transparency and hardness at a concentration of 0.5 M. In comparison to the free form, the chitinase production of immobilised *P. putida* Mb12 improved by 5-fold after optimising process parameters of immobilisation (sodium alginate concentrations of 3 % and 0.5 M calcium chloride, with 60 min curing time). El-sherif et al. (2013) reported that *B. lichenifomis* cells entrapped in 2% agar have higher chitinase activity (1.25 U/ml) than free cells. *A. hydrophila* SBK1 increased its chitinase specific productivity by 33.37 percent, when grown in 5.0 percent Na-alginate and 1.0 M $CaCl_2$.

From the early exponential phase of growth, the isolate could synthesise chitinase. A continuous increase in enzyme synthesis was observed with the progression of bacterial growth. After 24 h of fermentation, immobilised cells produced 3.8 times the amount of chitinase that free cells produced. The incubation time for production of chitinase varies with different bacteria; *Micrococcus sp*. AG84 (Annamalai et al., 2011) require 48 h of incubation to produce chitinase, while *Pseudomonas aeruginosa* (Wang et al., 2001) produced maximal chitinase after 72 h.

Maximum enzyme yields of 43 and 112 U/mL were obtained with free bacterial cells and immobilised *P. putida* Mb12 in culture medium containing 0.4 percent and 0.5 percent prawn shell powder, respectively. A larger concentration of prawn shell powder adversely affected enzyme synthesis by free cells. This would be due to the increased viscosity of culture broth at greater concentrations of prawn shell powder, which obstructs oxygen transfer and causes osmotic shock, thus limiting microbial growth. On the other hand, the Ca-alginate layer protected cells from these conditions. The Ca-alginate layer limited the translocation of polymeric prawn shell powder (mass transfer), thus making this process more suitable for liquefaction of higher concentrations of prawn shell powder in solution.

Agitation offers several advantages, including maintaining homogeneity throughout the fermentation process, promoting gas movement, increases bioavailability of micronutrients and dissolved oxygen to the organism, and facilitating heat transfer. But when the agitation rate was increased to 250, the yield of chitinase by immobilised *P. putida* Mb12 decreased dramatically. This could be owing to the influence of hydrodynamic stress, which could cause intracellular compound leakage (Chipeta et al., 2008).

Immobilized *P. putida* Mb12 exhibited increased production of chitinase under a range of temperature and pH. The decreased cell damage after the cells were entrapped inside the chitin-alginate may account for the improved stability of immobilised cells. Similarly, at 35 °C and pH 7.0, immobilised *Aeromonas hydrophila* SBK1 demonstrated increased chitinase synthesis. Detergents are thought to boost enzyme secretion by increasing cell membrane permeability (Chellapandi & Himanshu, 2008). This explains why the addition of detergents to the culture media increased chitinase synthesis in both free bacterial cells and immobilised *P. putida* Mb12.

One of the goals of immobilisation is to reuse the cells. They can be employed in a new fermentation process after previous fermentation. Even after 9 cycles of repeated hydrolysis of prawn shell powder, the immobilised *P. putida* Mb12 produced 84.5 percent chitinase compared to the first cycle. The subsequent decrease in chitinase activity could be attributed to a loss of bead stiffness, cell leakage, or the loss/utilization of entrapped colloidal chitin. Our findings on immobilised cell reusability were consistent with prior studies (El-Sherif et al., 2013). Immobilisation of *P. putida* Mb12 cells in Ca-alginate beads and employing prawn shell waste as an affordable substrate can result in high production of chitinase compared to free cell.

References

Annamalai, N., Rajeswari, M.V., Vijayalakshmi, S. and Balasubramanian, T. (2011) Purification and characterization of chitinase from *Alcaligenes faecalis* AU02 by utilizing marine wastes and its antioxidant activity. Ann. Microbiol. 61: 801-80

Balasubramanian, N., Annie Juliet, A., Srikalavani, P. and Lalithakumari, D. (2003) Release and regeneration of protoplasts from *Trichothecium roseum*. Can. J. Microbiol. 49: 263-268

Bladino, A., Macias, M. and Cantero, D. (2001) Immobilization of glucose oxidase with calcium alginate gel capsules. Process Biochem. 36: 601-606

Chandran, S., Parameswaran, B. and Pandey, A. (2007) Microbial Chitinases: Effective Biocontrol Agents. In: Biological Control of Plant Diseases, Chincholkar, S.B. and K.G. Mukerji (Eds.), pp. 381-382, The Haworth Press, Inc., New York

Chellapandi, P. and Himanshu, M.J. (2008) Production of endoglucanase by the native strains of *Streptomyces* isolates in submerged fermentation. Braz. J. Microbiol. 39: 122-127

Chipeta, Z.A., Du-Preez, J.C. and Christopher, L. (2008) Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. J. Ind. Microbiol. Biotechnol. 35: 587-594

Dervakos, G.A., Webb, C. (1991) On the merits of viable cell immobilization. Biotechnol. Adv. 9: 559-612

Dey, G., Singh, B. and Banerjee, R. (2003) Immobilization of á-amylase produced by *Bacillus circulans* GRS 313. Braz. Arch. Biol. Technol. 46: 167-176

Dhillon, G.S., Kaur, S., Brar, S.K. and Verma, M. (2012) Green synthesis approach: extraction of chitosan from fungus mycelia. Crit. Rev. Biotechnol. 33: 379-403

El-Sherif, M.F., Youssef, A.S., Hassan, M.A., Hassan, H.M. and El-Aassar, S.A. (2013) Immobilization and Solid-State Fermentation Methods for Chitinase Production from *Bacillus licheniformis*. Life Sci. J. 10(4): 3036-3043

Flach, J., Pilet, P.E. and Jolles, P. (1992) What's new in chitinase research? Experientia. 48: 701-716

Gohel, V., Singh, A., Maisuria, V., Phdnis, A. and Chatpar, H.S. (2006) Bioprospecting and antifungal potentialof chitinolytic microorganisms. Afr. J. Biotechnol. 5: 54-72

Gooday, G.W. (1995) Diversity of roles for chitinases in nature. In: Chitin and Chitosan. (Zakaria, M.B., Wan Muda, W.M., Abdullah, M.P., Eds), pp 191-202, Malaysia: Penerbit Universiti Kebangsaan

Jung, W.J., An, K.N., Jin, Y.L., Park, R.D. and Lim, K.T. (2003) Biological control of damping-off caused by *Rhizoctonia solani* using chitinase-producing *Paenibacillus illinoisensis* KJA-424. Soil Biol. Biochem. 35: 1261-1264

Kobayashi, D.Y., Guglielmoni, M. and Clarke, B.B. (1995) Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biocontrol agents for summer patch disease of turfgrass. Soil Biol. Biochem. 27: 1479-1487

Konsoula, Z. and Liakopoulou, M.K. (2006) Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from *Bacillus subtilis*. Process Biochem. 41: 343-349

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193: 265-275

Makino, A., Chmae, M. and Kohayashi, S. (2006) Synthesis of fluorinated chitin derivatives via enzymatic polymerization. Macromol. Biosci. 6: 862-872

Mamo, G. and Gessesse, A. (2000) Immobilization of alkaliphilic Bacillus sp. Cells for xylanase production using batch and continuous culture. Appl. Biochem. Biotechnol. 87: 95-101

Mendonsa, E.S., Vartak, P.H., Rao, J.U. and Deshpande, M.V. (1996) An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedes aegypti* mosquito. Biotechnol. Lett. 18(4): 373-376

Muzzarelli, R.A.A. (1977) Chitin, 252p, Pergamon Press Ltd, Oxford

Nampoothiri, K.M., Roopesh, K., Chacko, S and Pandey, A. (2005) Comparative study of amidase production by free and immobilized *Escherichia coli* cells. Appl. Biochem. Biotechnol. 120: 97-10

Paul, M.K., Mini K.D., Ally C Antony, Radhakrishnan, E.K. and Mathew, J. (2012) Utilization of prawn shell powder for the production of chitinase by *Kurthia gibsonii* MB126. Int. J. Pharma Bio Sci. 3(3): 163-172

Prabavathy, V.R., Mathivanan, N., Sagadevan, E., Murugesan, K. and Lalithakumari, D. (2006) Intra-strain protoplast fusion enhances carboxymethyl cellulase activity in *Trichoderma reesei*. Enzyme Microb. Technol. 38: 719-723

Ramakrishna, S.V. and Prakasham R.S. (1999) Microbial fermentations with immobilized cells. Curr. Sci. 77: 87-100

Rattanakit, N., Plikomol, A., Yano, S., Wakayama, M. and Tachiki, T. (2002) Utilization of shrimp shellfish waste as a substrate for solid-state cultivation of *Aspergillus* sp. S1-13: Evaluation of a culture based on chitinase formation which is necessary for chitin-assimilation. J. Biosci. Bioeng. 93: 550-556

Rojas-Avelizapa, L.I., Cruz-Camarillo, R., Guerrero, M.I., Rodríguez-Vázquez, R. and Ibarra, J.E. (1999) Selec-

tion and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis*, able to grow in shrimp waste media. World J. Microbiol. Biotechnol. 15(2): 299-308

Vyas, P.R. and Deshpande, M.V. (1991) Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its utilization to produce SCP. J. Appl. Gen. Microbiol. 37: 267-75

Wang, S.Y., Moyne, A.L, Thootappilly, G., Wu, S. J., Locy, R.D. and Singh, N.K. (2001) Purification and characterization of a *Bacillus cereus* exochitinase. Enzyme Microb. Technol. 28: 492-498

Zhang, Z. and Yuen, G.Y. (2000) The role of chitinase production by *Stenotrophomonas maltophilia* strain C3 in biological control of *Bipolaris sorokiana*. Phytopathology. 90: 384-389