

Research Paper

Enhancement of chitosanase production by cell immobilization of *Gongronella* sp. JG

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Abstract

Chitosanase production of *Gongronella* sp. JG cells immobilized in calcium alginate gel and polyurethane foam was compared with that of the free cells, there was a 60% increase in the enzyme yield (2429 U/L) compared to the highest yield obtained from free cells (1513 U/L). The optimal immobilization parameters (concentrations of sodium alginate, calcium chloride, bead inoculums, bead diameter, etc) for the enhanced production of chitosanase were determined as: sodium alginate 2% (w/v), 0.1 M calcium chloride, inoculum 10 mL beads to 100 mL production media and 2.7 mm bead diameter. Maximum chitosanase production was achieved with initial pH of 5.5 and temperature of 30 °C. The alginate beads had well stability, retained 85% ability of enzyme production even after 7 cycles of repeated batch fermentation. These results showed the immobilization technique was a feasible and economical method for chitosanase production by *Gongronella* sp. JG.

Key words: chitosanase production, *Gongronella* sp. JG, cell immobilization, sodium alginate.

Introduction

Chitosan, mainly obtained by deacetylation of chitin that has been extracted from an abundant of shrimp or crab shells, is a deacetylated derivative of chitin and is part of the most abundant renewable biomass, next to cellulose, of the earth. Applications of chitosan have been developed in industries, such as food production and pharmaceutical industry (Choi *et al.*, 2004; Streit *et al.*, 2009; Bento *et al.*, 2009). Moreover, chitosan oligosaccharides have recently received much attention because they perform various biological activities, such as inhibiting the growth of fungi and bacteria, activating immune response and exerting anti-tumour activity (Hadwiger *et al.*, 1994; Suzuki *et al.*, 1986; Ueno *et al.*, 1997). Although it would be desirable to utilize chitosan extensively as a biomaterial, most chitosans are not being utilized due to their high molecular weights and high viscosity. Deacetylated chitosans are customarily produced by treating chitin in a concentrated alkaline solution (50%, w/v) and boiling it for several hours, or enzyme catalyzing the cleavage of β -1,4-linked glycosidic linkage by

chitosanase (EC 3.2.1.132) which has been found in many micro-organisms, including viruses, bacteria and fungi (Alfonos *et al.*, 1992; Kim *et al.*, 2004; Park *et al.*, 1999; Price *et al.*, 1975; Sun *et al.*, 2006; Zhu *et al.*, 2003). Enzymatic processes to produce chitosan oligosaccharides are an ideal alternative compared to traditional chemical degradation which needs acid hydrolysis and results in low yields of oligosaccharides.

Microbial products are usually obtained from free or immobilized cells. The immobilized cell technology has several advantages over ordinary suspension culture systems, such as elimination the step of enzyme purification and extraction, higher enzyme activity after immobilization, greater resistance to environmental perturbations and lower cost. In addition, the immobilization support can be chosen with the desired geometry that provides greater ease of handling, better mass transfer characteristics and physical resistance (Hemachander and Bose, 2001). This has triggered a surge of research activity in this exciting and rapidly growing field. General immobilization techniques include the covalent attachment or adsorption to solid

supports, entrapment within a gel lattice, microencapsulation within semi-permeable membranes and cross-linking to insoluble aggregates. There are various natural and synthetic polymers that have been used, such as ion-exchange resins, activated carbon (Grishin and Tuovinen, 1988), polyurethane foam BSP (Armentia and Webb, 1992), nicked alloy fiber (Gomez and Cantero, 2000), calcium alginate (Dias *et al.*, 2000; Lancy *et al.*, 1984), PVA-boric acid (Long and Huang, 2004). However, each has its drawbacks. One of the most suitable methods for cell immobilization is entrapment in calcium alginate, because this technique is simple and cheap. Sodium alginate is a readily available non-toxic biological material and is therefore suitable as an immobilization matrix for bio-molecules and microorganisms. Beads of calcium alginate prepared under mild conditions have been used extensively for microencapsulating and entrapping cells (Jamuna and Ramakrishna, 1992).

A lot of work has been carried out on chitosanase production in submerged fermentation. However, little information is available about immobilization of microbial cells for extracellular chitosanase production (Wu and Xia, 2000). *Gongronella* sp. JG is a novel chitosanase producing fungus isolated by our group (Yuan and Zhou, 2007). The previous study found that strain *Gongronella* sp. JG produced at least two chitosanase isoenzymes and the two enzymes had been purified and characterized (Wang *et al.*, 2008; Zhou *et al.*, 2008). In the present investigation, a successful attempt has been made to identify the process conditions optimal for the immobilization of *Gongronella* sp. JG in calcium alginate gel and to determine the operational stability of immobilized beads in the production of chitosanase in repeated batch fermentation.

Materials and Methods

Chemicals

Chitosan with DA85 (deacetylate degree) was purchased from the local suppliers in China. D-glucosamine was purchased from Sigma-Aldrich. All other reagents were of analytical grade. Colloidal chitosan was prepared according to the method described by Kurakake *et al.* (2000) with a little modification. Commercial chitosan (1 g, 85% deacetylated) was dissolved in water (10 mL) by adding acetic acid (0.285 mL). Then, the pH was adjusted to 5.5 and water was added to a total volume of 100 mL.

Microorganism

Gongronella sp. JG used in the study was isolated in our laboratory from a soil sample collected from Hefei, Anhui Province, China. It was identified by characteristic of morphologic and molecular analysis of 18S rDNA sequence. The stock culture was maintained on potato dextrose agar (PDA) slants which were inoculated and kept at 30 °C for 120 h and then kept at 0-4 °C until further use.

Immobilization of *Gongronella* sp. JG in calcium alginate beads

Gongronella sp. JG subcultured on PDA medium was used to prepare the spore suspension. The spore crop from each slant was scrapped into 20 mL of sterile distilled water using a sterile glass rod. The spore count was measured using Neubauer's chamber and the spore suspension (1×10^7 spores/mL) was used for preparing the beads.

The spore suspension was made by mixing spores with an equal volume (1:1v/v) of sodium alginate solution and stirred for 5 min. The mixed solution obtained was then placed in a syringe and allowed to drop in to a sterile CaCl₂ solution that was stirred continuously. Alginate drops solidified upon contact with CaCl₂, forming beads and thus entrapping spore cells. The beads were allowed to harden for several minutes and then washed with sterile water to remove redundant calcium ions and free cells. The calcium alginate beads with immobilized cells of *Gongronella* sp. JG were then used for submerged fermentation. The whole procedure of immobilization was carried out under sterile conditions. Unless otherwise specified, the parameters of immobilization were kept constant.

Immobilization of *Gongronella* sp. JG in polyurethane foam

The polyurethane foam in 4 mm x 4 mm x 4 mm was used as the carrier of immobilized cell. 0.2 g polyurethane foam was added to 250 mL Erlenmeyer flasks containing 75 mL of PDA medium. The fermentation flasks were placed in an incubator shaker with an agitation rate of 150 rpm. After 10 h fermentation, washed the polyurethane foam with sterile water.

Fermentation

The immobilized cells prepared by the above methods (5, 10, 15 or 20 mL) were added to 100 mL of production medium (PM) in 250 mL Erlenmeyer flasks. Medium composition (g/L) was: glucose, 1; glutamic acid, 1.5; KH₂PO₄, 2; MgSO₄, 0.2; pH 5.5. The flasks were incubated on a rotary shake (150 rpm) at 30 °C for 84 h. Aliquots were withdrawn at regular time intervals of 12 h and the culture supernatant obtained after centrifugation at 10000 rpm for 20 min at 4 °C was used as the enzyme preparation. Simultaneous experiments with free cells equivalent to those used in immobilized cultures were also conducted. The effectiveness factor of the immobilized system was defined as the ratio of chitosanase activity of the immobilized system to that of the free cells.

Optimization of fermentation parameters

Effect of different concentrations of sodium alginate on chitosanase production

In order to evaluate the influence of sodium alginate concentrations to chitosanase production, four different

concentrations of sodium alginate (0.2-5%, w/v) were used for the preparation of beads. Fermentations were carried out using PM as described above.

Effect of different molarities of the calcium chloride on chitosanase production

To obtain beads with proper permeability and rigidity, the molarity of the calcium chloride needs to be optimized. For this purpose, different concentrations of sodium CaCl_2 (0.03-0.5 M) were used to beads preparation. The subsequent fermentation procedure was carried out as mentioned earlier.

Effect of bead inoculum on chitosanase production

100 mL PM was inoculated with varying volumes of gel beads (5, 10, 15 and 20 mL), known as bead inoculum, and the fermentation was carried out till 96 h as described earlier.

Effect of different bead diameter on chitosanase production

The effect of bead size was studied for various bead diameter 2.7, 3.2, 3.9 and 4.2 mm. The beads inoculum used in these processes was 10 mL beads to 100 mL production media, and the fermentation was carried out as described earlier.

Effect of different temperature on chitosanase production

The effect of temperature on fermentations was carried out at various temperatures of 24 °C, 27 °C, 30 °C, and 33 °C for 84 h. Initial pH of the fermentation medium was 5.5, 2% (w/v) of sodium alginate, 10 mL beads with 2.7 mm bead diameter was used in 100 mL production media, and the fermentation was carried out as described earlier.

Effect of different initial pH on chitosanase production

The effect of initial pH was studied by conducting fermentation at various initial pH of 4.5, 5.0, 5.5 and 6.0 with 2% (w/v) Calcium alginate. These flasks were incubated at 30 °C, 10 mL bead with 2.7 mm diameter was used in 100 mL production media, and the fermentation was carried out as described earlier.

Repeated batch fermentation

Repeated batch fermentation was conducted with the immobilized cells by running the fermentation for 84 h. At the end of each cycle, the PM was decanted, the beads containing immobilized cells were thoroughly washed with sterile distilled water, and a fresh PM was added to start a new round fermentation.

Determination of chitosanase activity

Chitosanase activity was assayed using colloidal chitosan as the substrate. The reaction mixture consisted of 1.8 mL 1% colloidal chitosan in 50 mM sodium acetate buffer (pH 5.5) and 0.2 mL enzyme solution. After the mix-

ture had been incubated at 55 °C for 30 min, hydrolysis reaction was terminated. Then 2 mL dinitrosalicylic acid reagent was added into the mixture and boiled for 10 min. After being chilled and centrifuged to remove insoluble chitosan, the resulting adducts of reducing sugar were measured by spectrophotometry at 540 nm. The amount of chitosanase which could produce 1 μM of reducing sugar per min was taken as one unit. D-glucosamine was used as the calibration standard to measure chitosanase activity (Dyger and Li, 1965).

Results and Discussion

Selection of immobilization materials

The results of chitosanase production from free and immobilized cells using various entrapment techniques were shown in Figure 1. Both free and immobilized cells showed maximum enzyme production at 84 h. The immobilized cells with calcium alginate as carrier had higher activity than those with polyurethane foam carrier and free cells. The beads were stable even beyond six cycles of reusability transfer. These results were in accordance with other studies on immobilization (Ellaiah *et al.*, 2004; Najafpour *et al.*, 2004). The immobilized cells with polyurethane foam have preferable life-time, but their activity is the lowest. The main reason might be that the agglomeration of PVA gel beads cause lower nourishment circulation and restrict the transfer of nutrients into the gel. Although PVA-boric acid method is simple and economical, two potential problems with this technique are the agglomeration of PVA gel beads and the toxicity of saturated boric acid which have not been completely eliminated until now (Lozinsky and Plieva, 1998). We did not choose PVA-boric acid method because chitosanase is mainly used in food and pharmacy industry. Since the enzyme production was a

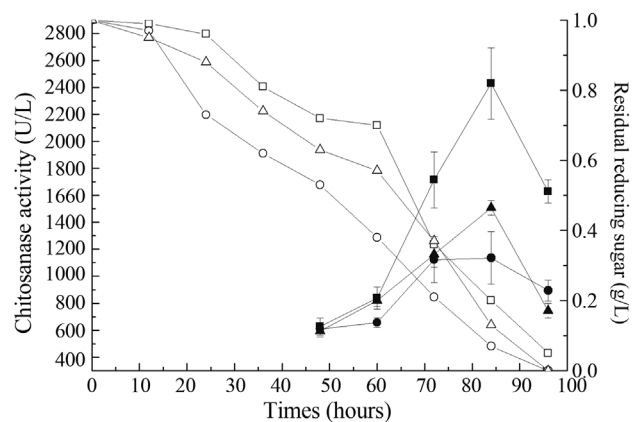


Figure 1 - Time course of chitosanase production by immobilized and free cells of *Gongronella* sp. JG ■ Chitosanase production of cells immobilized by calcium alginate ▲ Chitosanase production of free cells ● Chitosanase production of cells immobilized by polyurethane foam □ Residual reducing sugar of cells immobilized by calcium alginate Δ Residual reducing sugar of free cells ○ Residual reducing sugar of cells immobilized by polyurethane foam.

preferential target than the others in this study, sodium alginate was considered to be the best matrix for production of chitosanase. Therefore, cells entrapped by sodium alginate were used for further optimization studies in this work.

Optimization of immobilization in calcium alginate

Optimizing the parameters of immobilization offers the advantage of improving the bead characteristics, such as permeability and rigidity. Figure 2 showed the pattern of chitosanase production at various sodium alginate concentrations. The highest chitosanase yield was obtained with beads prepared using 2% (w/v) sodium alginate. At higher concentration of sodium alginate the pellet rigidity was improved but the chitosanase production decreased due to diffusion limitations that restricted the transfer of nutrients into the gel and enzyme out of the gel into the medium (Ellaiah and Prabhakar, 2004). On the other hand, beads prepared using lower concentrations of sodium alginate were fragile and the cells were easily leakage into the medium. These beads using 2% (w/v) sodium alginate were quite stable and did not show any tendency to dissolve.

The effect of CaCl₂ concentration on chitosanase production was shown in Figure 3. As in the case of sodium alginate, lower concentration of CaCl₂ may result in increased leakage of cells into the fermentation medium owing to the decreased rigidity of beads. On the other hand, an increase in CaCl₂ concentration from 0.03 M to 0.1 M minimized the cell leakage and consequently increased the chitosanase yield. But at higher concentration of CaCl₂, the enzyme yield was declined. Considering maximum enzyme yield, 0.1 M CaCl₂ was chosen as the optimum for the formation of beads with suitable rigidity and permeability. The mechanical strength of alginate beads is highly dependent on CaCl₂ concentration of the gelation solution and the use of concentrated CaCl₂ solutions has a higher effect

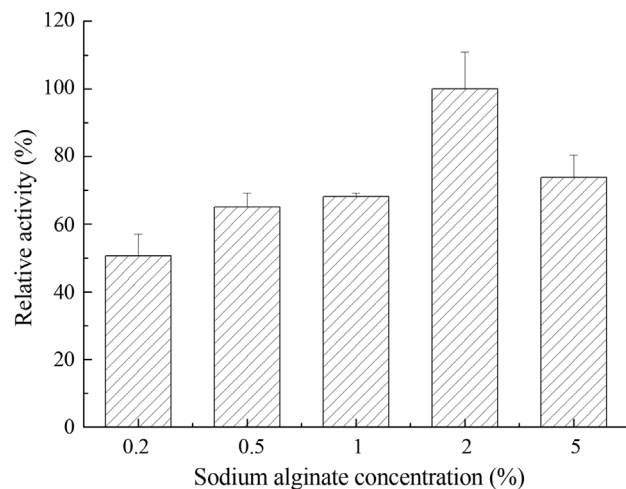


Figure 2 - Effect of sodium concentration on chitosanase production. The data presented here were the average of three independent experiments with standard deviation bars indicated. Fermentation was carried out at 30 °C and pH 5.5.

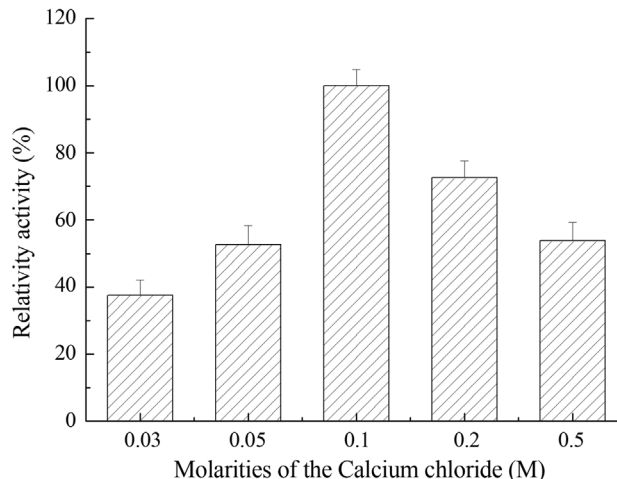


Figure 3 - Effect of molarities of the calcium chloride on chitosanase activity. 2% of sodium alginate was used for immobilization. Fermentation was carried out at 30 °C and pH 5.5.

on the efficiency of immobilized system (Konsoula and Liakopoulou-Kyriakides, 2006). The results can be explained by the fact that when the sodium alginate concentration is increased beyond 2%, the thickness of the membrane capsule decreases (Kurakake and Yo-u, 2000), and this is presumably due to the fact that increasing the number of biopolymer molecules per unit solution, the binding sites for Ca²⁺ ions also increase. As a result, a more densely cross-linked gel structure is probably formed, and consequently results in the formation of thinner walls. The dense membrane is expected to create diffusion resistance through the beads which result in lower product formation as nutrients and substrates are restricted to diffuse easily to the cells. The 2% sodium alginate concentration was observed to produce higher chitosanase probably be due to less cross-linking of the alginate molecules taking place forming less densely packed three-dimensional lattices from the outermost layer to the core of the drop and resulted in easy diffusion of nutrients through the beads, thus promoted growth of *Gongronella sp.* JG and increased productivity. Similar results were reported by Najafpour and Habibollah (2004) in ethanol production and Bandi *et al.* (2003) in neomycin production when using calcium alginate matrices.

In brief, the results indicated that chitosanase production depended on the rigidity and permeability of the beads. The constraints created inside the calcium alginate beads by the immobilization procedure led to changes in the micro environmental conditions which could modify the physiological and morphological behavior of a microorganism compared to that of the free cell cultures. By only adjusting the conditions of immobilization, it is possible to substantially influence the ratio in which the individual metabolites are synthesized. Proper rigidity and permeability promote chitosanase production. This means that there is a relationship between cell immobilization and chitosanase production.

Effect of initial pH and temperature

The effect of various initial pH on the chitosanase production of the immobilized *Gongronella* sp. JG during the batch fermentation was illustrated in Figure 4. The maximum chitosanase production for initial pH 5.5 was 12 h shorter compared with the other initial pH. It is possible that the higher initial pH bring too much stress on the organism metabolic abilities (Wu and Xia, 2000). At initial pH 5.5, cells start to utilize glucose earlier and at a faster rate than at other initial pH. Thus, initial environment of pH 5.5 encourages the *Gongronella* sp. JG to consume glucose rapidly contributing to the maximum chitosanase production. Further increase in initial pH beyond 5.5 does not improve the chitosanase production. This initial pH is also in agreement with the optimum pH of chitosanase from the organism *Gongronella* sp. JG.

The chitosanase production at temperature of 24, 27, 30, 33 °C was different: the highest chitosanase production was obtained at 30 °C, dropped to about 85% of the highest level at 27 and 33 °C, and the lowest was at 24 °C (lower than 20% of the highest). Temperature is one of the important factors that affect the growth of microorganism and its enzymes production. Most species have a charac-

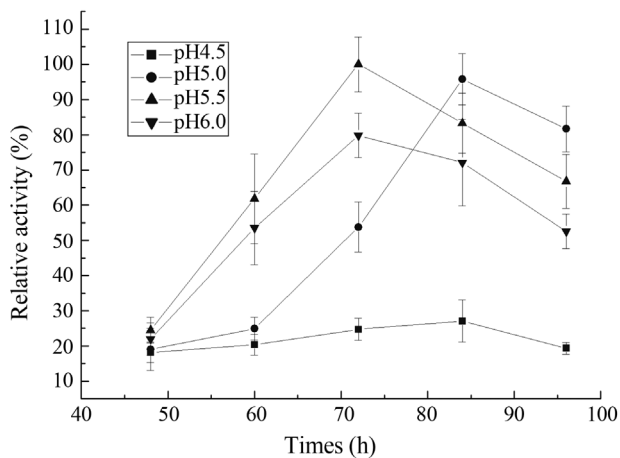


Figure 4 - Effect of initial pH on chitosanase production by calcium alginate immobilized *Gongronella* sp. JG (T = 30 °C, bead diameter = 2.7 mm, cultivate size = 10 mL, 2.0% sodium alginate and substrate concentration = 5 g/L).

teristic range of temperature in which they can grow, but they do not grow at the same rate over the whole of temperature range. In this case, the total enzyme activity is related to initial cell concentration, cell growth, enzyme production and enzyme stability in the process of fermentation, and the highest chitosanase activity was obtained at 30 °C.

Effect of bead diameter

The chitosanase production for the four different bead diameters was shown in Table 1. Maximum chitosanase production was attained for the 2.7 mm bead diameter. A further increase in the bead diameter resulted in a decrease of chitosanase production. Smaller bead diameter produced higher chitosanase production, might due to an increase in the surface volume ratio (Ellaiah and Prabhakar, 2004). The results seem to be in agreement with work performed by Abdel-Naby and Mok (1992) who found that the lactic acid increased as bead diameter decreased. But a balance must be reached among the ease handling of beads manufacture, better mass transfer characteristics, physical resistance and the fermentation production, and 2.7 mm was selected as our suitable choice.

Effect of bead inoculum

Different volumes of 2% alginate gel (5, 10, 15 and 20 mL) were added to 100 mL PM. Results indicated that the enzyme production increased from 5 mL to 10 mL, but decreased with increase in bead inoculum thereafter (Table 2). Dobрева *et al.* (1996) and Angelova *et al.* (1998) had also reported the decrease in enzyme production with increasing gel volume. The decreased enzyme activity with increase in bead inoculum might be due to competition between cells because of which the nutrient concentration available in the flasks might not have been sufficient for optimal growth, leading to low enzyme production. Moreover, the competition between cells would have lead to rapid use of nutrient resulting in smaller duration of enzyme activity. Hence, the variant with 10 mL bead inoculum appeared to present the best conditions for enzyme synthesis.

Table 1 - Effect of bead diameter on chitosanase production by calcium alginate immobilized *Gongronella* sp. JG (T = 30 °C, cultivate size = 10 mL/100 mL, 2.0% sodium alginate and substrate concentration = 5 g/L).

| Bead diameter (mm) | 2.7 | 3.2 | 3.9 | 4.2 |
|-------------------------|------------|--------------|--------------|--------------|
| Relative activity(100%) | 100 ± 7.90 | 87.42 ± 8.83 | 64.68 ± 5.55 | 63.13 ± 5.36 |

Table 2 - Effect of bead inoculum on chitosanase activity. 2% of sodium alginate was used for immobilization. Fermentation was carried out at 30 °C and pH 5.5.

| Bead inoculum (mL) | 5 | 10 | 15 | 20 |
|--------------------------|--------------|-------------|--------------|---------------|
| Relative activity (100%) | 66.51 ± 4.96 | 100 ± 13.50 | 77.89 ± 7.18 | 56.42 ± 13.21 |

Operational stability of immobilized cells

The possibility of re-using immobilized cells of *Gongronella* sp. JG for chitosanase production was studied seven cycles of fermentation. The duration of a fermentation cycle selected for repeated batch fermentation was 84 h. The immobilized system retained catalytic activity for a long time period and was reused successfully for the production of chitosanase (Figure 5). After the fifth cycle, there was about 5% loss in activity with each consecutive use till the seventh cycle. Even after seven successive and efficient fermentation operations, the alginate beads had good stability and maintained 85% of the enzyme yield obtained in the first cycle. The decrease in enzyme yield with successive fermentation might be due to the loss of cell viability. The reuse ability of the present immobilization system was in accordance with that reported in other findings (Angelova *et al.*, 1998; Hemachander *et al.*, 2001). The greater stability of the entrapped cells might be ascribed to the stabilizing effects of immobilization.

Conclusion

This study showed that our method was feasible to produce chitosanase using immobilized cell. Maximum chitosanase could be obtained with optimal immobilization parameters of 2% sodium alginate, 0.1 M calcium chloride, 2.7 mm bead diameter, initial pH of 5.5 and temperature of 30 °C.

Based on the above results, it could be concluded that calcium alginate served as a nice matrix for entrapment of fungal cells for chitosanase production. The alginate beads had well stability and also retained the ability of enzyme production up to 7 cycles of reusability transfer. The operational stability of this biocatalyst system indicated the possibility of the application of this immobilized system in semi-continuous processes for chitosanase production.

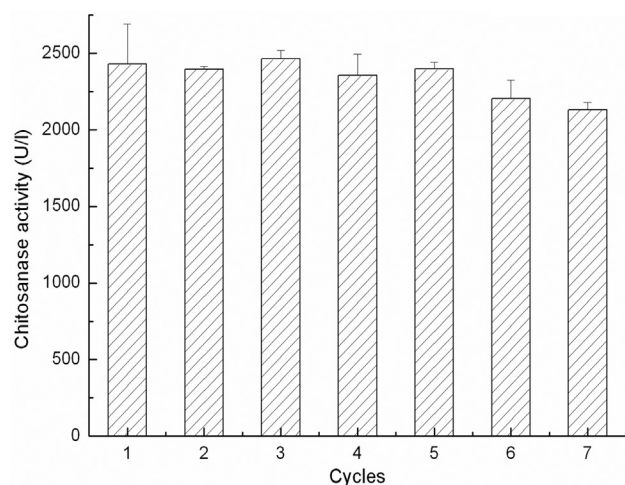


Figure 5 - Repeated use of the immobilized cells of *Gongronella* sp. JG for chitosanase production.

Acknowledgments

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