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Development of an Eco-Friendly Whole Cell Based Continuous System for the Degradation of Hydrogen Peroxide

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Abstract

Catalase (EC 1.11.1.6) is a metalloenzyme belonging to the class of oxidoreductases which catalyze the degradation of hydrogen peroxide into dioxygen and water. Catalase, an important enzyme in the world market has wide range of industrial applications. In one of the important application, it is used to degrade unused hydrogen peroxide from wastewater released after bleaching operations in the textile industry. The use of catalase enzyme in soluble form is not feasible because of many economical and technical limitations. Therefore, the use of immobilized whole cells of bacterium having intracellular catalase activity is advantageous due to their repeated use, but it suffers from a drawback of instability and leakage of cells after some time. In the present study, sodium alginate beads used to immobilize *Bacillus* sp. TE-5 cells was modified to increase their stability and tested for the degradation of H_2O_2 in batch and continuous mode. The effect of other critical factors like sodium alginate concentration, bacterial cell load, glutaraldehyde concentration and treatment time for hardening beads was investigated to test their suitability for long term repeated operations. Maximum catalase activity (15900 IU/g of cells) was achieved by using modified sodium alginate beads cross linked with glutaraldehyde (0.10%, w/v) with $CaCl_2$ treatment time of 18 hr. The reusability of beads in a batch mode was studied to test their potential for continuous operations and then these immobilized cells were further used in a packed bed reactor for the degradation of H_2O_2 in continuous mode. These results provide an indication that this method of gel entrapment followed by cross-linking could be an effective, stable and better alternative for degradation of H_2O_2 in continuous system.

Keywords: *Bacillus*; Catalase; Immobilization; Hydrogen peroxide; Degradation

Introduction

In textile industries, removal of natural colour and impurities on fabrics is usually carried out by using hydrogen peroxide under high alkaline conditions at temperatures above 50°C. Large amount of industrial wastewater containing toxic hydrogen peroxide is generated from these textile operations [1]. This high concentration of hydrogen peroxide being highly corrosive is lethal to flora and fauna of the water bodies. Industrial wastewater containing hydrogen peroxide is reported to be inhibitory for fishes and growth of phytoplankton's and bacterioplanktons in water bodies [2,3].

Consequently, it is critical to remove hydrogen peroxide from the bleaching wastewater before its release into the environment or any biological waste water treatment, because it has also the ability to damage the microorganisms used in wastewater treatment process. Current methods to remove hydrogen peroxide involve chemical treatment with sodium bisulphite and hydrosulphite which further result in the generation of large volumes of alkaline waste water in the process streams [4]. Hence, the presence of hydrogen peroxide in industrial wastewater constitutes serious disposal problems and requires special attention.

An eco-friendly alternative to the aforementioned problem is the development of enzyme based (catalase) bioremedial system which catalyses the degradation of H_2O_2 into water and oxygen. Catalase (EC 1.11.1.6), a ubiquitous enzyme with highest turnover number decomposes hydrogen peroxide to water and oxygen. It is widely distributed among variety of life forms including plants, animals and microorganisms with numerous applications as reviewed recently [4]. A number of studies pertaining to the use of free catalase for the degradation of H_2O_2 have been published, however, free enzyme has been found unsuitable due to additional purification costs [5], non-reusability [6] and undesirable interactions between the protein and dye [7]. In addition, microbial catalases with optimal activity at 20°C to 50°C and at neutral pH, are not able to withstand the adverse conditions of textile bleaching operations. Hence, enzyme based sustainable system is not suitable due to technical, economical and stability issues. Therefore, a system based on immobilized whole cells having sustainable catalase activity under adverse conditions is another eco-friendly alternative for the degradation of H_2O_2. Few immobilized systems using whole cells of *Bacillus halodurans* LBK 261 [7] and *Bacillus* sp. [8] cells have been demonstrated for the degradation of H_2O_2 but these too have operational limitations.

Hence, the present study has been accomplished to exploit a thermophilic catalase producing isolated bacterium (an isolate of our laboratory) belonging to genus *Bacillus* in immobilized form for the degradation of hydrogen peroxide from textile wastewater in batch and continuous system.

Materials and Methods

Chemicals

All the reagents used in the present study were procured from SD Fine Chemicals, Ranbaxy (SunPharma) Labs Ltd, Qualigens Fine Chemicals, Sigma-Aldrich, HiMedia and were of analytical grade.

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Maintenance and production of biomass

Bacillus sp. TE-5, an isolate of Enzyme Biotechnology Laboratory, Department of Biotechnology, Punjabi University, Patiala, India was used for the present studies. The bacterium was periodically maintained on nutrient agar slants at 4°C, until further use at fortnight intervals. Further, for the preparation of seed culture the bacterium was grown in a sterilized medium (50 ml) of pH 7.0 containing peptides digest of animal tissue (5.0 g/L), NaCl (5.0 g/L), beef extract (1.5 g/L) and yeast extract (1.5 g/L) at 50°C for 12 hrs. on a rotary shaker (100 rpm). The production of bacterial biomass having catalase activity was carried out in an optimized nutrient medium (50 ml in 250 ml conical flask) containing glucose (0.50%, w/v), meat extract (0.50%, w/v), peptone (0.25%, w/v), $FeSO_4$ (0.05%, w/v) having pH of 7.0 inoculated with 2.5% (v/v) seed culture by incubating under shaking conditions at 100 rpm for 36 hrs. at 50°C.

Whole cell immobilization of *Bacillus* sp. TE-5

The gel entrapment method [9] with slight modifications was used for the immobilization of *Bacillus* sp. cells. The cells were harvested after 24 hours of incubation by centrifugation (5000 g) at 4°C. The harvested biomass (0.6%, w/v) of *Bacillus* sp. TE-5 cells were suspended in phosphate buffer (0.01M, pH 7.0) after washing and mixed with 1.0% (w/v) sodium alginate to make slurry until or unless specified. Crosslinking of matrix to enhance the stability of beads was achieved by adding different volumes of glutaraldehyde to the alginate slurry. The resultant slurry was extruded into chilled $CaCl_2$ solution (0.1M) through syringe (10 ml) fitted with needle to obtain beads of 2 mm size and kept in $CaCl_2$ solution at 4°C overnight until or otherwise specified. The beads were washed and stored in phosphate buffer (0.01M, pH 7.0) till further use. The beads were also analysed for catalase activity [10].

The intracellular catalase activity of immobilized cells was determined by estimating residual H_2O_2 through colorimetric method by taking definite number of sodium alginate beads containing *Bacillus* sp. TE-5 cells at 37°C [10]. One unit of catalase is defined as the amount of enzyme required to degrade 1 µM of hydrogen peroxide per minute under standard assay conditions.

Optimization of immobilization parameters

Different immobilization parameters such sodium alginate concentration (0.5%, 1.0%, 1.5%, 2.0%, w/v), bacterial cell load (0.2%, 0.4%, 0.6%, 0.8%, 1.0%, w/v), glutaraldehyde concentration (0.05%, 0.10%, 0.15%, 0.20%, w/v) and $CaCl_2$ treatment time (1 hr, 6 hr, 12 hr, 18 hr, 24 hr) were optimized by the method of Sooch et al. [11] for obtaining maximum catalase activity.

Degradation of H_2O_2 in a batch process

Modified immobilized whole cells of *Bacillus* sp. were used for degradation of H_2O_2 in a batch mode and were investigated for the degradation of H_2O_2 at different incubation time periods i.e. 10 min, 20 min, 30 min, 40 min and 50 min at both stationary and agitation conditions [7]. These beads were also reused for multiple cycles to determine the effect of reusability of beads on H_2O_2 degradation.

Degradation of H_2O_2 in a continuous mode using modified immobilized cells of *Bacillus* sp. TE-5 cells

Catalase containing immobilized whole cells of *Bacillus* sp. TE-5 was tested for degradation of H_2O_2 in continuous mode [8]. A packed bed reactor (5.0 cm × 35 cm) with modified alginate beads containing immobilized *Bacillus* sp. TE-5 cells was set up for investigating the effect of flow rate on degradation of H_2O_2 for different time intervals. Different flow rates (2 ml/min, 5 ml/min, 7 ml/min and 10 ml/min) at 100 mM concentration of H_2O_2 were used to calulate the percentage degradation of H_2O_2.

Results and Discussion

The results of present investigations are expressed as an average of three independent experiments.

Modified sodium alginate beads containing *Bacillus* sp. TE-5 cells

An isolated bacterial strain of *Bacillus* sp. TE-5 was exploited for the production of catalase enzyme. The bacterial cells harvested by centrifugation were washed twice with phosphate buffer (0.01M, pH 7.0) and maximum catalase activity of 16600 IU/g of cells was obtained from these free cells.

The whole cells of *Bacillus* sp., source of intracellular catalase enzyme, were used for entrapment in sodium alginate matrix in the form of beads after improving their stability through cross linking approach using glutaraldehyde. Keeping in view the fact that concentration of gelling agent affects the pore size of the beads, the influence of sodium alginate at varied concentrations (0.5%, 1.0%, 1.5%, 2.0%, w/v) was tested to investigate its influence on catalase activity. The results depicted in Figure 1, clearly shows that maximum catalase activity of immobilized whole cells was found to be 13200 IU/g of cells at 1.5% (w/v) sodium alginate concentration. Further, increase in alginate concentration beyond 1.5% (w/v) causes hindrance for substrate and product movement, as these could not easily diffuse in and out of the alginate matrix due to small pore size, hence decrease in catalytic activity of cells was reported. Further, at lower alginate concentrations much softer beads were produced due to insufficient polymerization resulting in disruption of beads and leakage of cells after a short time.

Further, different amount of harvested bacterial biomass (0.2%, 0.4%, 0.6%, 0.8%, 1.0%, w/v) of *Bacillus* sp. TE-5 were immobilized in 1.5% (w/v) sodium alginate to form 2.0 mm diameter beads. It was found that with increase in cell load, catalase activity increases with maximum enzyme activity achieved at 0.8% (w/v) and thereafter, a decline in this function was observed (Figure 2). This may be attributed

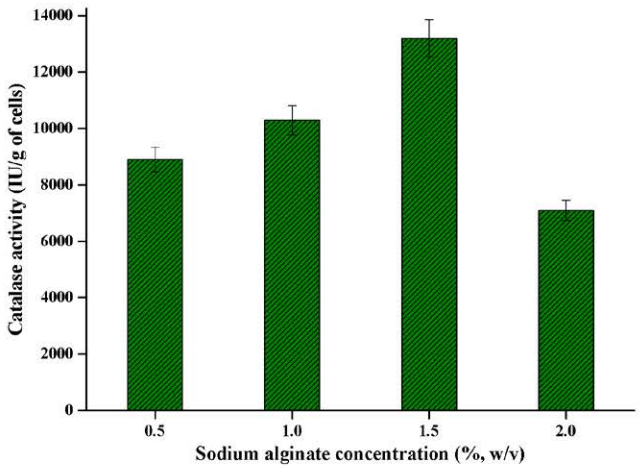

Figure 1: Influence of sodium alginate concentrations on catalase activity (IU/g of cells) by modified immobilized cells of *Bacillus* sp. TE-5.

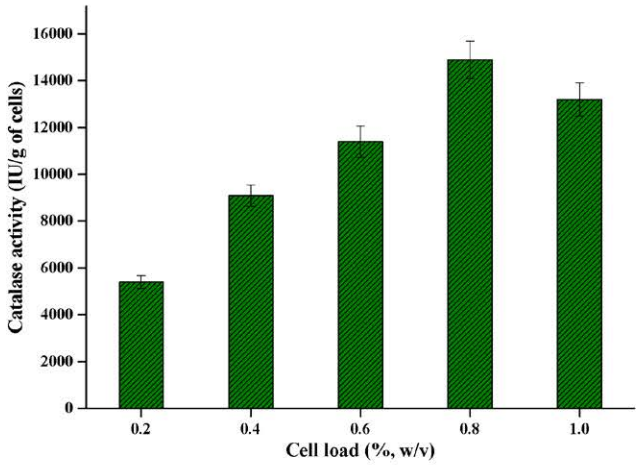

Figure 2: Influence of concentrations of cell biomass on catalase activity (IU/g of cells) by modified immobilized cells of *Bacillus* sp. TE-5.

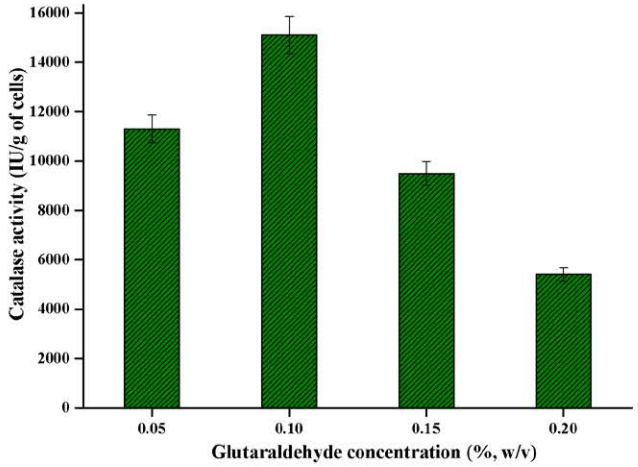

Figure 3: Influence of glutaraldehyde concentrations on catalase activity (IU/g of cells) by modified immobilized cells of *Bacillus* sp. TE-5.

due to the breakage of capsule structure of beads at high concentration of biomass, which increases the exposure of the cells to the external environment and further leads to cell leakage, and hence causes decrease in enzyme activity.

Furthermore, it has been observed that as the concentration of glutaraldehyde increases above 0.10% (w/v), a decrease in catalase activity was recorded. Maximum enzyme activity of 15100 IU/g of cells (Figure 3) was reported at this concentration of cross linking agent. It has been reported that the modification of alginate formulation by means of cross-linking agent like glutaraldehyde changes the matrix performance with improved catalytic efficacy of immobilized cells [12]. The decrease in efficacy of the immobilized cells at higher concentrations of glutaraldehyde may be due to dense cross-linking which could have occurred inside the beads. It has also been reported that with increase in concentration of glutaraldehyde accessibility of substrate to the active site of the immobilized enzyme decreases [13]. However, at lower concentrations of glutaraldehyde, leakage of cells from the beads results in disintegration of alginate beads and hence, decline in catalase activity [14].

The optimum time for hardening of alginate beads without affecting their enzyme activity in calcium chloride solution (0.1M) was found to be 18 hr, where maximum catalase activity (15900 IU/g of cells) was recorded (Figure 4). Thereafter, decline in this parameter was observed due to the formation of tight junction zones with increase in hardening time between the calcium ions and the active sites on the gluconic acid chain in alginate matrix. The tight junctions may have produced high strength and inflexible polymeric chains which further create substrate and product diffusion limitations.

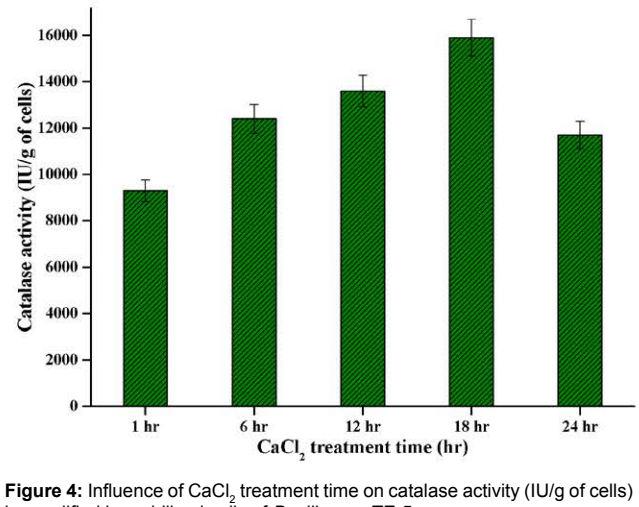

Figure 4: Influence of $CaCl_2$ treatment time on catalase activity (IU/g of cells) by modified immobilized cells of *Bacillus* sp. TE-5.

Batch process for H_2O_2 degradation

Immobilization of a biocatalyst often improves operational stability and makes its long-term use easier. Modified immobilized whole cells of *Bacillus* sp. TE-5 were used to study the degradation of H_2O_2 in a batch process by adjusting initial concentration of H_2O_2 at 100 mM. Immobilized whole cells were tested for the maximum degradation of H_2O_2 at different incubation times i.e. 10 min, 20 min, 30 min, 40 min and 50 min in batch system at stationary and agitation (100 rpm) mode. The results are presented in Figure 5. It was found that maximum degradation has been achieved at agitation mode in

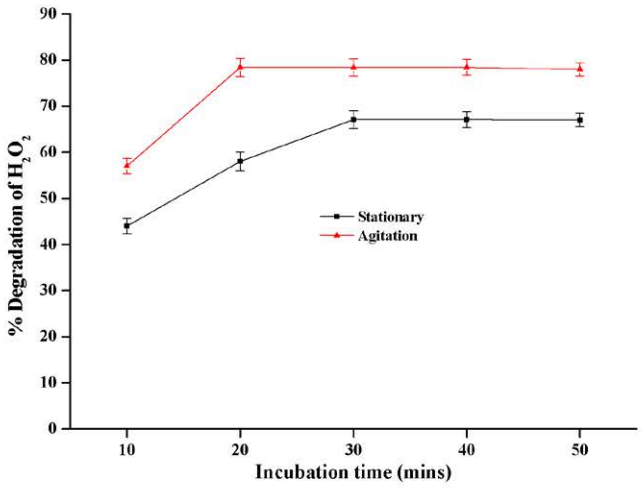

Figure 5: Comparative percentage degradation of H_2O_2 using modified immobilized cells of *Bacillus* sp. TE-5 at stationary and agitation mode in a batch system.

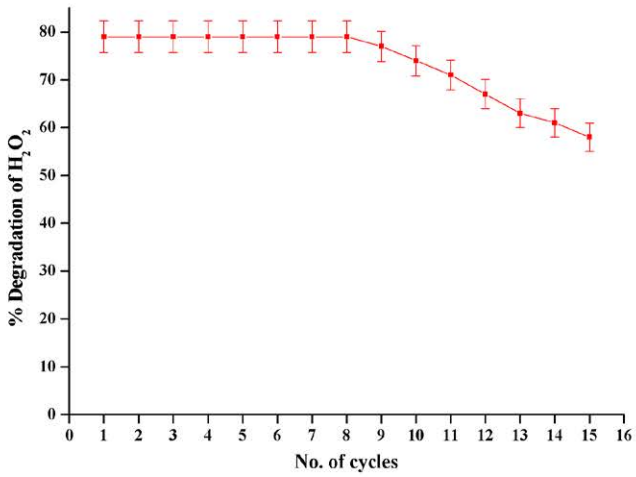

Figure 6: Effect of reusability of modified sodium alginate beads on percentage degradation of H_2O_2.

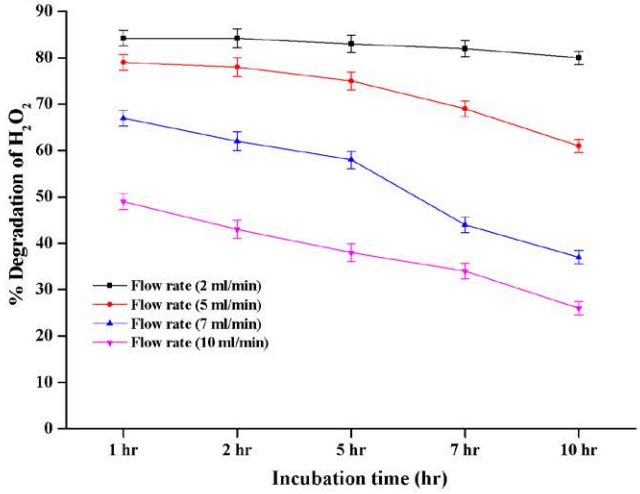

Figure 7: Percentage degradation of H_2O_2 with time at different flow rates in a continuous system.

comparison to stationary. The maximum H_2O_2 degradation at agitation mode may be due to the increased mass transfer between beads and H_2O_2 solution, which prevent accumulation of inhibitory product near the bead surface [15]. It was also reported that mass transfer resistances significantly affected the performance of immobilized biomass and such resistances can be minimized by mechanical agitation [16]. Further, there was a progressive increase in the degradation of H_2O_2 (78.40%) up to incubation time 20 min and thereafter, no significant increase in degradation of H_2O_2 was observed. Since, maximum degradation has been achieved at agitation mode after 20 mins; hence this parameter was selected for further studies.

Operational stability of modified immobilized whole cells in a batch mode

To investigate the effect of reusability on the stability and efficiency for degradation of H_2O_2, the modified immobilized cells of *Bacillus* sp. TE-5 were reused up to 15 cycles in agitation mode (100 rpm) in a batch system. The beads were washed with buffer (0.01M, pH 7.0) after each cycle. The results have been shown in the Figure 6.

It was observed that degradation of H_2O_2 was decreased after 8th cycle. There was 79.0% degradation of H_2O_2 in the first cycle and the degradation was reduced to 58.0% up to 15th cycle. It can be clearly interpreted from the results that immobilized cells of present strain can be reused for 10 batches efficiently without any significant decrease in activity. Hence, it has been concluded that operational stability of alginate beads is significantly improved by modification of alginate matrix with glutaraldehyde treatment and alteration in hardening time of beads with $CaCl_2$. Improvement in alginate beads with crosslinking agents has also been investigated by Birnbaum et al. [17]. The reuse of immobilized *Bacillus halodurans* cells for degradation of H_2O_2 was also studied earlier [7], however, the system could work for only four batches.

Degradation of H_2O_2 in a continuous system using modified immobilized cells of *Bacillus* sp. TE-5

Modified immobilized whole cells of *Bacillus* sp. TE-5 having catalase activity were also used for the degradation of H_2O_2 in a packed bed reactor in continuous mode. A packed bed reactor (2.5 × 20 cm) containing modified immobilized *Bacillus* sp. TE-5 cells was set up for investigating the effect of flow rate on degradation of H_2O_2 for different time intervals. Different flow rates (2 ml/min, 5 ml/min, 7 ml/min and 10 ml/min) at 100 mM concentration of H_2O_2 were used to estimate the percentage degradation of H_2O_2 at different time intervals (1 hr-10 hr). The results have been shown in Figure 7. It was depicted from the Figure 7 that maximum percentage degradation of H_2O_2 (84.19%) was reported at 2 ml/min flow rate at 1 hr of operation, when 100 mM concentration of H_2O_2 solution was used. An optimum flow rate of 2.5 ml/min has also been recorded to degrade hydrogen peroxide solution (0.03%, w/v) in 1 hr by immobilized whole cells of *Bacillus* sp. [8]. It has been observed from the studies that there was a decrease in total degradation of H_2O_2 with time. This may be due to presence of oxygen bubbles liberated during the degradation of H_2O_2, which causes hindrance for the enzyme substrate proximity. It has been found that maximum percentage degradation of H_2O_2 (84.19%) was achieved at lower flow rates (2 ml/min) due to the increase of residence time of substrate within the reactor. The immobilized whole cells of *Bacillus halodurans* LBK 261 [7] and *Bacillus* sp. [8] were reported to degrade H_2O_2 efficiently till 5 days and 6 days of continuous operation, respectively.

These results provide an indication that this method of gel entrapment followed by cross-linking could be an effective, stable and better alternative for degradation of H_2O_2 in continuous system.

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