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Entrapment of white-rot fungus *Phanerochaete chrysosporium* in calcium alginate beads- its preparation and kinetics for decolorization of the dye basic green

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ABSTRACT

Phanerochaete chrysosporium, the white rot fungus is widely used in decolorization studies. The fungus was immobilized by entrapment technique using calcium alginate beads. Various factors that affect the decolorization on immobilization of the fungus were studied. The parameters include the reaction condition, size of the immobilized bead, pH and temperature. It was observed that the immobilized beads of size 1mm were found to be most effective for decolorization. The optimum pH and temperature was found to be 4.5 and 37°C respectively. The apparent activity was found to be 25.6U/g of beads. Along with these Kinetics parameters of the Michaele's - menten model, K_m and V_{max} were estimated. On study with operational parameters, the immobilized cells showed a high operational stability by retaining almost 45 % of the initial activity after fourth use. © 2008 Trade Science Inc. - INDIA

KEYWORDS

Phanerochaete chrysosporium;
Entrapment;
Calcium alginate;
Basic green;
Kinetics.

INTRODUCTION

The application of microorganisms in the treatment of effluents is potentially very interesting. It has been reported that *Phenerochaete chrysosporium* is a highly utilized organism in the treatment of various dyes[1]. This is due to the versatile ability of the fungus to degrade, partially or completely various dyes such as heterocyclic, azo, anthraquinone, vat and polymeric dyes[2-5]. *P.chrysosporium* displayed color reduction abilities for all such dyes including the dyes used in newsprint, writing and printing paper industries[6]. Recently the application of immobilized cells has been receiving increasing attention in the field of wastewater decolorization. Many researchers have studied the effect of immobilized whole cells and enzymes on decolorization characteristics since immobilization provides distinct stability over free cells[3,7].

Though the studies involved in the decolorization and degradation is wide, the studies on the use of the microorganism in immobilized form are minimal. For continuous decolorization of the effluents immobilized *P.chrysosoprium* would offer several advantages like easy operation, repeated use of the immobilized product, providing opportunities for scaling-up and allowing the development of processes based different reactor

configurations. A wide variety of carriers have been used for immobilization procedures. However, other binding techniques involve chemical modification of the cells. It is preferable that the method employed for immobilization of cells should cause as little trauma to the cells as far as possible. Entrapment of the whole cells fulfills this criterion.

The advantage of whole cell immobilization is that many other enzymes that are active in the whole cell might also take part in the conversion and leads to better decolorization.Hence, in the present study an attempt was made to immobilize whole cells by entrapment, as entrapping method leads to immobilized biocatalyst with high retention of specific activity.

Natural polymers such as alginate, chitosan, chitin and cellulosic derivatives have been mostly used as the matrix for the immobilization of microbial cells via the entrapment technique. Among the many matrices available, one of the most frequently used is entrapment within porous matrices, such as alginate often in the form of beads. This sort of system is reasonably safe, simple and cheap offering good mechanical strength.

The present study deals with the immobilization of whole cells of *P.Chrysosoporium* by entrapment in calcium alginate beads. The conditions of entrapment like concentration of sodium alginate and bead size were optimized for highest apparent activity. The kinetics of the immobilized cells entrapped in different bead size was analyzed. The process parameters, pH and temperature, affecting the performance of the immobilized cells were optimized. Lastly, the catalytic properties and reusability of the immobilized cells were also studied.

MATERIALS AND METHODS

1. Materials and methods

Microorganism

The white-rot fungus *P.chrysosporium* MTCC 787 was obtained from the Culture collection of Institute of Microbial Technology, Chandigarh, India and the stock cultures were maintained by periodic subculture on malt agar medium at 4^0C.

Inoculum

The fungus *P.chrysosporium* was inoculated on malt agar (20.0g/l, 2.0g/l peptone, 10.0g/l agar) and incubated at 35^0C until extensive mycelia growth occurred. The liquid medium[8] used to study the fungal biomass and decolorization test is D-glucose 5.0g/l; KH_2PO_4 2.0g/l; NH_4CL 0.050g/L; $MgSO_4.7H_2O$ 0.5g/l; $CaCl_2.2H_20$ 0.1g/l; Thiamine HCl 100µg; Distilled water 1l; The final pH of the medium was maintained at pH 4.5.

Chemicals and dye

Commercial dye belonging to the Basic group, Basic green was used. Sodium alginate, calcium chloride and soluble starch were obtained from E Merck; all the other chemicals used were of analytical grade.

Decolorization determination

After harvesting mycelium from the liquid cultures, decolorization of the filtered broth was assessed spectrophotometrically by measuring absorbance with respect to the dye. The concentration of the dye in the samples was determined in a UV/VIS Shimadzu Spectrophotometer model U 2000 which was measured at regular intervals which took place for a period of maximum 200 hours. Decolorizing activity was calculated using standard curves and expressed in terms of percentage. Decolorization is determined by monitoring the decrease in absorbance at the absorbance maximum of 455 for the dye λ_{max} [9]. The experiments were carried out in triplicates and averaged for better results. Control experiment for each tests were carried out using uninoculated medium with dye addition.

Immobilization

P.chrysosporium were grown into the stationary phase in malt agar slants. Spore suspension of 1ml (approximately 3.2×10^5cells/ml) was added to 2% sodium alginate. The mixture was gently stirred at room temperature to produce a uniform suspension. Different nozzles were used to form beads of different sizes using pastuer pipettes. The beads so obtained were stored in calcium chloride solution at 4^0C for 2 hours to complete gel formation[10]. The calcium alginate beads containing the cells were thoroughly washed with distilled water. The insoluble and stable immobilized beads thus obtained were further used for decolorization studies. For experiments with free cells the same amount of cells were used.

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Variation in sodium alginate was made in order to find the effect of the polymer. Here the concentration of the spore suspension was constant whereas the sodium alginate concentration was varied from 1 to 6 grams in 100ml of water.

Determination of immobilization efficiency

Immobilization efficiency was determined from the difference in decolorization percentage in the solution before and after the immobilization.

Immobilization yield (%)= (I/ A-B)×100

Where A=added cells /gram of bead; B=unbound cells/gram of bead; I=immobilized cells/gram of bead

Scanning electron microscopy

Samples of the free calcium alginate and immobilized *P.chrysosporium* were analyzed to study the uniform growth of the fungus onto the beads. A fixative solution of 6% glutaraldehyde was added to the sample kept in Petri plate on a cover slip and allowed for fixation for an hour. Fixed samples were then washed with 0.1M phosphate buffer for 2 hours and then dehydrated with a series of ascending concentrations of ethanol in distilled water(50, 70 and 95%). A dehydration time of 15 minutes was allowed for the samples. Care was taken to ensure that the volume of the solution used was at least ten times the volume of the sample. The dehydrated samples were then dried to critical point with liquid CO_2, mounted on a metal holder with conductive carbon paint, sputter coated with gold prior to viewing, and photographed using a scanning electron microscope (JEOL JSM 5300).

On studies using SEM, the amount of the fungus in the support was measured to be 0.201g/bead. This was determined at the end of the five days cultivation period and no fungal biomass increase was observed after this period.

Optimization of curing time of calcium alginate beads

The calcium alginate beads were cured in 2%(w/v) calcium chloride solution for different time intervals of 30, 60, 90, 120, 150 and 180min. After curing, the beads were washed thoroughly with distilled water, wiped dry and their hardness was measured using a Texture- analyzer (Stevens-LFRA), L6512. The hardness was measured for 25beads one by one and averaged. The hardness of the beads was expressed as the load(g force) that the beads could withstand for 1milimeter compression.

Determination of kinetic constants

Kinetic studies were carried out at 37^0Cand at pH 4.5 for different substrate concentrations. The kinetic constants K_m and V_{max} were calculated according to Lineweaver-Burk plot. For this rate parameters were estimated experimentally from the initial rate of the decolorization process. A plot of 1/V vs. 1/S yields a linear line with a slope of K_m/V_{max} and an intercept of $1/V_{max.}$

Study on operational stability

The calcium alginate beads containing the cells were thoroughly washed with distilled water after single use. The insoluble and stable immobilized beads thus obtained were further used for next set of decolorization studies. The study was thus carried out further five times. The pH and temperature was maintained at 4.5 and 37^0C.

RESULTS AND DISCUSSION

1. Optimization of parameters for immobilization on alginate gel

Effect of sodium alginate concentration

It has been reported that the porosity of the calcium alginate beads depend upon the alginate type and the gelling agent concentration[11]. So, various concentrations of sodium alginate solution were used for preparation of calcium alginate beads in order to vary the relative degree of cross linking, which would create dif-

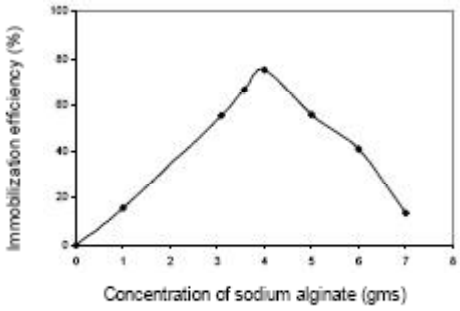

Figure 1: Effect of calcium alginate on immobilization efficiency

ferent pore size. The immobilization efficiency was found to be highest (75%) for a concentration of 4% (w/v) sodium alginate solution which later on started to decrease(Figure 1)

Higher immobilization efficiency could not be attained due to some leakage of the enzyme into the solution. Although, in practice reducing the size of the pores can reduce leakage, some initial leakage of the cells is certain to occur[12]. The lower immobilization efficiency in case of lower percentage sodium alginate solution might be due to larger pore size and consequently greater leakage of the cells from the matrix.

Effect of curing time of calcium alginate beads

Time required for the gel to set is an important step in immobilization as it affects the stability of the resulting calcium alginate beads. The effect of curing time on the hardness of the calcium alginate beads was evaluated (Figure 2). The treatment of the beads in a calcium chloride bath for 2hrs gave a hardness of 85g. Prolonged curing of the beads with calcium chloride solution did not improve the structural stability of the beads.

SEM studies

The SEM micrographs of the plain calcium alginate beads and *P.chrysosporium* immobilized form were presented in figures 3 and 4 respectively. The calcium alginate bead was spherical shaped. The SEM micrograph of fungus-immobilized alginate beads were completely different from the plain beads and revealed a uniform growth on the beads surface. There was visible biomass growth, which was seen from the SEM micrographs, as there were dense branching hyphae. This uniform distribution is an important criterion for the proper biosorption and biodegradation surface area of the fungus immobilized beads. Due to the growth of the mycelia on the surface, it is clear that there is oxygen and nutrient diffusion on the polysaccharide and they are not restricted to growing[13].

Optimum conditions for using *P.chrysosporium*-immobilized beads

In the immobilized cell system, the substrate has to diffuse for the reaction to take place; hence, the size of the final lattice (bead) has significant effect on the rate of decolorization. Moreover, the bead size determines the suitability for reactor configuration. In situations

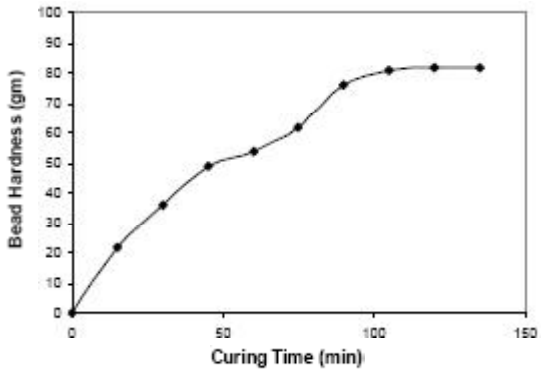

Figure 2: Effect of curing time on bead hardness

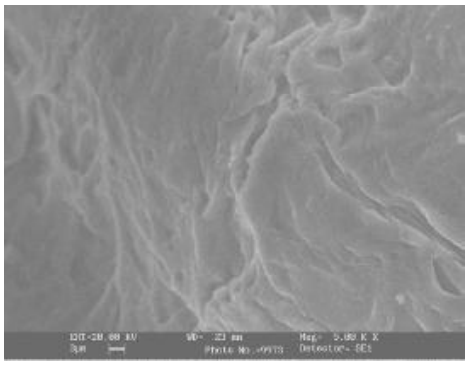

Figure 3: SEM photograph of Plain bead

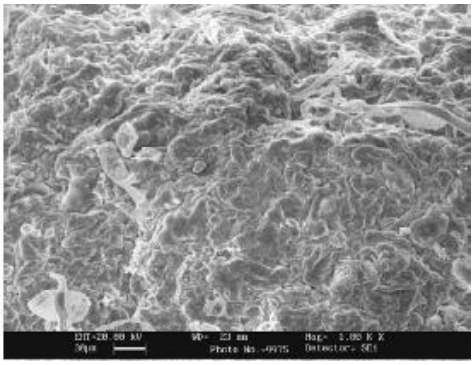

Figure 4: SEM photograph of immobilized bead

where the substrate has to be transported from the bulk solution to the outer surface of the matrix, both the intraparticular diffusion and the external mass transfer should be taken into consideration. However, in the present study, the external transport has not been considered on the assumption that greater contribution is from the intraparticle mass transfer.

Effect of diameter of the immobilized beads

Experiments were carried out, varying the diameter of the immobilized beads. As shown in figure 5, the

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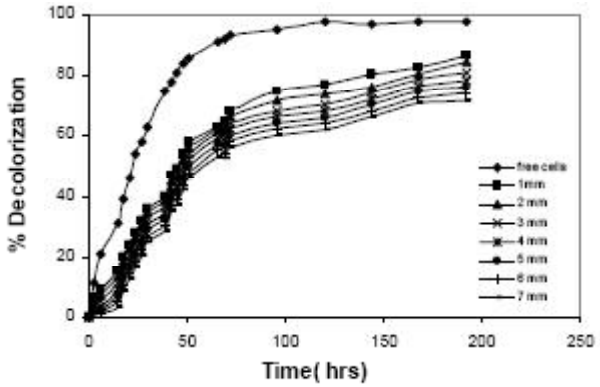

Figure 5: Effect of diameter of immobilized beads

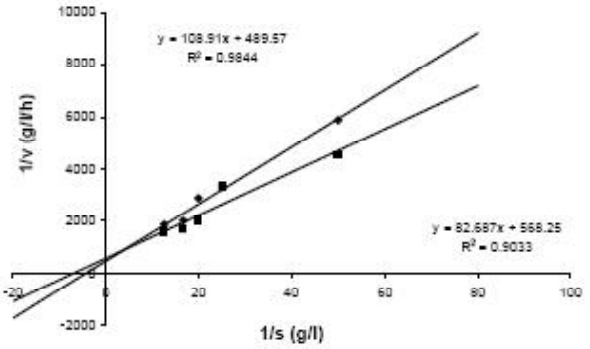

Figure 6: Arrhenius plot

TABLE 1 : Kinetic constants obtained using Line weaver-Burke plot

Diameter of the immobilized bead(mm)	K_m/V_{max}	$1/V_{max}$	V_{max}(g/l/h)	K_m(g/l)
Free cells	108.91	489.57	0.0020	0.2225
1	81.561	522	0.0019	0.1562
2	82.687	568.25	0.0018	0.1455
3	83.706	572	0.0017	0.1463
4	91.683	632	0.0016	0.1451
5	83.69	687	0.0015	0.1218
6	82.66	695	0.0014	0.1189
7	81.55	721	0.0014	0.1131

highest rate of decolorization was observed with bead size of 1mm and the bigger sizes showed lower rate. From the above finding it may be concluded that the beads of 1mm offered lesser diffusion resistance compared to the larger beads. In order for reference the free cells data is also plotted. Depending on the diameter of the beads, the percentage decolorization also varied to from 86.71 to 71.05 %. For the free cells, it was around 97.99% decolorization whereas for the immobilized beads it was lesser owing to the mass transfer effects on the bead surface. As the size of the beads increases the surface area decreases and due to this there may be diffusional limitations making the mass transfer resistance to be more prominent. Similar results of decreased ethanol productivity on increasing the size of immobilized bead have already been discussed[14]. They have also reported that as the biomass concentration increases, the diameter of the beads plays an important role in decreasing the productivity. This is entirely matching our results as the decolorization decreases as the bead size increases.

Kinetic analysis: Line weaver burke plot for free cells and entrapped cells

The intrinsic kinetics of an entrapped cell is defined as being to those that of the soluble cells in free form. Nevertheless, the immobilization of whole cells onto porous particles imposes additional resistances and the kinetics of the immobilized biocatalyst in the presence of modifying factors is called the apparent kinetics. The kinetic parameters of the immobilized cells may be different from those of the suspended cells because of diffusional restrictions and interactions with the carrier or deactivation due to immobilization. The rate parameters were estimated experimentally from the initial rate of the decolorization process. A plot of 1/V vs. 1/S yields a linear line with a slope of K_m/V_{max} and an intercept of $1/V_{max}$. (Figure 6)

$$-\frac{1}{V} = \left[\frac{K_m}{V_{max}}\right]\frac{1}{s} + \frac{1}{V_{max}}$$

The kinetic parameter, V_{max} is not a fundamental characteristic of cell, because it depends on the purity and concentration of the fungal cells[15]. The Michaelis-Menten constant, K_m, is more a fundamental parameter. Although, K_m is not dissociation constant it is often used as an affinity parameter in particular for a comparison of enzyme affinity to various substrates: the smaller is K_m the larger is said to be the affinity. Thus, K_m is customarily considered roughly reciprocal to the enzyme-substrate affinity, which follows from the steady state derivation of K_m in classical Michaelis- Menten kinetics as the sum of the constants for breakdown of enzyme-substrate complex.K_{-1} and K_2 divided by the constant for its formation, K_1.

The results of the kinetic constants are tabulated in TABLE 1. K_m of the immobilized *P.chrysosporium* increased with increase in the size of the beads. The re-

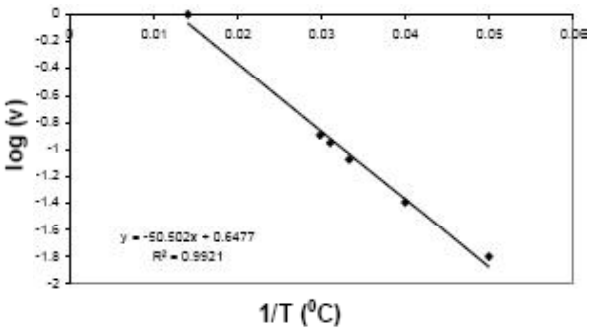

Figure 7: Lineweaver burke plot for the dye basic green

TABLE 2: Operational stability for the immobilized cells

Number of cycles	% Decolorization: Diameter of the immobilized beads(mm)						
	1	2	3	4	5	6	7
1	97.54	95.67	94.25	93.27	92.15	91.28	91.23
2	88.23	84.23	83.98	82.91	81.83	80.56	80.18
3	74.56	65.12	64.66	63.93	63.45	62.06	61.59
4	68.49	44.87	44.03	43.55	42.22	41.85	40.67
5	45.81	42.41	41.12	40.51	39.28	38.06	37.53

sults obtained in the present study were similar to those reported for immobilized glucoamylase (Cabral, 1982). Koji et al.(1999) also observed that the K_m for the immobilized urease became higher with increasing the fiber diameter for entrapment-immobilized urease. In the present study, the internal diffusion effects are present in the operational conditions used and as the beads of larger size offer more internal diffusion resistance, the K_m value increases with the size of the bead. Concomitantly, as the diffusional limitations were eliminated by reducing the size, the maximal activity, V_{max}, increased with the decrease in the size of the beads.

For practical application, an immobilized system with lower K_m value and faster rate of reaction is preferred. In this respect, immobilized enzyme particle of 2mm size gave satisfactory results and was used for further investigations.

Activation energy of the immobilized enzyme

The logarithm of decolorization percentage was plotted as a function of inverse of temperature and the activation energy was calculated from the Arrhenius equation (Figure 7). The E_a of the immobilized whole cells was found to be 6.08kcal/mole.

Kinetic constants obtained for temperature variation

In order to make a rational reactor design for immobilized whole cell system it is important as well as necessary to evaluate the kinetics of the system for properties of immobilized cells. Hence, variations in temperature were made and it was found that the immobilized cells showed very less decolorization percentages at extreme temperatures. The reasonable temperatures at the two extremes were 30^0C and 37^0C which are reported in our results. The kinetic constants, K_m, and V_{max} of the cells entrapped in 1mm size bead was evaluated at 37^0C and 30^0C .

The estimated V_{max} at 37^0C was found to be 0.0019g/l/h and that at 30^0C was 0.0012 g/l/h. However, with the increase in temperature, V_{max} decreased from 0.0019g/l/h to 0.0009g/l/h of beads which indicated that the increase in temperature lessened the activity of the immobilized cells. Similarly, with the decrease in temperature from 30^0C, V_{max} decreased from 0.0012g/l/h to 0.0005g/l/h of beads which indicated that the decrease in temperature also lessened the activity of the immobilized cells.

Operational stability

The operational stability of immobilized cells is one of the most important factors affecting the utilization of an immobilized cell system. The operational stability of the immobilized beads was evaluated in batch process. The results (TABLE 2) indicated that on repeated use of the immobilized cells, 45% of the initial activity was retained upto four cycles. After fifth cycle, there was loss of enzyme activity, which may be due to enzyme denaturation and due to physical loss of all essential parameters from the carrier.

CONCLUSION

The immobilized *P.chrysosporium* showed a high decolorization capacity for the dye Basic green. The kinetics of the decolorization was studied using Lineweaver Burke plot and a size of 1mm diameter bead showed a higher effectiveness for decolorization than larger beads. Kinetics constants, K_m and V_{max} were estimated and were found to be affected by the bead size. The reaction conditions, pH, temperature, was optimized for the immobilized condition. At the optimum pH and temperature of 4.5 and 37^oC, the apparent

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activity was 25.6U/g of beads, resulting in almost 2-fold increase in activity. The immobilized cells showed a high operational stability and retained 45 % of the initial activity after fourth use.

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