International Journal of Microbiological Research 3 (2): 104-108, 2012
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2012.3.2.56280

Production of Alkaline Protease by Adsorbed Cells of *Bacillus circulans* MTCC 7906 under Batch Conditions

[1]Shefali Mishra, [2]G.S. Kocher, [3]Prem Sagar and [1]Pesal Savitha

[1]Department of Agricultural Microbiology,
University of Agriculture Sciences, Dharwad (Karnataka), India
[2]Department of Microbiology, Punjab Agricultural University, Ludhiana-141004, India
[3]Scientist in-charge, R & D, Briiliant Biopharma Ltd, Hyderabad-502307, India

Abstract: Alkaline protease production by immobilized cells of *Bacillus circulans* MTCC 7906 was studied using activated charcoal and kieselguhr as adsorbing materials. The production was optimized with respect to adsorbing material concentration and inoculum's size. A concentration of 75mg/5 ml activated charcoal and kieselguhr respectively produced minimum leakage of cells. The inoculum's size was optimized as 20% (w/v) that produced protease activities of 3225.05 and 3147.33 U/ml in activated charcoal and kieselguhr respectively after 120 h. The surface structure of adsorbed cells was studied under the scanning electron microscope. The adsorbed cells depicted a random distribution of *Bacillus circulans* cells on the surface of adsorbing material with pockets of high cell density that were the areas of cell growth. The kieselguhr adsorbed cells showed presence of cells in pores of cork like kieselguhr structure.

Key words: Adsorption · Alkaline Protease · *Bacillus circulans* · Immobilization

INTRODUCTION

Enzymes have attracted attention from researchers all over the world because of the wide range of physiological, analytical and industrial applications, especially, from microorganisms, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for a two third share of the detergent industry [1]. In view of its possible applications, alkaline proteases from extreme organisms should be produced commercially in high yield at low cost method [2]. At present, use of alkaline proteases has increased remarkably with their large proportion derived from *Bacillus* strains. However, these producing strains have disadvantages in submerged cultures in that the enzymes have low shelf life and are susceptible to rise in temperature, pH etc [3].

Thus there is a need to look for alternate technologies that can overcome these problems. The use of immobilized biocatalysts is one such technology which has recently gained attention of many biotechnologists. For industrial applications, the immobilization of protease on a solid support can offer several advantages including repeated use of enzyme, ease of product separation, improvement of enzyme stability and continuous operations in packed bed reactors [4]. Activated charcoal is well known as an adsorbent and thus finds increased application in different industrial processes, viz. biofiltration[5], in medical applications as an oral antidote for drug overdoses and poisoning [6], detoxification [7] etc. In the present study, the productivity of cells of *Bacillus circulans* MTCC 7906 (enriched from vegetable waste and identified by MTCC lab, IMTECH, Chandigarh) immobilized in activated charcoal and kieselguhr as adsorbing material was optimized under batch conditions.

MATERIALS AND METHODS

B. circulans MTCC 7906 was grown in 50 ml inoculum's medium consisting (gl^{-1}) of glucose, 10.0; casein, 5.0; Yeast Extract, 5.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.20 and Na_2CO_3, 10.0 with a pH of 9.5 added to a 5ml of 72 hold inoculum activated charcoal/kieselguhr and

Corresponds Athur: Shefali Mishra, Department of Agricultural Microbiology,
University of Agriculture Sciences, Dharwad (Karnatka) India.
Tel: +919379625393.

incubated at 37°C in a water bath with gentle agitation for 2 h. The mixture was then centrifuged at 600xg for 5 minutes, supernatant was discarded and activated charcoal/kieselguhr with adsorbed cells- were washed with sterile water to remove free cells.

The enzyme production was carried out under batch conditions and all experiments were conducted in triplicate. The production media for immobilized cells of *Bacillus circulans* consisted of (gl^{-1}) cotton deoiled meal (CDM), 15.0; Yeast Extract, 5.0 and salt solution (gl^{-1}) of K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.20 and Na_2CO_3, 10.0 with a pH of 10 [8].

The different concentrations i.e. 50-100 mg/5ml of adsorbing materials selected were mixed with inoculum and incubated in a water bath (37°C) for 2h under shaking conditions. The mixture was centrifuged at 600xg for 5 minutes to remove activated charcoal/ keiselguhr. After centrifugation, supernatant was plated on Reese agar to enumerate the free cells. The inoculum size was varied between 2.5 to 20%(w/v) by taking respective amount of culture broth and mixing with activated charcoal or kieselguhr at 75mg/5ml.The different inoculum sizes so produced and adsorbed cells were used to inoculate the production medium (50 ml) which was incubated at 28°C for 120 h. Samples were withdrawn at regular intervals of 24 h and assayed for cell leakage and alkaline protease activity. Cell leakage was determined by viable cell plate count method [9]. Periodic samples from the enzyme production media were serially diluted and plated on Reese medium and incubated at 28±2°C for 48 h.

The protease activity of the periodic samples was determined in a reaction mixture consisting 0.1 ml of crude enzyme, 2 ml of 0.5% casein (in carbonate-bicarbonate buffer, 0.1M, pH 9.5) and 0.9 ml of distilled water and incubated at 60°C for 15 minutes. The proteins were precipitated out thereafter by adding 3 ml of 5% ice-cold trichloroacetic acid (TCA) and free amino acids released by crude protease from casein hydrolysis were estimated [10]**.** The protease activity was defined as µg of tyrosine released per minute per ml of crude enzyme.

The *Bacillus circulans* cells immobilized in activated charcoal and kieselguhr were examined for their distribution pattern on the immobilizing material surface. The samples were fixed with 2% glutaraldehyde and dehydrated in graded series of ethanol. Finally, the samples were dried by critical point drier (EMS-850, Japan), coated with gold and observed with a Scanning Electron Microscope (Hitachi S-3400 N, Japan) under different magnifications.

RESULTS AND DISCUSSION

The production of alkaline protease by activated charcoal and kieselguhr immobilized cells of *B. circulans* 7906 was carried out in the modified Reese broth using already optimized conditions of pH 10, temp 28°C, agitation 120 rpm with cotton deoiled meal (CDM) at 1.5% as C and N source [8].

The effect of different concentrations of activated charcoal and kieselguhr on adsorption of *B. circulans* cells was studied. The cell count adsorbed by different concentrations of activated charcoal i.e. 50, 62.5, 75, 87.5 and 100 mg/5ml was 40, 55.45, 96.06, 84.85 and 87.27% respectively while by kieselguhr it was 53.94, 88.18, 96.67, 95.15 and 95.76% respectively for the same concentrations (Table 1). Thus, the results revealed that 75mg/5ml concentration of activated charcoal as well as kieselguhr produced minimum number of free cells which meant their maximum adsorption at this concentration. Activated charcoal was used for decolorization and purification of alkaline protease from *Bacillus sp.* and a concentration of 7.5g/l was reported for maximum protease decolorization as well as protease activity [11].

The effect of inoculum size was studied for alkaline protease production and the results analyzed statistically by Random block design revealed a maximum activity of 3225.05 and 3147.33 U/ml at 120 h with 20% of inoculum's size in activated charcoal and kieselguhr adsorbed cells respectively. However, the enzyme activity was insignificantly different with 15% and 20% inoculum's size and the activities obtained at 72, 96 and 120 h were statistically same in case of activated charcoal. But with kieselguhr, inoculum's size of 20% was significantly better than other inoculum's sizes (Table 2). Both adsorbing materials were also significantly better than free cell for enzyme production.

Table 1: Effect of different concentrations of activated charcoal and kieselguhr on adsorption of *B. circulans* cells

	Free cells (CFUml) in	
Concentration (mg/5ml)	Activated Charcoal	Kieselguhr
50	198×10^4	152×10^4
62.5	147×10^4	39×10^4
75	13×10^4	11×10^4
87.5	50×10^4	16×10^4
100	42×10^4	14×10^4

Initial cell count = 330×10^4 CFU/ml

Table 2: Effect of inoculum's size on alkaline protease production by adsorbed cells of *B. circulans*

	a. Activated charcoal					
	Protease activity (U/ml) at time (h)					
Inoculum's size (%)	24	48	72	96	120	Mean
2.5	58.28	169.99	252.56	485.67	577.93	308.876
5	67.99	263.14	895.43	1330.81	1622.24	835.920
10	198.17	405.98	1636.25	2185.64	2336.22	1352.452
15	339.96	931.24	2675.13	2923.87	3006.45	1975.330
20	355.72	1163.43	2796.54	3079.33	3225.05	2124.014
Mean	204.018	586.750	1651.182	2001.064	2153.578	
Control (free cells)	72.85	189.42	410.72	1258.55	1387.69	
	CD(5%) for both Inoculum's size and time = 629.562					
b. Kieselguhr						
2.5	77.71	137.14	235.98	427.38	587.68	293.178
5	97.14	239.36	831.43	1471.65	1661.07	860.130
10	135.97	313.97	1287.50	1687.37	1867.94	1058.550
15	427.38	659.04	1487.96	2044.76	2520.73	1427.974
20	461.93	904.25	1711.38	2797.63	3147.33	1804.504
Mean	240.026	450.752	1110.850	1685.758	1956.950	
Control (free cells)	67.99	169.99	480.84	1199.67	1257.95	
	CD(5%) for both Inoculum's size and time = 481.164					

Table 3: Cell leakage during alkaline protease production by adsorbed cells of *B. circulans*

	(CFU/ml)					
	Activated Charcoal			Kieselguhr		
Inoculum's Size (%)	0 h	72 h	120 h	0 h	72 h	120 h
2.5	0×10^4	2×10^4	39×10^4	0×10^4	31×10^4	65×10^4
5	4×10^4	9×10^4	46×10^4	2×10^4	22×10^4	47×10^4
10	1×10^4	5×10^4	329×10^4	1×10^4	6×10^4	13×10^4
15	1×10^4	16×10^4	267×10^4	0×10^4	12×10^4	26×10^4
20	1×10^4	31×10^4	127×10^4	0×10^4	19×10^4	24×10^4
Initial count (CFU/ml)		560×10^4			482×10^4	

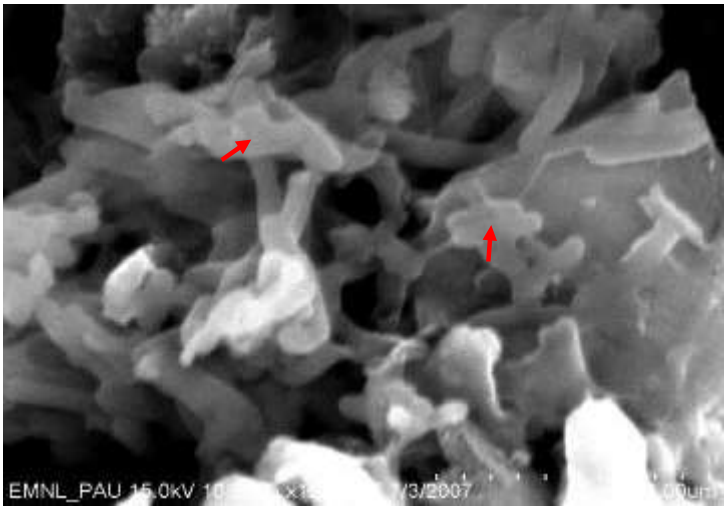

Fig. 1: SEM observation of activated charcoal adsorbed cells of *B. circulans*.

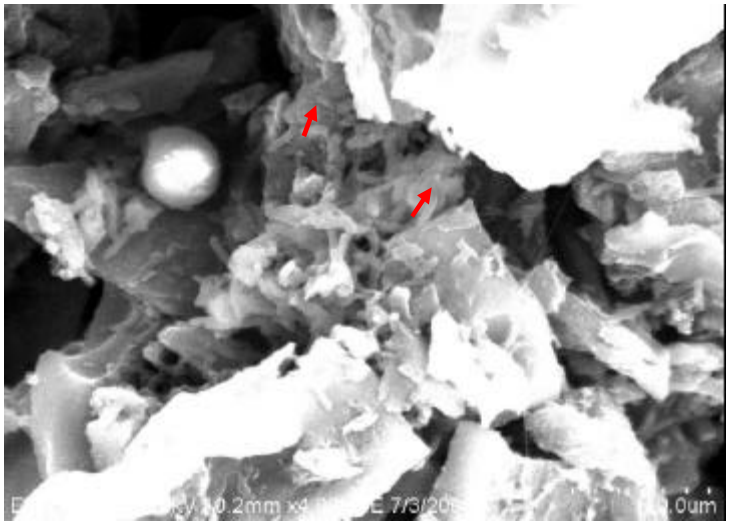

Fig. 2: SEM observation of kieselguhr adsorbed cells of *B. circulans*.

The cell leakage during alkaline protease production by activated charcoal at 2.5, 5, 10, 15 and 20% of inoculum's sizes was 6.96, 8.21, 58.75, 47.67 and 22.68%, respectively after 120 h whereas in kieselguhr it was 13.49, 9.75, 2.69, 5.39 and 4.98% with inoculum's sizes of 2.5, 5, 10.15 and 20%, respectively reported after 120 h of batch process (Table 3).

The surface of immobilized *B. circulans* cells was studied under SEM. It was observed from the SEM photographic plates that the cells were randomly distributed on the surface with pockets of high cell density that were the areas of cell growth. The activated charcoal and kieselguhr adsorbed cells reflected a loose arrangement of cells (Fig. 1,2). Among the adsorption materials, the cumulative growth of *Rhodococcus* cells was observed in pores of activated carbon [12].

In the present study, the adsorbing materials activated charcoal and kieselguhr were found to be suitable for alkaline protease production than free cells by immobilizing the cells of *B. circulans* MTCC 7906. Further, this strain produced high protease activity at pH of 10 and may be considered as a potential candidate for commercialization.

REFERENCES

1. Gupta, M.N. and I. Roy, 2002. Applied biocatalysis: an overview. Indian J. Biochem. Biophys., 39: 220-228.
2. Elibol, M. and A.R. Moreira, 2003. Production of extracellular alkaline protease by immobilization of the marine bacterium *Teredinibacter turnirae*. Process Biochem., 38: 1445-1450.
3. Beshay, U. and A.R. Moreira, 2001. Effect of medium compostion on the production of alkaline protease by Teredinobacter turnirae. *proceedings 7th conference on Food Engineering*, American Institute for Chemical Engineering, pp: 311-317.
4. Abdel Naby, M.A., A.M.S. Ismail, S.A. Ahmed and A.F. Abdel Fattah, 1998. Production and immobilization of alkaline protease from *Bacillus mycoides*. Biosource Technology, 64: 205-210.
5. Ye, L., N.N. Khandan and F.G. Edwards, 1994. Biological treatment of airstreams contaminated with organic Vapours. Water Science and Technol., 30: 71-74.
6. Cooney, D.O., 1995. Methods for treating poisoning and drug overdose. In: Activated Charcoal in Medical Applications, Ed., Cooney, D.O., New York, Marcel Dekker, ISBN 0-8247-9300-5, pp: 110-149.
7. Galvano, F., A. Pietri, T. Bertuzzi, G. Fusconi and M. Galvano, 1996. Reduction of carryover of aflatoxin from cow feed to milk by addition of activated carbon. J. Food Prot., 59: 551-554.
8. Kumar, R., 2005. Production and characterization of bacterial alkaline protease. M.Sc. thesis, Punjab Agricultural University, Ludhiana, India.
9. Aneja, K.R., 2003. Experiments in microbiology, Plant pathology and biotechnology. 4TH ed. New age international (P) Ltd publishers.

10. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem., 193: 265-75.
11. Kumar, C.G. and P. Parrack, 2003. Activated charcoal a versatile agent for the recovery and purification of alkaline protease. World J. Microbiol Biotechnol., 19: 243-246.
12. Pai. S.L., Y.L. Hsu, N.M. Chong, C.S. Sheu and C.H. Chen, 1995. Continuous degradation of phenol by *Rhodococcus sp.* immobilized on granular activated carbon and in calcium alginate. Bioresource Technol., 51: 37-42.